The Effect of Eicosapentaenoic Acid on Biomarkers of Growth and Vascularity of Human Colorectal Cancer Liver Metastases

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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My own contributions, fully and explicitly indicated in the thesis, have been the trial design, the application for regulatory approvals, the recruitment and consent of all trial participants, the completion of clinical record forms for each participant, the collection of all tissue samples, the laboratory analysis of all samples except those attributed to others in the paragraph below, the statistical analysis of all data and the preparation of the thesis manuscript.

The other members of the group and their contributions have been as follows. Prof Mark Hull (Section of Molecular Gastroenterology, Leeds Institute of Molecular Medicine, University of Leeds) had the original idea for the trial and was Chief Investigator. Mr Giles Toogood (Department of Hepatobiliary Surgery, St James' University Hospital, Leeds) assisted with the trial design and facilitated access to patients for recruitment into the trial. Mr Raj Prasad and Prof Peter Lodge (Department of Hepatobiliary Surgery, St James' University Hospital, Leeds) facilitated access to patients for recruitment into the trial. Dr Paul Loadman and Ms Amanda Race (Institute of Cancer Therapeutics, University of Bradford) performed all liquid-chromatography tandem mass-spectrometry analyses. Dr Andrea Belluzzi (Department of Gastroenterology, Saint Orsola-Malpighi Hospital, University of Bologna, Italy) and Dr Alessandra Munarini (Endocrinology Unit and Centre for Applied Biomedical Research, Saint Orsola-Malpighi Hospital, University of Bologna, Italy) performed the gas-chromatography mass spectrometry analyses. Dr Adrian Jubb (Genentech Inc, CA, USA) ran the automated image recognition

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Abstract

Background: The omega-3 fatty acid eicosapentaenoic acid (EPA) has been demonstrated to be incorporated into tumours and inhibit tumour growth in pre-clinical models of colorectal cancer liver metastases (CRCLM).

Aims: To test the safety, tolerability and effect on tumour biomarkers of growth and vascularity of orally administered EPA in patients awaiting liver resection surgery for CRCLM.

Methods: In a Phase II randomised, double-blind, placebo-controlled trial, patients with CRCLM received EPA 2g daily (n=43) or placebo (n=45) prior to surgery. CRCLM tissue was analysed for fatty acid content, PGE_2 content, proliferation index (Ki-67), apoptosis index and vascularity. Blood was collected for platelet function and monocyte NFkB binding studies, and urine for measurement of PGE-M. Supplementary *in vitro* endothelial cell studies investigated the effects of EPA on angiogenesis.

Results: The two treatment groups were well matched for burden of disease and previous chemotherapy exposure. Mean duration of EPA treatment was 30 days (range 12-65 days). EPA was safe and well tolerated, with a small excess of diarrhoea (p=0.09), and no excess of post-operative complications. Tumours from the EPA group had a 40% higher EPA content (p<0.01), no difference in proliferation or apoptosis, and a trend to reduced vascularity. EPA treatment was associated with a 36% reduction in urinary PGE-M (p=0.03) compared to placebo, and reduced monocyte NFκB DNA binding compared to baseline (p=0.03). EPA inhibited angiogenesis *in vitro*.

Conclusions: EPA 2g daily is safe and well-tolerated in patients with CRCLM before liver resection. EPA incorporates into CRCLMs, exhibits systemic anti-inflammatory effects, and may have anti-angiogenic activity. Phase III clinical

evaluation of prolonged EPA treatment is warranted in patients with, or at risk of, CRCLM.

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Chapter 1: Introduction

1.1 Colorectal cancer (CRC) and colorectal cancer liver metastasis (CRCLM)

Colorectal cancer (CRC) remains a major cause of morbidity and mortality in the western world, responsible for approximately 10% of all UK cancer deaths (1). In 2008, there were 31,846 new CRC cases in the UK with an incidence of 57 cases per 100,000 in men and 37 per 100,000 in women.(1) Approximately 5% of CRCs are clearly linked to hereditary syndromes, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), with studies suggesting that a further 15-25% of CRCs have a familial link.(2-4) Other CRCs arise on a background of chronic intestinal inflammation such as ulcerative colitis. The majority, however, are believed to be caused by a complex interaction of environmental exposures and non-Mendelian genetic predisposition. These are known as "sporadic" CRCs. Smoking, alcohol, obesity, dietary patterns and exercise levels have all been linked to the development of CRC.(5) The impact of dietary patterns on the development of cancer has been extensively reviewed by the World Cancer Research Fund and the American Institute for Cancer Research.(6) In their Second Expert Report into Food, Nutrition, Physical Activity and the Prevention of Cancer they conclude that there is convincing evidence that the consumption of red meat and processed meats increases the risk of CRC, that the consumption of dietary fibre, garlic and calcium probably reduces the risk of CRC, and that the consumption of fish may also reduce the risk of CRC.(6)

Most deaths from CRC are due to metastatic disease. Metastasis is the spread of cancer from one part of the body to another, with the most common site of metastasis from CRC being the liver. Around 50% of all CRC patients will eventually develop metastatic disease of the liver. Liver metastases are present at the time of diagnosis of CRC in 15-20% of patients (known as synchronous metastases),(7-9) and a further 6-20% of patients with CRC will develop CRCLM at

a later date (known as metachronous metastases).(7, 10) The metastatic process is complex (Figure 1.1). Not all cells that break away from a cancer develop into metastatic tumours. Only subpopulations of cells with particular combinations of genetic mutations and gene expression patterns will have the necessary characteristics to proceed through each sequential step of the metastatic process to form metastatic colonies. This involves the acquisition of a mesenchymal as opposed to an epithelial phenotype, the so called epithelial-mesenchymal transition. Several reviews are available which provide a detailed overview of epithelial-mesenchymal transition (11, 12) and the metastatic pathway.(13, 14) Briefly, to metastasise cells must first escape their normal structural constraints, acquire motile behaviour, and invade and traverse the basement membrane and extracellular matrix to enter the circulation. Cells must survive within the circulation then arrest in the capillary bed of a distant organ such as the liver. Here they must escape from the circulation (extravasate) by adherence to and migration through the subendothelial basement membrane into the liver parenchyma. Once in the liver parenchyma, cells must respond to growth factors in the liver microenvironment to proliferate, evade the signalling pathways that promote apoptosis, and stimulate the growth of new blood vessels (angiogenesis) to sustain the growth of the metastatic colony.

The growth of any tissue beyond a size of 1-2 mm^3, being the threshold for nutrient diffusion across cells, is dependent on angiogenesis to deliver oxygen and nutrients, and this occurs in both physiological and pathological processes. The critical role of angiogenesis in the development of tumours has long been recognised,(15) with extensive research into understanding the mechanism of tumour angiogenesis and its manipulation as a potential therapeutic target.(16-18) Of the many pro-angiogenic factors and signalling pathways identified, vascular endothelial growth factor (VEGF) has been the most intensely studied.(17, 19, 20) It is expressed in approximately 50% of CRC and CRCLM,(21-23) and has been

shown to stimulate endothelial cell proliferation, migration and invasion and increase vascular permeability.(20)

It is increasingly apparent that this overview of metastasis is simplistic, and that cancer cells interact with cells in both the tumour microenvironment and in the circulation to facilitate this process. For example, macrophages at the tumour periphery can be activated and facilitate invasion by the secretion of matrix metalloproteinases (MMP) to degrade extracellular matrix.(24, 25) An interaction between cancer cells and platelets has also been shown. Platelets aggregate with circulating tumour cells and may act to shield cancer cells from immune responses as well as protecting cells from physical shear forces, thereby promoting survival within the circulation.(26) These aggregations may also promote arrest of cancer cells in capillary beds both by physical plugging, and through platelet adhesion to the endothelium.(26) More recently, a direct interaction between platelets and cancer cells has been shown in which platelet-derived transforming growth factor β (TGFβ) activates epithelial-mesenchymal transition related genes in the cancer cell, promoting extravasation and metastasis formation.(27) Different types of cancers have a predisposition to metastasise to different sites. This is affected by circulatory patterns, differences in capillary wall structural features or the endothelial cell surface expression of molecules such as integrins and adhesins, the favourability of tissue microenvironments to support the growth of different cancer cell types, as well intrinsic properties of the circulating cancer cell itself.(28, 29) In the case of CRC, venous blood from the colon drains directly to the liver via the portal vein, and CRC cells are likely to become trapped in the narrow hepatic sinusoids (approximately 7μm diameter).(30) This, together with the rich vascularity, nutrient supply and growth factor production of the liver which makes it an amenable microenvironment for the establishment of metastatic colonies, probably explains why CRC typically form metastatic colonies in the liver.

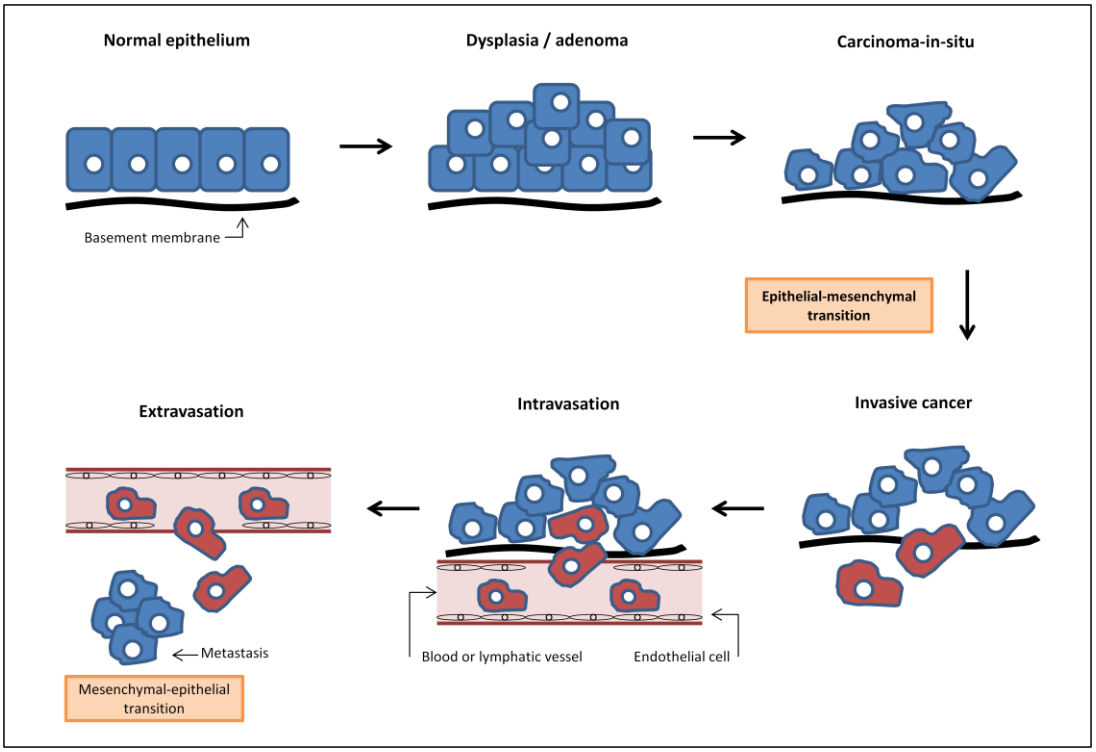


Figure 1.1. The carcinogenesis, epithelial-mesenchymal transition and metastasis pathway. Adapted from Thiery JP, *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. 2(6): p. 442-54.

The accumulation of genetic mutations by a **normal epithelial cell,** each conferring a growth advantage, results in uncontrolled proliferation of that cell to become a focus of disordered epithelial proliferation (**dysplasia**). Dysplasia is identified by characteristic cellular and architectural features, with the loss of normal morphological appearance and disordered tissue architecture. In colorectal carcinogenesis, the earliest dysplastic feature may be an aberrant crypt focus, a cluster of abnormal tube like glands in the colonic epithelium. As dysplasia progresses, a discrete lump or polyp (**adenoma**) may form on the lining of the colon. The severity of dysplasia is often classified as *low-grade* or *high-grade* dysplasia. High grade dysplasia is often referred to as **carcinoma-in-situ** in certain cancer types, although high grade dysplasia is the preferred terminology in CRC. These cells have all the features of cancer, but have not yet breached the basement membrane. Once cells have escaped their normal structural constraints and acquired motile and invasive characteristics (the epithelial to mesenchymal transition) they can invade through the basement membrane. At this stage the tumour is an **invasive carcinoma.** The next steps on the metastatic pathway involve invasion into an adjacent blood or lymphatic vessel (**intravasation**) and then **extravasation** from the circulation at a distant site. Cells may then establish a metastatic colony at a distant site. This may involve reversion back to an epithelial phenotype (mesenchymal to epithelial transition).

1.2 The molecular basis of colorectal carcinogenesis

1.2.1 The Wnt/β-catenin pathway, APC, and other genetic mutations

Activation of the Wnt/β-catenin signaling pathway is believed to be key in the initiation of colorectal carcinogenesis.(31) Cytoplasmic β-catenin levels are ordinarily regulated by a multi-protein complex containing the adenomatous polyposi coli (APC) protein, Axin and glycogen synthase kinase.(32) This complex binds to β-catenin and promotes proteosomal degradation. Mutations in the tumour suppressor gene *APC* occur in 60-80% of sporadic CRCs.(33-35) *APC* mutations typically cause truncation of the APC protein with loss of the β-catenin binding site, and result in cytoplasmic accumulation of β-catenin.(32) Similarly, activation of the proto-oncogene Wnt which leads to inhibition of the APC-protein complex, mutation in the *Axin* tumour suppressor gene, or a mutation in the β-catenin gene can all lead to cytoplasmic accumulation of β-catenin.(32) With increasing cytoplasmic levels of β-catenin, there is the potential for translocation of β-catenin into the nucleus where it acts as a co-factor for the transcription of genes associated with cell-cycle regulation, such as *c-MYC* (36) and *CCND1,*(37) and other genes involved in carcinogenesis such as *MMP-7* (38) and *VEGF*.(39) However, whilst *APC* loss contributes initially to adenoma formation, further mutations are required for progression from adenoma to carcinoma.(40) These typically include inactivation of the p53 tumour suppressor pathway as a subsequent step in colorectal carcinogenesis, inactivation of tumour-suppressing TGF-β signaling, and activation of the oncogenes *KRAS*, *BRAF* and *PI3K*.(31) The mutations in these pathways are described in more detail in the review by Markowitz and Bertagnolli.(31)

1.2.2 Cyclooxygenase and prostaglandins

Upregulated cyclooxygenase (COX) expression and the COX-dependent synthesis of prostaglandins (PG)s and thromboxanes are also believed to play a critical role in the early stages of colorectal carcinogenesis.(41, 42) Arachidonic acid is the principle substrate for COX. It is a polyunsaturated fatty acid (PUFA) found in cell phospholipid membranes. Fatty acids are carbon chains with a methyl group at one end and a carboxyl group at the other. Saturated fatty acids contain only carbon-carbon single bonds, whereas unsaturated fatty acids contain one (monounsaturated) or more (polyunsaturated) carbon-carbon double bonds. Arachidonic acid is a 20-carbon chain with 4 carbon-carbon double bonds. It is an omega (ω)-6 PUFA, so named by the position of the first double bond at the 6^{th} carbon from the methyl (omega) end. Arachidonic acid can therefore be represented by the notation "20:4 ω-6 " (Figure 1.2). Arachidonic acid is liberated from phospholipid membranes by phospholipase A_2 and catalysed by COX to produce PGH_2, via the unstable intermediate PGG_2. PGH_2 is further catalysed by prostaglandin synthases to produce a range of prostaglandins, including PGE_2 (Figure 1.3), and thromboxane (Tx) A_2. AA can also be metabolised by the lipoxygenase (LOX) pathway to leukotrienes (LT) such as LTB_4. COX exists as two isozymes. COX-1 is constitutively expressed in most human tissues, including platelets, and is responsible for the basal level of PG synthesis required for normal tissue homeostasis. Meanwhile, COX-2 is not normally expressed in most tissues but is induced in response to inflammation and carcinogenesis.(43, 44) Increased COX-2 expression has been found in 50-77% of adenomas,(45, 46) 61-84% of adenocarcinomas,(45, 47-49) and 100% of CRCLM.(50, 51)

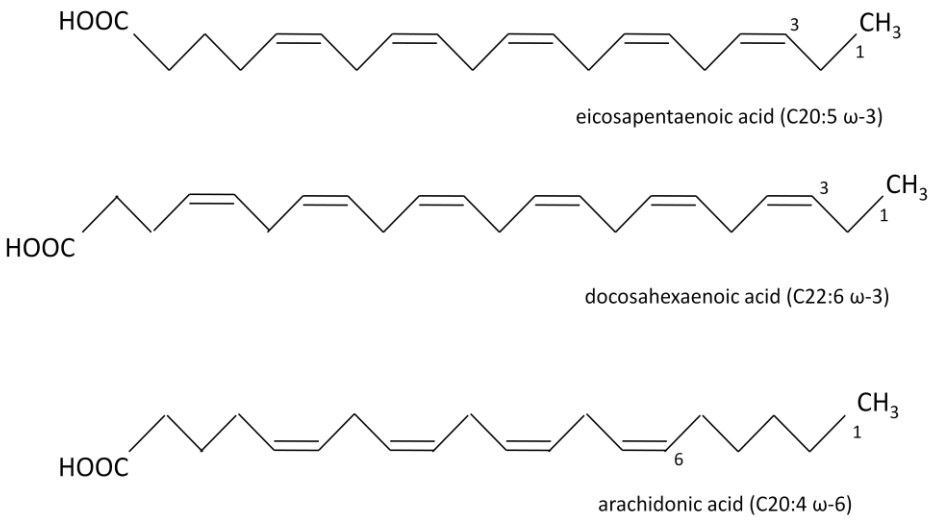


Figure 1.2. Structure and nomenclature of polyunsaturated fatty acids (PUFAs). Cx:y ω-z refers to the chemical structure where x is the number of carbon atoms, y is the number of carbon-carbon double bonds and z is the position of the first carbon-carbon double bond away from the methyl (ω) end of the hydrocarbon chain. Note that position of the first carbon-carbon double bond which defines arachidonic acid as an ω-6 PUFA, in contrast to the position of the first carbon-carbon double bond in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which defines them as ω-3 PUFAs. EPA and DHA are discussed further in section 1.6.

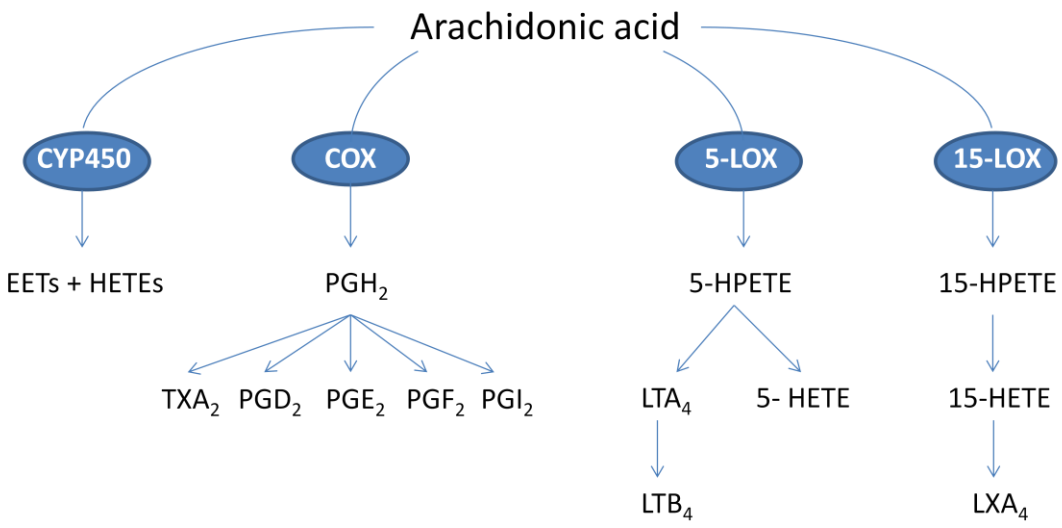


Figure 1.3. Metabolism of arachidonic acid by the COX, LOX and CYP450 pathways. **COX**=cyclooxygenase, **LOX**=lipoxgenase, **CYP450**=cytochrome P450, **EET**=eicosatetraenoic acid, **HETE**=hydroxyeicosatetraenoic acid, **PG**=prostaglandin, **TX**=thromjboxane, **HPETE**=hydroperoxyeicosatetraenoic acid, **LT**=leukotriene, **LX**=lipoxin

Prostaglandins mediate their effects through specific cell-surface G-protein coupled receptors, with PGE_2 acting through the receptors EP1-EP4.(52) EP1 signals via phospolipase C / inositol triphosphate signalling, EP2 and EP4 signal predominantly through an increase in intracellular cyclic adenosine monophosphate (cAMP), whereas EP3 is regarded as an inhibitory receptor, signaling via a reduction in cAMP.(52) Prostaglandins may also exert an effect by acting as direct ligands for peroxisome proliferator-activated receptors (PPARs). Prostaglandin catabolism is regulated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a nicotinamide adenine dinucleotide (NAD)+ linked dehydrogenase that oxidizes the 15-hydroxy group of a prostaglandin to a ketone group, rendering it inactive.(53) Suppressed 15-PGDH has been demonstrated in up to 80% of colorectal adenomas and carcinomas.(54, 55)

Prostaglandins and thromboxanes have wide-ranging roles in inflammation, platelet aggregation and tissue homeostasis, and can act in an autocrine or paracrine manner to effect changes in their immediate environment. Increased PG signaling, through an upregulation of the COX-2/PGE_2 pathway is believed to play a key role in colorectal carcinogenesis, with PGE_2 having been shown to promote CRC cell proliferation *in vitro* (56, 57) and stimulate intestinal adenoma formation *in vivo*.(58, 59) Elevated PGE_2 levels have been demonstrated in human CRC and adenomas (60). The pro-tumourgenic effects of PGE_2 are mediated through a number of signaling pathways involved in tumour proliferation, apoptosis, migration, invasion and angiogenesis, which have not been clearly elucidated but which may be interlinked.(41, 44) These include promotion of tumour proliferation through the activation of the β-catenin/Wnt pathway (61, 62) and promotion of cell proliferation, migration and survival through the activation of epidermal growth factor receptor (EGFR) signaling.(63-65) PGE_2 has been shown to suppress apoptosis by increasing *BCL-2* expression (66, 67), activating EGFR and nuclear PPARδ signaling,(59, 64) and activating the Ras-MAPK/ERK and PI3K/AKT pathways.(59,

65, 68) PGE_2 has also been associated with inactivation of E-cadherin and increased expression of MMPs, thereby promoting cell migration and invasion.(66, 69) Finally, PGE_2 has been shown to stimulate the production of pro-angiogenic factors including VEGF and basic fibroblast growth factor (bFGF).(70-73). PGE_2 has been demonstrated to promote tumour angiogenesis in pre-clinical models of CRC, (74, 75) whilst the expression of COX-2 and PGE_2 has been shown to correlate with VEGF expression and tumour vascularity in human CRC,(71, 76, 77) CRCLM (78) and gastric cancer.(79)

Whilst PGE_2 levels can easily be measured *in vitro*, measurement of systemic PGE_2 production in humans is more challenging. Sampling techniques, such as venepuncture, can cause platelet activation and prostaglandin release, and prostaglandin production and metabolism can continue in samples *ex vivo*. Measurement of PGE_2 in the urine is similarly unreliable as this primarily reflects local production of PGE_2 in the kidney.(80) It is widely accepted that the most accurate index of systemic PGE_2 production is the measurement of PGE-M, a stable metabolite of PGE_2 that is excreted in the urine.(81) Elevated levels of urinary PGE-M have been associated with colorectal adenomas and carcinomas in humans,(82-84) suggesting PGE-M as a potential biomarker for the detection of CRC and other cancers.(85)

1.3 Surgery for CRCLM

Untreated, the prognosis from CRCLM is poor, with a median survival of 6-12 months.(86) Current chemotherapy regimens for inoperable disease extend median survival up to almost 24 months in those that respond to treatment,(87) but this remains a palliative treatment. Surgery is the only potentially curative treatment for patients with CRCLM, with 5 year survival rates after surgery ranging from 30-60%.(88-92) Of all patients who undergo liver resection surgery for CRCLM, 40-60% will develop disease recurrence, at a median of 13.8-16.3 months.(91, 93)

Just under half of all recurrences will be in the liver alone, one-third extra-hepatic alone, and the remaining one-fifth will be both hepatic and extra-hepatic recurrences.(91) Patients with recurrent hepatic metastases may be offered repeat liver resection, so long as the disease is technically resectable. This is true for first, second, and even third recurrences of CRCLMs. Such a strategy of aggressive redo-resections of recurrent CRCLMs is justified by a 5-year overall survival rate after repeat resections of almost 30%.(93)

1.3.1 Liver anatomy

The liver is the largest solid organ in the body, situated in the right upper quadrant of the abdomen and weighing approximately 1.5kg. The liver has a dual blood supply, with approximately 75% of blood flow from the portal vein and 25% from the hepatic artery. The portal vein delivers venous blood from the small intestine to the liver, ensuring that substances absorbed from the gut are metabolised and detoxified by the liver before entering the systemic circulation. The portal circulation provides a direct route for the haematogenous spread of CRC cells to the liver, and explains why the liver is the most common site of CRC metastases. It is the first capillary bed in which CRC cells escaping into the circulation will arrest and "plug".

Whilst the liver might be divided on external anatomic appearances by the falciform ligament into a right and left lobe, it is more common to divide the liver based on its functional anatomy into two lobes and eight functionally independent segments (Figure 1.4), as described by Couinaud in 1957.(94) The middle hepatic vein divides the liver into a larger right lobe (segments 5-8) and a smaller left lobe (segments 2-4). This plane runs in an approximate plane between the gallbladder fossa and the inferior vena cava (IVC). The right hepatic vein divides the right lobe into an anterior section (segments 5&8) and posterior section (segments 6&7), whilst the left hepatic vein divides the left lobe into a lateral section (segments 2&3)

and medial section (segment 4a and 4b). All three hepatic veins meet to drain into the IVC at the postero-superior aspect of the liver. The liver is then further divided by the right and left portal veins into superior and inferior sections. The right portal vein separates segments 7&8 superiorly from segments 5&6 inferiorly, and the left portal vein separates segments 4a&2 superiorly from segments 4b&3 inferiorly. Segment 1, also known as the caudate lobe, lies posteriorly adjacent to the IVC.

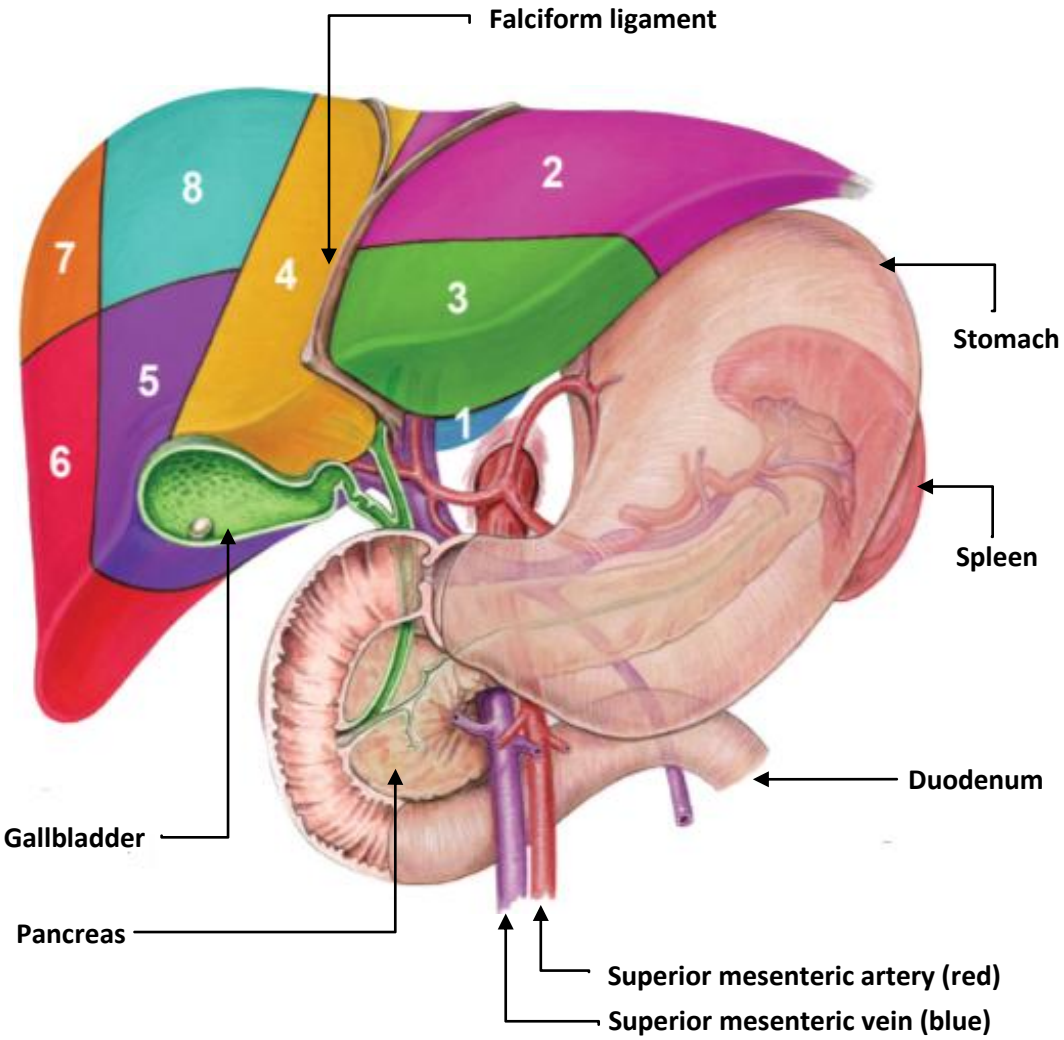


Figure 1.4. Anatomy of the liver, and its anatomical relations. (Adapted from a Leeds Teaching Hospitals NHS Trust patient education leaflet). The liver is divided into eight segments (numbered). Segments 1-4 form the left lobe of the liver, and segments 5-8 form the right lobe of the liver. The hepatic artery (red) and portal vein (blue) can be seen immediately below segments 3 and 4, where they are branching into their respective left and right branches before entering the liver. The inferior vena cava lies behind segment 1 and is not shown.

1.3.2 Liver resection and other surgical adjuncts

Because each segment of the liver has its own branch of the hepatic artery and portal vein (vascular inflow), its own branch of the hepatic vein (vascular outflow), and its own branch of the bile duct, each segment can be individually resected without compromising the viability of the remaining segments. Following liver resection hyperplasia of hepatocytes in the remaining segments occurs,(95) and the remaining liver begins to grow within days of surgery.(96, 97) Liver functional capacity is restored within days to weeks of surgery,(98) and growth will continue over a period of weeks and months until the original liver volume is restored. The liver may recover 75% of its original volume within 1-3 months (98, 99) and 100% of its original volume within 12 months after a major resection.(96)

Resectability of CRCLMs is dependent on the ability to obtain a complete macroscopic clearance of tumour, preserve at least 2 contiguous segments of liver with adequate vascular inflow and outflow, and preserve sufficient liver volume (known as the liver remnant) to be able to continue all of the liver functions. Up to 75-80% of the liver can be resected whilst still preserving sufficient liver function in the liver remnant for the patient not to develop liver failure.(100) However, the extent of resection that is feasible depends not only on the residual volume, but also the functional capacity of the predicted remnant. Functional capacity may be impaired in patients with steatotic or cirrhotic livers, such as in chronic liver diseases or following chemotherapy,(100) and their rate of liver regeneration may be slower.(101, 102) This may mean that a larger remnant of diseased liver would be required to achieve the same function as a smaller remnant of healthy liver. Concerns about the functional capacity of the liver remnant may limit the extent of resection that can safely be performed in patients with underlying liver disease.

Historically, only 20-25% of patients with CRCLM presented with resectable CRCLMs.(103, 104) However, over the last 10 years advances in neoadjuvant

chemotherapy regimens to reduce the burden of disease has meant that an increasing number of patients have a good response to chemotherapy, effectively "downsizing" their disease to within operable limits. Similarly, in patients who may have been deemed inoperable on the basis of an inadequate remnant liver volume, techniques such as portal vein embolisation or two-stage resections are now used to allow the remnant liver to hypertrophy before resection of the main tumour mass. For example, when a right hemihepatectomy is planned in a patient with a small left lobe, embolisation of the right portal vein 6-8 weeks preoperatively will divert portal flow away from the right lobe. The right lobe will begin to atrophy and the small left lobe begins to hypertrophy in response, providing a left lobe of sufficient volume to allow the resection to be performed safely without fear of inducing liver failure. Alternatively, when a patient presents with disease in both the right and left lobes of the liver which cannot all be resected safely for fear of small remnant volume, resection of the disease in one side, an interval to allow the remnant liver to hypertrophy, followed by a second operation to resect the disease in the other half of the liver may also allow resection of all disease whilst leaving an adequate remnant volume (a so-called "2-stage resection").(105, 106) As a result of these techniques, and improvements in the peri-operative surgical and anaesthetic management of liver resection patients, there has been a change in many surgeons' interpretation of "resectability", and a further 10-40% of patients previously considered unresectable may now be suitable for potentially curative surgery.

1.4 Chemotherapy for CRC and CRCLM

Chemotherapy can be given at any one of a number of stages in the natural history of CRC and CRCLM. In patients presenting with CRC, "adjuvant" chemotherapy may be given following resection of the primary CRC to control microscopic disease with the aim of reducing the risk of CRC recurrence or CRCLM in the future.

Chemotherapy may also be given to patients with established CRCLMs. In those patients presenting with operable CRCLMs "adjuvant" chemotherapy may be given either just before or just after surgery, with the same aim of reducing the risk of disease recurrence and improving patient survival following surgery. By contrast, those patients presenting with inoperable CRCLMs may be offered neoadjuvant chemotherapy if there is a chance that they have a distribution or burden of disease which may become operable if they respond to chemotherapy and the burden of disease is reduced. The aim of such neoadjuvant chemotherapy is to convert patients from inoperable to operable disease and thus to be able to perform potentially curative liver resection following chemotherapy. In those patients with inoperable CRCLM where neoadjvant chemotherapy is not appropriate, be it due to patient factors or the burden of disease, palliative chemotherapy may be offered with the aim of slowing disease progression and extending life. Finally, other drugs may be given as long term therapies for the chemoprevention of both CRC and CRCLM. These will be discussed in section 1.5.

1.4.1 Adjuvant chemotherapy for CRC

The mainstay of CRC treatment is complete surgical resection, with adequate margins (>5cm) and lymphadenectomy (>12 nodes). Survival is influenced by the extent of tumour spread. The TNM classification describes the extent of local, lymph node and metastatic disease and is used to assign an overall tumour "stage" (Table 1.1 and 1.2). Staging of tumours helps to plan further treatments such as chemotherapy and can give an estimate of prognosis (Table 1.2).

Adjuvant chemotherapy aims to improve patient disease-free survival (DFS) and overall survival (OS) by killing residual tumour cells, either those persisting locally or those that have metastasised to distant sites. The role of adjuvant chemotherapy in Stage III disease is well established. FOLFOX (folinic acid + fluorouracil + oxaliplatin) chemotherapy is now the standard regimen for Stage III

disease, following the publication of the MOSAIC and NSABP C-07 trials demonstrating a clear survival advantage (7% absolute increase in OS at 3 years) with the addition of oxaliplatin to original fluorouracil (FU) based regimens.(107, 108) Capcitabine, an oral fluoropyrimidine, can be substituted in place of FU without loss of efficacy.(109) Studies of the addition of irinotecan or newer biologics such as the monoclonal antibodies cetuximab (against EGFR) and bevacizumab (against VEGF) have not demonstrated any survival benefit.(110-113)

The evidence for adjuvant chemotherapy in stage II disease is more contentious. The QUASAR study demonstrated a 2.9% increase in OS with FU + folinic acid compared to no chemotherapy in patients with stage II CRC.(114) Some, including the American Society of Clinical Oncology, advocate adjuvant chemotherapy in medically fit patients with “high-risk” stage II CRC, i.e. T4 lesion or tumour perforation, poorly differentiated tumour on histology, or less than 12 lymph nodes sampled (i.e. an inadequate sampling which may understage lymph node status).(115) However, the MOSAIC study demonstrated no improvement in 6yr OS with oxaliplatin + FU in patients with Stage II CRC, and a non-significant 2.3% increase in OS in those deemed “high-risk”.(107) On balance, the risks and benefits of chemotherapy need to be discussed with individual patients with Stage II disease, although it is clear from the QUASAR and MOSAIC studies that chemotherapy does not offer a survival advantage to those aged 70 years and older, either with Stage II or Stage III disease.(107, 108)

	T (Tumour)	N (lymph node)	M (metastases)
0	No evidence of tumour	No lymph nodes involved	No metastases
1	Into mucosa	1-3 lymph nodes	Metastases present
2	Into muscularis propria	4 or more lymph nodes	
3	Into serosa		
4	Through serosa		

Table 1.1. The TNM staging of colorectal cancer. T describes the layer of the bowel wall that the tumour has extended into. The mucosa is the innermost lining of the bowel wall, the muscularis propria is the thick muscle layer, and the serosa is the outermost layer of connective tissue covering the colon. Once a tumour has grown through the serosa, it either directly invades adjacent tissue (either other parts of the bowel or adjacent organs) or there is perforation of the bowel. N describes the number of lymph nodes that are involved, and M describes the presence or absence of metastatic disease.

Stage	Definition	5yr survival after surgery
I	T1 or T2, no lymph node or metastatic involvement	93%
II	T3 or T4, no lymph node or metastatic involvement	77%
III	Any T, with lymph node involvement (N1 or N2) but no metastases	48%
IV	Metastatic disease (any T, any N)	6%-40%*

Table 1.2. Staging of CRC. Staging is a means of defining and describing the spread of CRC and is based on the TNM classification of tumour, nodal and metastatic extent of disease. Staging groups CRCs with a similar extent of disease, and is used to plan treatment. It can also help to give an estimate of prognosis. *Survival in stage IV disease depends on the extent of metastatic disease. If metastatic disease can be fully resected, the prognosis is much better than in patients with inoperable disease.

1.4.2 Adjuvant chemotherapy for CRCLM

The evidence for adjuvant chemotherapy for CRCLM is less well established than that for primary CRC, with only limited randomised controlled trial (RCT) evidence for the use of adjuvant chemotherapy in patients presenting with resectable CRCLM. The EORTC 40983 trial (n=364) compared 6 cycles of FOLFOX pre- and post- liver resection to surgery alone and demonstrated an increase in 3yr DFS from 28.1 to 36.2%.(116) Other studies have demonstrated a non-significant increase in DFS with postoperative FOLFOX or FOLFIRI (folinic acid + fluorouracil + irinotecan).(117, 118) Whilst the results of the EORTC trial has led to many centres advocating pre-operative rather than post-operative adjuvant chemotherapy for earlier control of micrometastatic disease, there is currently no evidence supporting one over the other, and pre-operative chemotherapy is associated with increased steatosis, steatohepatitis, liver vascular lesions, and significantly higher postoperative complications.(119, 120)

1.4.3 Neoadjuvant chemotherapy for CRCLM

Neoadjuvant chemotherapy to downsize disease is based on FU in combination with either irinotecan or oxaliplatin. In a recent review by the United Kingdom National Institute for Health Research, resection rates increased from 7-9% to 35-51% with these regimens.(121) More recently cetuximab has been approved by the National Institute for Health and Care Excellence (NICE) for first line combination therapy with either FOLFOX or FOLFIRI for patients with unresectable CRCLM if disease is confined to the liver, the primary tumour has already been resected or is potentially operable, and the patient is fit for resection if they demonstrate adequate response to chemotherapy. This approval is based on the results of two studies in patients with inoperable CRCLM. The CRYSTAL study (n=1198) demonstrated that addition of cetuximab to FOLFIRI was associated with increased DFS, and a non-significant increase in OS from 21 months to 24.9 months. Post-hoc subgroup-analysis of patients who were KRAS wildtype demonstrated an increased median

DFS from 8.7 to 9.9 months and increased response rate from 43.2% to 59.3%.(122) Similar results were seen in the OPUS study (n=337), with the addition of cetuximab to FOLFOX increasing response rates from 36% to 45% (37% to 60.7% in KRAS-WT) and a statistically significant increase in DFS from 7.2 months to 7.7 months, although this small increase in DFS is of limited clinical relevance.(123) A further study has evaluated the effect of giving both oxaliplatin and irinotecan in combination with folinic acid and FU (FOLFOXIRI) compared to FOLFIRI alone for patients with unresectable disease.(124) The addition of oxaliplatin was associated with an increase in response rate from 34% to 60%, an increase in R0 resection rate from 6% to 15%, and significantly increased DFS (6.9 months to 9.8 months) and OS (16.7 months to 22.6 months). The FOLFOXIRI combination was however associated with significantly increased peripheral neurotoxicity and neutropaenia.(124)

1.5 Chemoprevention of CRCLM

One further approach to treating CRCLM is through chemoprevention, with the aim of preventing the development of CRC and CRCLM in the first place. A large body of evidence has accumulated to support COX inhibition as a potential target for prevention of CRC in humans.(41, 125, 126) Large population-cohort and case-control studies have consistently demonstrated a reduction in CRC incidence with regular use of aspirin or other non-steroidal anti-inflammatory drugs. In a recent meta-analysis of 12 such studies, including over 405,000 patients, the pooled relative risk (RR) of CRC in patients taking regular non-aspirin NSAIDs was 0.74, and RR 0.5 for patients taking regular NSAIDs including aspirin.(127) Three RCTs of the chemopreventative efficacy of the selective COX-2 inhibitors rofecoxib and celecoxib in patients with recently removed sporadic colorectal adenomas all demonstrated significant reductions in polyp incidence at 3 years follow up of between 25-37%.(128-130) A fourth RCT demonstrated a 28% reduction in polyp

number after 6 months of celecoxib supplementation in patients with FAP.(131) However, analysis of safety data from these trials demonstrated an increased risk of cardiovascular events in the COX-2 inhibitor groups, primarily myocardial infarction and ischaemic cerebrovascular events. A meta-analysis of 145,373 patients in 138 placebo controlled COX-2 inhibitor trials demonstrated an increased risk of myocardial infarction (hazard ratio HR 1.86), but no difference in thrombotic strokes in the COX-2 inhibitor group.(132) This increased cardiovascular risk led to rofecoxib being withdrawn in 2004, and the premature closure of another large RCT (anticipated n=7000) comparing rofecoxib to placebo for chemoprevention following CRC resection, which would have more specifically included CRCLM development in their endpoints of DFS and OS.(133) Further study into the toxicity and tissue specificity of COX-2 inhibitors is required if they are to have a role in CRC chemoprevention.(134)

RCTs have also investigated the chemopreventative effect of aspirin. In patients with previously resected colorectal cancer (n=635), 325mg daily aspirin reduced adenoma incidence at 3yr colonoscopy by 37% compared to placebo, together with a 39% reduction in mean adenoma number and an increase in time to development of first adenoma.(135) A similar study by the same group compared daily aspirin 81mg vs. 325mg vs. placebo in patients with recently documented adenoma (n=1121). At 3 year colonoscopy the 81mg aspirin group had a 19% reduction in adenoma incidence compared to placebo. There was a 4% reduction in adenoma risk (non-significant) in the aspirin 325mg group compared to placebo.(136) In the latter study, risk of advanced adenomas (>1cm or with tubulovillous or villous features, severe dysplasia or invasive cancer) was reduced in both aspirin groups, although again this did not reach statistical significance in the high dose aspirin group (HR 0.59 and 0.83 respectively, 40% and 18% reduction respectively), whereas no difference in advanced adenomas was seen in the former study. Aspirin 600mg daily has also been shown to reduce the risk of colorectal cancer in

patients with HNPCC (n=861). At 4 year follow-up CRC incidence in the aspirin group was 4.2% compared to 6.9% in the placebo group (39% reduction).(137) Recent meta-analyses of five studies of aspirin in primary and secondary cardiovascular protection have shown a reduction in 20-year CRC incidence of up to 70% in patients taking aspirin for 5 years or more (n=14,033). There was no increased benefit of taking aspirin doses above 75mg daily.(138). A second meta-analysis by the same group of 8 studies of aspirin vs. placebo for at least 4 years, demonstrated a reduction in all-cancer death at 20 years follow-up in the aspirin group compared to placebo group.(139) Again, there was no increased benefit to aspirin doses greater than 75mg daily. The benefit for reduced CRC incidence was only apparent after 10 years follow-up, with a HR 0.51 at 10-20yrs follow-up. This is consistent with the lag phase in colorectal carcinogenesis, widely taken to be approximately 10 years for the development of CRC from a colorectal adenoma. As with COX-2 inhibitors, the side-effect profile of aspirin with an increased tendency for gastrointestinal bleeding (140) may limit its attractiveness as a long-term chemopreventative agent, especially in those without cardiovascular disease or cardiovascular risk factors. However, based on the findings of these recent meta-analyses there is renewed interest in the risk-benefit ratio of long-term low-dose aspirin for the chemoprevention of CRC.(141, 142)

1.6 Omega-3 Polyunsaturated Fatty Acids (PUFAs)

'Essential' fatty acids are those which are required for biological processes, but which humans are unable to synthesize and must therefore obtain from dietary sources. The parent ω-3 PUFA α-linolenic acid (ALA, 18:3) and parent ω-6 PUFA linoleic acid (LA, 18:2) are both found in vegetable oils. Humans can easily metabolise LA to form the ω-6 PUFA arachidonic acid (AA, 20:4). However, endogenous production of the ω-3 PUFAs eicosapentaenoic acid (EPA, 21:5) and docosahexaenoic acid (DHA, 22:6) from ALA by humans is so small as to be

insignificant.(143) Therefore, the main ω-3 PUFAs EPA and DHA are considered 'essential' and are obtained predominantly from cold water, oily fish such as mackerel, salmon and sardines. White fish, such as cod and haddock, and shellfish typically have much lower ω-3 PUFAs levels.(144) Table 1.3 details the EPA and DHA content of commonly consumed fish.

Fish	**EPA+DHA (mg) per 140g (5oz) serving**
Salmon	1500 - 3000
Anchovies, Herring	2900-3000
Mackerel	1700 - 2650
Tuna: Bluefin	2150
Sardines	1400 - 2000
Oysters: Pacific	1950
Trout: Freshwater	1250 - 1400
Mussels	1150
Squid	950
Crab	250 - 700
Tuna: Skipjack and Yellowfin	200 - 450
Plaice, and Sole	450
Tuna: Light canned	200 - 400
Cod	250
Scallops	250
Haddock	250
Shrimp	150

Table 1.3. EPA and DHA content of common fish and seafood. Figures are rounded to the nearest 50mg per typical 140g cooked weight portion.

PUFAs are biologically important, with roles in phospholipid membrane structure and function, as well as cellular signalling and lipid metabolism. PUFAs can be liberated from phospholipid membranes by the phospholipase A2 family of enzymes and are metabolised by three main pathways: i) the cyclooxygenase (COX)

pathway, ii) the lipoxgenase (LOX) pathway and iii) the cytochrome P450 monoxygenase (CYP450) pathway. Metabolites derived from the ω-6 PUFA AA, such as prostaglandin (PG)E_2, are typically pro-inflammatory and have been linked with initiation and progression of colorectal carcinogenesis, whereas those derived from ω-3 PUFAs (e.g. PGE_3) are less pro-inflammatory, and may even have anti-cancer properties. Excellent reviews are already available on the metabolism of ω-3 PUFAs,(145) the implications of inhibition of AA metabolism on cell proliferation (146) and the effects of ω-6 and ω-3 PUFAs metabolites on colorectal carcinogenesis.(147)

1.7 Mechanisms of the anti-neoplastic activity of ω-3 PUFAs

Current knowledge of the anti-neoplastic activity of ω-3 PUFAs has been comprehensively reviewed in articles by Calviello, Chapkin and Smith.(145, 147, 148) The main mechanisms that have been proposed for anti-neoplastic activities of ω-3 PUFAs are:

i) modulation of COX activity

ii) alteration of membrane dynamics and cell surface receptor function

iii) increased cellular oxidative stress

iv) production of novel anti-inflammatory lipid mediators including resolvins, protectins and maresins

The relative contributions of and interactions between these activities to the anti-cancer properties of ω-3 PUFAs, however, remains unclear. A summary of each of these mechanisms focusing on more recent findings is provided here. Two further mechanisms by which ω-3 PUFAs may also have anti-neoplastic activity will also be briefly discussed; the direct activation of G protein-coupled receptors (GPCRs), and the effect on nuclear factor kappa beta (NFκB) signalling.

1.7.1 Modulation of COX activity

EPA can act as an alternative substrate for COX-2, instead of AA, leading to a reduction in formation of pro-tumorigenic '2-series' PGs (e.g. PGE_2) in favour of '3-series' PGs (e.g. PGE_3) in several cell types including CRC cells (Figure 1.5).(145, 149, 150) PGE_3 has anti-tumorigenic activity against human lung cancer cells *in vitro* (150) and inhibits pro-tumorigenic PGE_2-EP4 receptor signaling in human CRC cells.(149) Recently, a 'PGE_2 to PGE_3 switch' has been demonstrated in colorectal mucosa of rats treated with fish oil.(151) However, reduction of PGE_2 synthesis and/or generation of PGE_3 following EPA treatment remains to be demonstrated in human CRC tissue. Meanwhile, one study demonstrated that *in vitro* and *in vivo* growth of HCT-116 CRC cells was inhibited by ω-3 PUFAs irrespective of whether the cells expressed COX, suggesting that inhibition can occur via COX-independent mechanisms.(152)

1.7.2 Alteration of membrane dynamics and cell surface receptor function

There is some evidence that the incorporation of ω-3 PUFAs into cell phospholipid membranes alters the fluidity, structure and/or function of lipid rafts or calveolae.(153) These are sphingolipid and cholesterol rich microdomains that float freely in the cell membrane. The localization of cell surface receptors, such as EGFR,(154) in lipid rafts is believed to be crucial for downstream receptor signaling controlling proliferation and apoptosis.(155, 156) In a recent study DHA, but not EPA, suppressed CRC cell proliferation via an EGFR dependent mechanism, with a reduction in EGFR localisation to lipid rafts, suppression of EGFR signaling, and an increase in EGFR degradation. A reduction in EGFR signaling was also seen in the colonic epithelium of mice supplemented with DHA.(157) ω-3 PUFAs could alter EGFR function by changing receptor behaviour in lipid rafts but EPA could also decrease *trans*-activation of EGFR by reduction in PGE_2 synthesis.(63)

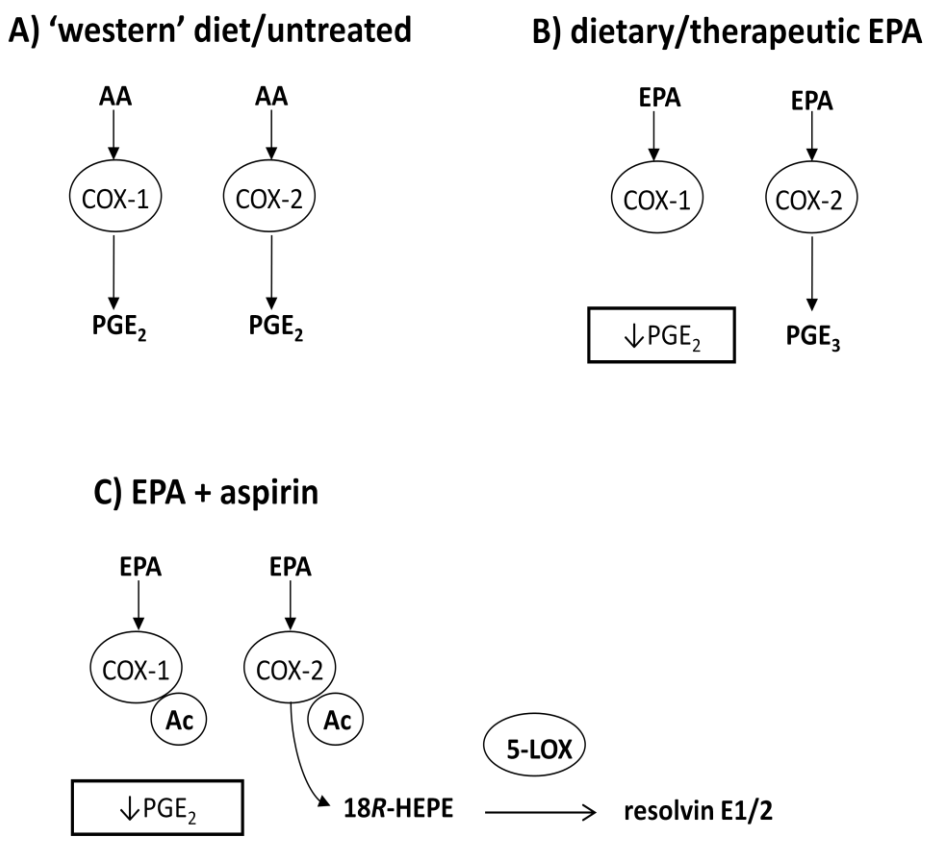


Figure 1.5 The effect of eicosapentaenoic acid (EPA) with or without concurrent aspirin on cyclooxygenase (COX) activity. Adapted from: Cockbain *et al*. Omega-3 polyunsaturated fatty acids for the treatment and prevention of colorectal cancer. Gut 2012;61:135-149

A) When arachidonic acid (AA), derived mainly from the dietary ω-6 polyunsaturated fatty acid (PUFA) linoleic acid in "western" diets is the main substrate for COX-1 and COX-2, prostaglandin(PG)E_2 is the predominant metabolite in colorectal tissue. **B)** EPA can act as an alternative substrate for both COX-1 and COX-2. It effectively inhibits COX-1 activity, but modulates COX-2 activity such that PGH_3 is the predominant metabolite.(145) This is then converted to other three-series PGs, including PGE_3, by PG synthases. Enzymatic turnover of EPA is approximately three-fold less than that of AA, so the net result is a reduction in COX-2 synthesis of PGE_2 and the production instead of (smaller quantities of) PGE_3.(145) **C)** Aspirin irreversibly acetylates COX-1 and COX-2. COX-1 is effectively inhibited. However, acetylated COX-2 can metabolise EPA to produce 18*R*-hydroxyeicosapentaenoic acid (HEPE) and 18*S*-HEPE instead of PGH_3. HEPEs can be further metabolised by 5-lipoxygenase (5-LOX) to produce E-series resolvins, in combination with a reduction in PGE_2 production.(158, 159) D-series resolvins are produced via docosahexaenoic acid metabolism by LOX-dependent pathways.

1.7.3 Increased oxidative stress

Omega-3 PUFAs may have an anti-neoplastic effect through alteration in the cellular redox state and increased oxidative stress. PUFAs are highly peroxidisable, which generates reactive oxygen species (ROS) such as the superoxide radical. Many tumour cells display altered cellular pathways for the handling of ROS including depletion of the major intracellular antioxidant, glutathione. A subsequent elevation of intracellular ROS levels by ω-3 PUFAs has been hypothesised to induce cancer cell apoptosis.(160) A potential beneficial interaction between ω-3 PUFAs and dietary fibre leading to induction of colonocyte apoptosis has been elegantly studied by Chapkin and colleagues.(161-163).

1.7.4 Novel anti-inflammatory lipid mediators

In the presence of aspirin, which irreversibly acetylates the COX enzyme, EPA drives COX-2-dependent production of resolvin (Rv) E1 (5*S*,12*R*,18*R*-trihydroxyeicosapentaenoic acid) via metabolism of 18*R*-hydroxyeicosapentaenoic acid by 5-LOX (Figure 1.5).(164) 18*R*-RvE1 has been detected in plasma of healthy volunteers in ng/ml quantities after aspirin and EPA ingestion.(158) More recently, synthesis of the 18*S* enantiomer of RvE1 has been demonstrated after EPA and aspirin treatment in healthy volunteers.(159) The precursors of E-series resolvins may also be produced independently of COX by direct CYP450 metabolism of EPA.(165)

Metabolism of DHA can produce D-series resolvins, via a LOX-dependent pathway to produce 17*S*-resolvins, or via acetylated-COX-2 leading to 17*R*-resolvin synthesis.(166) DHA can also be metabolised by leucocyte-mediated pathways to produce 17S-docosatrienes termed protectins (167) or by macrophage-mediated pathways to produce 14-LOX-derived products termed maresins.(168) These newly described families of EPA- and DHA-derived lipid mediators all share anti-

inflammatory and inflammation resolution activity in animal models of acute inflammation.(168, 169)

Cell signalling via these novel lipid mediator families is best characterised for RvE1. Both 18*R* and 18*S* enantiomers of RvE1 are ligands for ChemR23 and BLT1 GPCRs.(159) RvE1 has been demonstrated to induce expression of intestinal alkaline phosphatase in human CRC cells in a ChemR23-dependent manner and abrogate chemically-induced colitis in mice.(170) It is currently not known whether ω-3 PUFA-derived resolvins exhibit anti-neoplastic activity. However, it is known that ChemR23-dependent RvE1 signalling inhibits NFκB activation, which is a critical regulator of early-stage colorectal carcinogenesis.(171)

1.7.5 Direct signalling via G protein-coupled receptors

Omega-3 PUFAs are known to bind and directly activate GPCRs such as GPR120(172) and GPR40.(173) Whilst the GPR120 signalling pathway has been shown to mediate potent anti-inflammatory effects,(172) the potential role of GPCR signalling in mediating an anti-cancer activity of ω-3 PUFAs has yet to be studied.

1.7.6 NFκB signalling

The transcription factor NFκB is a key component of immune and inflammatory signalling pathways, and has been implicated as a regulator of oncogenesis.(174) The NFκB family contains five proteins (p50, p52, p65, RelB and c-Rel) which exist as homo- or hetero-dimers. The p50/p65 and p50/p50 dimers are the most common.(175) These dimers are retained in cytoplasm bound to specific inhibitors (IκBs). Cell stimulation activates IκB kinase (IκK) which in turn phosphorylates IκB inducing its degradation. This releases the NFκB dimer which translocates to the nucleus and coordinates gene transcription. NFκB activation promotes the expression of anti-apoptotic genes such as *Bcl-2*, proinflammatory cytokines such as IL-1B and TNFα, inducible enzymes such as COX-2, cell adhesion molecules such as ICAM-1 and proteases such as MMP-9.(176, 177) The ability of NFκB to

promote cell proliferation, migration, metastasis and angiogenesis and to inhibit apoptosis has led to interest in the role of NFκB in carcinogenesis, and in its inhibition as a possible chemotherapeutic target.(Karin 2004) Omega-3 PUFAs have been shown to inhibit IκB phosphorylation and reduce NFκB activation in CRC cell lines (178, 179) and inhibit NFκB activation in endothelial cells.(180) Omega-3 PUFA supplementation has also been associated with reduced expression of IKK and NFκB in a transgenic mouse prostate cancer model.(181) In a breast cancer xenograft model, ω-3 PUFAs reduced NFκB DNA binding and NFκB-dependent transcription of the anti-apoptotic genes *Bcl-2* and *Bcl-XL*.(182) Modulation of NFκB signalling therefore represents a further mechanism by which ω-3 PUFAs might exert an anti-CRC effect.

1.8 Pre-clinical studies of ω-3 PUFAs in the treatment of colorectal cancer

1.8.1 *In vitro* studies

Many *in vitro* studies have explored the anti-neoplastic activity of ω-3 PUFAs against human CRC cells, with both EPA and DHA treatment being associated with reduced cellular proliferation (152, 183-189) and increased apoptosis.(183, 184, 187, 190) It remains unclear from the few studies comparing EPA and DHA whether there is any significant difference in anti-proliferative and/or pro-apoptotic activity. Calviello *et al.* reported a more pronounced reduction in cell number with EPA than with DHA,(186) whereas Chen *et al.* have reported a lower cell number with DHA than with EPA, and that only DHA induced apoptosis.(187) Both ω-3 PUFAs have been shown to reduce COX-2 expression and PGE_2 production.(149, 180, 184, 186) Human CRC cell lines treated with ω-3 PUFAs have demonstrated increased membrane fluidity (188) and lipid peroxidation,(188, 189) reduced levels of VEGF, β-catenin, PPARγ, BCL-2 and matrix metalloproteinase, and reduced extracellular signal-related kinase-1/2 signalling.(156, 184, 186, 187, 190, 191)

Suppression of angiogenic factors by ω-3 PUFA treatment has also been demonstrated in a number of *in vitro* studies. Calviello *et al* (2004) demonstrated that EPA supplementation of HT-29 cells resulted in a significant reduction of VEGF expression (90% reduction at 30μM EPA), COX-2 expression (37% reduction at 30 μM EPA), and PGE_2 production.(186) The addition of excess PGE_2 restored VEGF expression, demonstrating that one mechanism of inhibition of VEGF by ω-3 PUFAs is via the COX-2 pathway. EPA also reduced the phosphorylation of ERK-1 and ERK-2 which has previously been shown to induce VEGF expression in response to PGE_2 stimulation.(192) Other *in vitro* studies have investigated the effects of ω-3 PUFAs on endothelial cells. Studies culturing endothelial cells in the presence of ω-3 PUFAs, typically EPA or DHA added to culture medium at concentrations between 15μM and 100μM, have shown increased endothelial cell membrane EPA/DHA content,(193) reduced COX-2 expression and PGE_2 production,(180) reduced cell proliferation,(194, 195) reduced cell migration (194, 196) and reduced VEGF-stimulated endothelial cell microtubule formation.(193, 194, 196) Omega-3 PUFAs have therefore been shown to have both direct and indirect anti-angiogenic properties through effects on endothelial and epithelial cells respectively.

1.8.2 Animal studies

The effect of ω-3 and ω-6 PUFA supplementation on the growth of human CRC cell lines grown as xenograft tumours in immunocompromised mice has been studied widely (Table 1.4). There has been a consistent 40-60% reduction in xenograft size in rodents supplemented with ω-3 PUFAs compared with controls.(152, 186, 197) Similar findings have been reported for studies of rodent CRC cell allograft tumours (Table 1.4).(198-202) The ω-3 PUFA content of tumours increased, at the expense of AA, in animals supplemented with ω-3 PUFA,(152, 201-203) together with a reduction in expression of COX-2 (186, 199) and a reduction in tissue PGE_2 levels.(186) In the one study which measured tumour vascularity, oral ω-3 PUFA

supplementation (1g/kg body weight) was associated with a 46% reduction in tumour vascularity compared to controls.(186) Reduced tumour vascularity has also been demonstrated in mammary tumours grown in mice given ω-3 PUFA supplemented diets.(204, 205) Some studies have investigated the effects of conjugated EPA. Conjugated EPA is a mixture of positional and geometric isomers of EPA, where the carbon-carbon double bonds form a "conjugated" chain of alternating double and single carbon-carbon bonds (Figure 1.6). Conjugated EPA has been shown to suppress the growth of DLD-1 human CRC cell tumours in nude mice, which was associated with increased lipid peroxidation in the phospholipid membrane, increased levels of oxidative stress and increased apoptosis.(206)

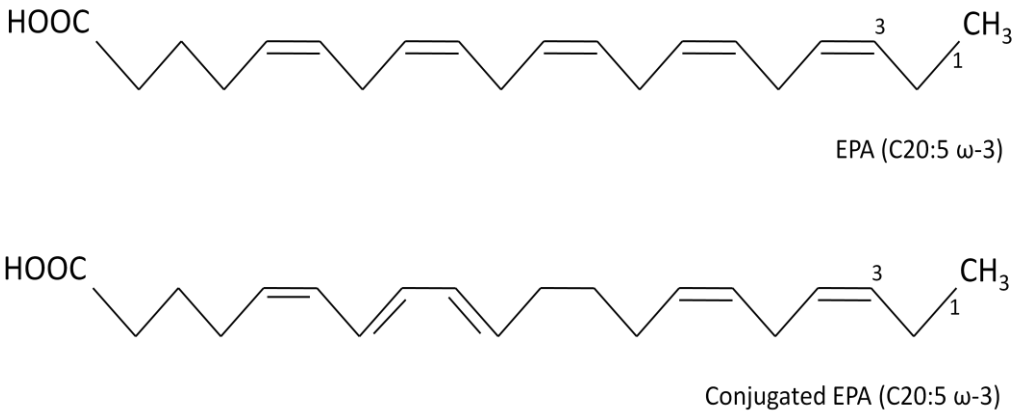


Figure 1.6. Comparison of the structure of EPA and conjugated EPA.
Note the spacing of the carbon-carbon double bonds in EPA (double bonds at carbons 3,6,9,12,15), compared to the presence of alternating carbon-carbon single bond and carbon-carbon double bonds in conjugated EPA (double bonds at carbons 3,6,11,13,15). In chemistry, conjugation is the overlapping of p-orbital electron fields, made possible in compounds with alternating single and double bonds. The overlapping p-orbitals bridge the intervening single bond and electrons can delocalise across the aligned p-orbitals. The system is thus said to be "conjugated". Usage of the term "conjugated" in this sense is therefore different form the use of the term "conjugated" in biology where it is often used to describe the joining together of two compounds.

Study	Model	N=	Treatment Groups	Timing/Duration	Outcome measure(s)	Results (changes are for ω-3 PUFA group vs. control group unless stated)
Cannizzo 1989 (198)	BALB/c mouse, CT26 CRC cells into 1) descending colon 2) tail vein	330	5% & 25% MO_1 vs. 5% & 25% SAFO	30 days pre- and 28 days post- injection	1) Tumour size 2) No. lung mets	3-5x↓ tumour size MO vs. SAFO grps ↑ lung mets in 25% SAFO grp vs. all other grps
Iigo 1997 (202)	CDF1 mouse Co 26Lu CRC cells subcut	230	0.1/0.2ml EPA vs. DHA vs. OA vs. LA ig daily	Day 5-21 post-injection	Tumour size, no. lung mets Plasma/tumour PUFA	EPA/DHA grps dose dependent ↓tumour size and ↓lung mets ↑EPA/DHA & ↓AA in plasma & tumour
Calder 1998 (203)	Nude mouse HT-29 CRC cells subcut	90	20% CO/SAFO/MO_2/OO vs. 2.5% CO Ctrl	3 wk pre- and 2 wk post- injection	Tumour size and PUFA content	65-74% ↑tumour size in high fat grps except MO ↑tumour EPA/DHA + 57%↓AA in MO grp
Boudreau 2001 (152)	Nude mouse HCT116 CRC cells subcut	102	18% MO_3 vs.20% SAFO	2 wk pre- and 3 wk post- injection	Tumour size Tumour PUFA content	50% ↓ tumour size ↑ tumour EPA and 50% ↓ tumour AA
Kato 2002 (197)	Nude mouse WiDR CRC cells subcut	24	8% & 24% CO 16% MO_4 vs. 16% GAO	53 days post-injection	Tumour wt	Tumour wt vs. 8% CO tumour wt: 87%↓ (GAO), 54%↓ (MO), 36%↑ (24% CO)
Togni 2003 (201)	Wistar rat Walker 256 tumour cells subcut	78	1g/kg body wt. MaxEPA vs. CO vs. Ctrl	10 wk pre- and 2 wk post- injection	Tumour wt & PUFA content Cachexia biomarkers (serum glucose, chol, lactate, & liver/muscle glycogen)	40/60% ↓ tumour wt vs. CO/Ctrl 63/42%↓ tumour AA:EPA vs CO/Ctrl ↓cachexia biomarkers
Calviello 2004 (186)	Nude mouse HT-29 CRC cells subcut	45	1g/kg body wt. EPA vs. DHA vs. H_2O Ctrl ig	1wk pre- and 4 wk post-injection	Tumour size, AI, PI, MVD Tumour COX-2 expression Tumour PGE_2	No difference in effect EPA vs DHA EPA/DHA vs. Ctrl both showed 55%↓ tumour size, 50%↓ PI, 1.5x↑ AI, ~50%↓ MVD, 40%↓ PGE_2, 30%↓COX2 and ↓VEGF
Tsuzuki 2004 (206)	Nude mouse DLD-1 CRC cells subcut	40	50mg 20% CLA/EPA/CEPA daily vs. 50mg SAFO Ctrl	4 wk post-injection	Tumour wt, DNA fragmentation Membrane phospholipid peroxidation	Tumour wt ↓80% CEPA vs Ctrl. DNA fragmentation ↑4x CEPA vs Ctrl ↑ phospholipid peroxidation CEPA vs. all grps
Pizato 2005 (200)	Wistar rat Walker 256 tumour cells subcut	60	20% FO_1 vs. 20% SUNO vs. Ctrl	8 wk pre- and 2 wk post- injection	Tumour wt Lipid peroxidation products Cachexia biomarkers (blood glucose, TAG, chol)	60% ↓tumour wt 34% ↑ lipid peroxidation products ↓cachexia biomarkers

Study	Model	N=	Treatment Groups	Timing/Duration	Outcome measure(s)	Results (changes are for ω-3 PUFA group vs. control group unless stated)
Mund 2007 (199)	Wistar rat Walker 256 tumour cells subcut	70	1g/kg FO_2 vs CO vs Ctrl	70 days pre- and 14 days post- injection	Tumour weight, AI & COX-2 Lipid peroxidation products Plasma PGE_2	Tumour weight 50%↓(FO) & 30%↑(CO) AI 4x ↑(FO) & 50% ↓ (CO) COX2 45%↓(FO) & 50%↑(CO) Plasma PGE_2 ↓ (FO) & ↔ (CO) FO 3x↑ lipid peroxidation products

Table 1.4 ***In vivo*** **pre-clinical studies of ω-3 PUFA supplementation for the treatment of CRC**

Abbreviations: ↑ = increase, ↓ = decrease, ↔ = no significant difference, **AI** = apoptosis index, **chol** = cholesterol, **CO** = corn oil, **Ctrl** = control, **FO**= fish oil (**FO_1** = 13%EPA/20%DHA, **FO_2** = 18% EPA/12%DHA), **GAO** = golden algae oil (DHA only), **grp** = group, **grps** = groups, **ig** = intragastric, **LA** = linoleic acid, **MaxEPA** = 18%EPA/12%DHA, **mets** = metastases, **MO** = menhaden oil (MO_1, MO_3, MO_4 ω-3 PUFA content unspecified, MO_2 11%EPA+5.2%DHA), **MVD** = microvessel density, **OA** = oleic acid, **OO** = olive oil, **PI** = proliferation index, **SAFO** = safflower oil, **SAFO** = safflower oil, **subcut** = subcutaneous, **CLA** = conjugated linoleic acid, **CEPA** = conjugated EPA,**SUNO** = sunflower oil, **TAG** = triacylglycerol, **VEGF** = vascular endothelial growth factor, **wt** = weight

1.8.3 Models of CRC metastasis

Few pre-clinical studies have investigated the effect of ω-3 PUFA supplementation on the development of metastatic disease, with only four studies using a liver metastasis model, and a further two using a lung metastasis model. Iwamoto *et al.* supplemented F344 rats with EPA (9.5% w/w as the ethyl ester) for 1 week pre- and 3 weeks post-injection of ACL-15 cells into the superior mesenteric vein. They demonstrated a 40% reduction in number and 44% reduction in size of liver metastases in rats supplemented with EPA compared to controls fed a standard commercial diet containing 5.1% total fat . This was associated with a reduction in tumour cell proliferation index, but no change in apoptosis index, and down-regulation of vascular cell adhesion molecule 1 (VCAM-1). Rats fed a diet high in ω-6 PUFA (10% w/w LA) showed a 3-fold increase in number and 1.5-fold increase in the size of metastases.(207)

Gutt *et al.* supplemented WAG/Rij rats with an EPA/DHA mixture (3.23% ω-3 PUFA; EPA:DHA ratio 3:2) for 3-days pre- and 4 weeks post-injection of CC531 cells into the spleen. They demonstrated a 70% reduction in incidence and 50% reduction in size of hepatic and extra-hepatic metastases, as well as a 30% reduction in tumours expressing VCAM-1.(208) More recently, a study by Hawcroft *et al.* supplemented BALB/c mice with 5% w/w EPA (95% pure free fatty acid) for 14 days pre- and 14 days post- injection of MC-26 cells into the spleen. Notably in this study, injection was performed under anaesthesia percutaneously using ultrasound guidance rather than through a midline laparotomy as in other studies. They demonstrated a 36% reduction in liver weight (used as a measure of tumour burden) in mice supplemented with EPA compared to controls fed a diet containing 5% corn oil. This was associated in the EPA group with a 9-fold increase in tumour EPA content, 54% reduction in tumour AA content, and 19% reduction in tumour proliferation index. There was also a 60% reduction in tumour PGE_2 together with a

significant increase in tumour PGE_3, the first *in vivo* demonstration in tumour tissue of a PGE_2 to PGE_3 switch with ω-3 PUFA supplementation.(209)

By contrast, Griffini *et al* supplemented WAG/Rij rats with an EPA/DHA mixture (20% fish oil v/w; EPA:DHA ratio approximately 3:2) 3 weeks pre- and 3 weeks post-injection of CC531 cells into the portal vein. They demonstrated a 10-fold increase in liver metastases in rats supplemented with ω-3 PUFAs compared to 5% soybean controls and a 4-fold increase compared to those animals supplemented with 20% safflower oil (high in ω-6 PUFA).(210) This was associated with an increase in liver:body weight ratio and an increase in mitotic tumour cells in the ω-3 PUFA group. These results are difficult to reconcile with the studies of Gutt, Iwamoto and Hawcroft, and with the extensive *in vitro* and *in vivo* data suggesting an anti-CRC activity of ω-3 PUFAs. The 20% fish oil preparation used in the Griffini study was a much higher dose than that used in any other *in vivo* study, and is far in excess of any clinically attainable dose in humans.

Two further studies have modelled the effect of ω-3 PUFA supplementation on the development of pulmonary metastases, by measuring lung colonization after injection of CRC cells into the tail vein.(198, 202) One demonstrated that supplementation of either EPA or DHA (0.1 ml aliquots daily of 98% pure EPA or DHA ethyl ester) was associated with significantly fewer lung colonisations at 12 days compared to controls (54% and 58% fewer colonies respectively).(202) The other study found that whilst supplementation with high-fat safflower oil (24.7% by weight for 30 days pre-inoculation) caused a 5-fold increase in the number of pulmonary colonies, there was no difference in low (5%) or high (24.7%) ω-3 PUFA intake on the number of metastases compared with controls.(198)

1.9 Clinical trials of ω-3 PUFAs in patients with CRC

1.9.1 Single agent therapy

Despite strong *in vitro* and *in vivo* evidence for direct anti-CRC activity of ω-3 PUFAs, only two trials have investigated the anti-neoplastic effect of ω-3 PUFAs in patients with CRCLM and no such trial has been performed in patients with CRC. The first of these two trials was a recently published double-blind RCT by Dennison's group in Leicester. They randomized 20 patients to receive a continuous 72hr infusion of total parenteral nutrition (TPN) at 1.5ml/kg/hr. For the ω-3 PUFA supplementation group, the standard 2000ml TPN was compounded with 500ml of Lipidem® (B Braun) which contained a 50:40:10 (vol/vol/vol) mix of medium-chain fatty acids, soybean oil and fish oil, including approximately 3.7g EPA + 2.55g DHA. TPN in the control group was compounded with 500ml of Lipofundin® (B Braun) which contains a 50:50 (vol/vol) mix of medium-chain fatty acids and soybean oil. Patients underwent resection of liver metastases a mean of 9 days (range 5-12 days) after completion of TPN. There was a rapid uptake of ω-3 PUFAs into plasma phospholipids, but no change in AA levels, in the 72 hours of TPN administration. However, there was also a rapid return to baseline ω-3 PUFA levels by the time of surgery.(211) There was no significant difference between the two groups in tumour EPA, DHA or AA content, although there was a trend in the ω-3 PUFA group for a higher total tumour ω-3 PUFA content and lower tumour ω-6:ω-3 ratio in those patients with a shorter interval between finishing TPN and resection of their tumour.(212) This interval between TPN and surgery limits the interpretation of changes in tumour ω-3 PUFA content. It is not clear how quickly tumoural ω-3 PUFA levels rise or fall after iv supplementation, and extrapolation of tumoural PUFA data back to the time of finishing TPN, when plasma levels of ω-3 PUFA were elevated, may be unreliable.

The second trial of ω-3 PUFA supplementation in patients with CRCLM is a Phase II double-blind RCT trial of oral EPA supplementation prior to resection of CRCLM, which is the basis of this MD work (clinicaltrials.gov NCT01070355).

1.9.2 Adjuvant therapy with traditional chemo-radiotherapy

In vitro and *in vivo* studies have shown that ω-3 PUFAs can potentiate the anti-proliferative and pro-apoptotic effects of chemotherapy and radiotherapy used to treat CRC and other solid tumours.(213-217) Despite this, only one published human study of the anti-cancer effects of combining ω-3 PUFAs with chemotherapy exists. A Phase II study evaluated addition of 1.8 g DHA daily to an anthracycline-based chemotherapy regimen for metastatic breast cancer. Patients were dichotomized based on high or low DHA incorporation into plasma phospholipids. The high DHA-incorporation group had a significantly longer time to disease progression (median 8.7 months vs. 3.5 months) and overall survival (median 34 months vs. 18 months).(218) Similarly, only one clinical study of combining ω-3 PUFAs with radiotherapy was identified. This retrospective study of 143 patients who had been prescribed ω-3 fish oil (0.9 g EPA, 1.5 g DHA daily) for 18 weeks following radiotherapy for brain metastases found reduced radionecrosis (3.5% vs. 14.1%) and improved overall survival (median survival 88.8 wks vs. 54.1 wks) compared to the 262 patients who had not been prescribed fish oil.(219) Whilst un-blinded and non-randomized, this study nevertheless demonstrated a clear survival advantage linked to ω-3 PUFA supplementation. One further study demonstrated improved tolerability of chemoradiation for oesophageal cancer in patients taking ω-3 PUFA supplements, with a reduction in the incidence of grade 2-4 neutropenia, diarrhoea and pharyngitis compared to those not taking supplements.(220)

A paper published in *Cancer Cell* found that endogenous mesenchymal stem cells (MSCs) can become activated during platinum-based chemotherapy treatment and secrete fatty acids that induce resistance to chemotherapy.(221) Mesenchymal

stem cells are known to be recruited to the stroma of tumours and help to mediate tumour growth, angiogenesis and metastasis by the secretion of growth factors and cytokines.(222-224) In this paper, MSCs activated by platinum-based chemotherapy were found to secrete two fatty acids, including the ω-3 fatty acid hexadeca-4,7,10,13-tetraenoic acid (16:4, ω-3), that abolished the effect of a variety of chemotherapy drugs on C26 (colon cancer cell line) and LLC (lung cancer cell line) tumours *in vivo.* These fatty acids were only secreted by MSCs exposed to platinum-based and not other types of chemotherapy drugs. Additionally, other PUFAs including EPA, did not confer resistance to chemotherapy drugs in this model. Inhibition of thromboxane synthase or COX-1, but not COX-2, prevented the MSC-induced resistance of chemotherapy. The two fatty acids implicated were subsequently identified in humans treated with platinum-based chemotherapy. Interestingly, 16:4 ω-3 is also found in commercially available fish oil products. Such fish oil products fed to mice also induced chemotherapy resistance. This study demonstrates an important new mechanism of chemotherapy resistance which is mediated by platinum-based chemotherapy activation of mesenchymal stem cells, via the production of two specific fatty acids, and suggests a possible negative effect of the use of mixed ω-3 PUFA supplements in patients being treated with platinum-based chemotherapies.

There is a clear need for further human studies to evaluate the role of ω-3 PUFA supplementation in improving the efficacy and/or tolerability of chemotherapy and radiotherapy. Two ongoing studies are investigating the combination of fish oil and chemotherapy in patients with advanced pancreatic cancer (clincialtrials.gov NCT01019382, and International Clinical Trials Registry Platform JPN-UMIN000003658) and one in advanced oesophagogastric cancer (clincialtrials.gov NCT01870791).

1.10 Omega-3 PUFAs for the prevention of colorectal cancer

1.10.1 Animal studies

The differential effect of ω-3 and ω-6 PUFAs on the prevention of CRC has been demonstrated in a number of animal models of early-stage colorectal carcinogenesis. Studies of rodents fed an ω-3 PUFA-supplemented diet *versus* an equivalent ω-6 PUFA-supplemented diet or low-fat control diet control have consistently reported a 20-50% reduction in chemically-induced tumour incidence,(161, 225-233) together with a 30-70% reduction in tumour multiplicity, in both carcinogen and $Apc^{Min/+}$ mouse studies (Table 1.5).(226, 227, 230, 232, 234-237) Studies using the number of aberrant crypt foci (ACFs) as the primary end-point have reported a similar magnitude effect with ω-3 PUFA supplementation (Table 1.5).(228, 231, 235, 238, 239) These effects are directly related to ω-3 PUFA supplementation rather than simply a reduction in ω-6 PUFA intake.(199, 230, 240) Whilst most *in vivo* studies have compared a mixture of EPA and DHA, attention is drawn to the few studies either directly comparing EPA and DHA (241), or using EPA (227, 232, 236) or DHA (237, 242, 243) as single agents. In general, similar results have been demonstrated with each of the two main ω-3 PUFAs. Analysis of mucosal PUFA content has consistently demonstrated incorporation of ω-3 PUFA, at the expense of AA content, in rodents supplemented with ω-3 PUFA compared to controls,(226, 227, 242) together with a reduction in mucosal PGE_2 ,(151, 227, 236, 241, 244) reduction in mucosal cell proliferation (225, 226, 238) and an increase in mucosal cell apoptosis (Table 1.5).(151, 161, 162, 238, 244).

Study	Model	N=	Treatment Groups	Timing/Duration	Outcome measure(s)	Results (maximal changes ω-3 PUFA group vs. Ctrl unless stated)
Carcinogen-induced models						
Nelson 1988 (234)	Sprague-Dawley rat DMH	50	17% MO_1 vs. 17% CO vs. Ctrl	7 wk pre- & 17 wk post-DMH	Tumour incidence & multiplicity Incidence of metastases	↔ tumour incidence 35% ↓ tumour multiplicty ↔ incidence of metastases
Minoura 1988 (227)	Donryu rat AOM	100	4.7% EPA vs. 5% LA	15 wk pre- & 20 wk post-AOM	Tumour incidence Tumour & colonic mucosal PUFA + PGE_2 content	50%↓tumour incidence, 75%↓multiplicity ↑ EPA & ↓ AA in tumour & mucosa 80%↓tumoural PGE_2
Reddy 1988 (245)	F344 rat AOM	234	4% - 12% MO_1 vs. 24% & 5% CO	38 wk post- AOM	Tumour incidence Tumour PUFA content	50% ↑ tumour incidence + multiplicity in 24% CO vs. all other grps ↑ tumoural EPA+DHA with MO diets
Deschner 1990 (225)	CF1 mouse AOM	300	4%-16% MaxEPA vs. 4% & 20% CO	2 wks pre- & 1-45 wk post- AOM	Colonic mucosa PI & FAD 1wk Tumour incidence 45 wk	Dose dependent ↓ FAD 38%↓ PI in 16% MO grp vs. 20% CO 50%↓ tumour incidence 16%/10% MO grp
Reddy 1991 (229)	F344 rat AOM	273	18% MO_2 vs 23.5% & 5% CO	2 wk pre- & 36 wk post-AOM. Diet crossover 3/7 post- AOM.	Tumour incidence	↓ tumour incidence and multiplicity when MO vs. 23.5% CO given in either initiation or post-initiation phases
Takahashi 1993 (242)	F344 rat DMH	97	0.7ml DHA vs 0.7ml H_2O ig daily	1 day pre- & 4/8/12 wk post-DMH	No. of colonic ACFs Serum chol & PUFA content	60% ↓ ACFs (↓ ACFs seen when DHA given in either initiation or post-initiation phase) 50%↓ AA, 50%↑ DHA & EPA, 20%↓ chol.
Hendrickse 1995 (226)	Wistar rat + colon anastomosis vs sham AOM	160	20% FO_1 vs. 20% CO	3 wk pre- & 15/23 wk post- AOM	Colonic mucosal PI Tumour incidence, size, no. Mucosal PUFA content	40%↓ tumour incidence, 50%↓ multiplicity 60%↓ peri-anastamotic tumours. ↓PI ↑EPA/DHA & 90%↓ AA in tumour/mucosa
Chang 1998 (161)	Sprague-Dawley rat AOM	260	11.5% FO_2 vs. 15% CO +/- 6% cellulose vs. 6% pectin	1 week pre- & 16/32 wk post- AOM	Colonic tumour incidence Crypt PI/AI/cell differentiation	20%↓ tumour incidence ↔ PI , 20%↑AI, ↑ cell differentiation ↑AI FO-pectin grp vs all other fat-fibre grps
Takahashi 1997 (243)	F344 rat AOM	96	1ml DHA vs 1ml water ig daily	4/12/36 wk	No. of ACF and tumours Plasma PUFAs & PGE_2	25%↓ ACF (wk 4/12) & 35%↓ tumour multiplicity, 50%↓ plasma PGE_2, 75%↓ plasma AA, 30x↑ EPA, 6x↑DHA (wk 36)

Study	Model	N=	Treatment Groups	Timing/Duration	Outcome measure(s)	Results (maximal changes ω-3 PUFA group vs. Ctrl unless stated)
Carcinogen-induced models (continued)						
Good 1998 (233)	F344 rats AOM	161	18% MO_3 vs. 5% & 23% CO	5% CO 12 wk post-injection then 6-12 wk experimental diet	No. ACF No. + size colonic tumours	15-20%↑ ACF vs. both CO grps 25%↓ tumour incidence but 50%↑ tumour size MO vs. CO grps (both NS)
Singh 1998 (230)	F344 rat AOM	144	21% FO_3 vs. 24% CO vs. Ctrl	1/12/36 wk post- AOM	Tumour incidence & multiplicity	FO grp 30%↓ tumour incidence & multiplicity. 23.5% CO grp 33%↑tumour incidence & 90% ↑multiplicity
Latham 1999 (238)	Wistar rat DMH	68	8% FO_4 vs. 8% CO	FO vs. CO 24/48hrs post-DMH then 18 wk CO	Crypt cell AI & PI (24/48hr) No. ACF (18wks)	↑ AI and ↓ PI at 24/48hrs 50% ↓ACF at 18 wk
Rao 2001 (228)	F344 rat AOM	360	17% FO_3 vs. 5% CO vs. 20% mixed lipids	CO 2 wk pre-AOM Experimental diets for 8-38 wk post-AOM	No. ACF, tumour incidence Colonic mucosa AI	44%↓ ACFs , 30%↓ tumour incidence, 60% ↓ tumour multiplicity & 2x ↑AI vs. 20% mixed lipid diet.
Crim 2008 (162)	Sprague-Dawley rat AOM	80	11.5% FO_2 vs 15% CO +/- 5% butyrate	3 wk pre- & 8 wk post-AOM	No. ACF, colonic mucosa AI,	↑ ACFs CO + butyrate vs all grps ↓ large ACFs and ↑AI in FO + butyrate grp vs. FO alone or control.
Vanamala 2008 (151)	Sprague-Dawley rat AOM	20	15% FO_5 + pectin vs. 15% CO + cellulose	32 days pre- & 31 wk post- AOM	Colonic mucosa AI Colonic mucosa PGE_2/PGE_3 β-catenin & PPARδ expression	2x↑ AI & 78%↓ mucosal PGE_2 ↑PGE_3 (PGE_3 not detected in CO group) ↓ β -catenin & PPARδ expression
Moreira 2009 (231)	Wistar rat DMH	20	18% FO_6 vs. 18% SOYO	2 wk pre- & 36 wk post-DMH	No. ACFs, adenoma incidence Colon/liver PUFA content	47%↓ ACF, 80%↓ adenoma incidence 5 x↑ ω-3-PUFA in colon + liver 60%-75%↓ n-6 PUFA in colon + liver
Woodworth 2010 (246)	*SMAD3*$^{-/-}$ mouse Helicobacter induced colitis	122	0.75% - 6% DHA vs. 6% SAFO vs. 7% CO vs. Ctrl	1. 8 wk pre- infection 2. 8 wk pre- & 4 wk post-infection	Colon inflammation/dysplasia Hepatic PUFA content Body wt.	↑ inflammation/dysplasia 2.25%-6% DHA vs Ctrl. 5x↑ hepatic DHA content, 85% less wt. gain & 10-18% ↓4wk survival in 6% DHA grp vs CO/SAFO/Ctrl
Burlamaqui 2012 (239)	Wistar rat AOM	36	8.2% lipid diet (4.5:3:1 ω-9:6:3) vs. 1.6% lipid diet (3.2:8:1 ω-9:6:3)	16wk pre- & 15wk post-AOM	No. ACFs Body wt.	12% ↑ body wt No difference in total no. ACFs 33% ↓ no. of ACFs with >4 crypts

Study	Model	N=	Treatment Groups	Timing/Duration	Outcome measure(s)	Results (maximal changes ω-3 PUFA group vs. Ctrl unless stated)
***Apc* mouse models**						
Oshima 1995 (237)	$Apc^{\Delta 716}$	20	3% DHA vs Ctrl	7wk	Colonic polyp no. and size	69%↓ polyp no. in females mice only ↔ polyp no. in male mice ↓ polyp size, more marked in females
Paulsen 1997 (235)	$Apc^{Min/+}$	51	0.4%-2.5% FO_7 vs 12% CO	17 wk	No. ACFs and adenomas	48-66%↓ no. and 26-38%↓ size of tumours ↓ACFs in female mice on 2.5% diet only.
Petrick 2000a (241)	$Apc^{Min/+}$	77	3.1% EPA vs. 3.1% DHA vs. Ctrl	7 wk	Tumour size + no. Mucosal PUFA + PGE_2	30%/50%↓ tumour no. DHA/EPA grp vs Ctrl 15%↓tumour size EPA/DHA grp vs Ctrl 50%↓ PGE_2 in EPA/DHA grp vs Ctrl
Petrick 2000b (236)	$Apc^{Min/+}$	20	1.5% EPA vs.1.5% AA vs. Ctrl	8 wk	Tumour size + no. Mucosal PUFA + PGE_2	54-68%↓ tumour no. & 18%↓ tumour size EPA vs. Ctrl & AA grps. 74%↓ mucosal PGE_2 EPA vs AA grp
Bose 2007 (244)	$Apc^{Min/+}$	95	12% MO_4 vs. 20% mixed lipid diet	9 wk	Tumour no. + size Tumour PI, AI, PGE_2, β-catenin	↔ tumour no, 50%↓ no. tumours >2cm 3.7x↑AI, ↔ PI, 89%↓ PGE_2, 62%↓β-catenin
Fini 2010 (247)	$Apc^{Min/+}$	48	2.5% & 5% EPA vs. Ctrl	12wk	Mucosal PUFA + COX-2 Polyp no. + size, body weight	72%/79%↓ polyp size (2.5%/5% EPA grp) ↓COX-2 & ↑ EPA in EPA grps, ↓weight Ctrl grp

Table 1.5 ***In vivo*** **pre-clinical studies of ω-3 PUFA supplementation for the prevention of CRC**

Abbreviations: ↔ = no significant difference, ↓ = decrease, ↑= increase, **ACF** = aberrant crypt foci, **AI** = apoptosis index, **AOM** = azoxymethane, **chol** = cholesterol, **CO** = corn oil, **Ctrl** = control, **DMH** = 1,2-dimethylhydrazine, **FAD** = focal area of dysplasia, **FO** = fish oil (FO_1= 18% EPA/15% DHA; FO_2= unspecified ω-3 PUFA content; FO_3 = 31% ω-3 PUFA; FO_4= 18% EPA/8% DHA; FO_5= 18%EPA/11%DHA; FO_6= 24%EPA/20%DHA; FO_7= 54% EPA/30% DHA), **ig** = intragastric, **LA** = linoleic acid, **MaxEPA**= 18%EPA + 12% DHA, **MO** =menhaden oil (MO_1 16% EPA + 11% DHA; MO_2 2.4% EPA + 11% DHA; MO_3= unspecified ω-3 PUFA content; MO_4 = 13%EPA/12%DHA), **PI** = proliferation index, **PPAR** = peroxisome proliferator-activated receptor, **SAFO** = safflower oil, **SOYO** = soybean oil, **wk** = weeks, **wt** = weight

1.10.2 Epidemiological observations

A link between dietary ω-3:ω-6 PUFA balance and CRC risk first emerged from epidemiological studies that observed reduced rates of CRC in Greenland and the Far East compared to Western populations.(248) Whilst the results of epidemiological studies have been variable, they have tended, in general, to report a small reduction in CRC risk with increasing dietary fish intake, a view supported in 2007 by The Second Expert Report into Food, Nutrition and the Prevention of Cancer, a meta-analysis by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR).(6) Interpretation of epidemiological studies has been hampered by heterogeneity in study design. The use of food questionnaires to record dietary intake is subjective, and does not always discriminate between oily fish such as sardines (high in ω-3 PUFAs) and lean fish such as cod (lower ω-3 PUFA content). Moreover, studies do not always discriminate between processed (smoked or salted) and non-processed fish, or the cooking method, which may confound observational data on CRC risk.(6, 249) Meta-analyses have also been hampered by the wide variability in both the frequency of fish intake and the choice of reporting measures (e.g. grams/day, portions/week, ω-3 PUFA g/day).(250)

Since the WCRF/AICR meta-analysis in 2007,(6) a prospective study of 53,988 patients found no association between fish consumption and CRC risk, but did find a risk reduction with increased fish consumption in patients who also had low fibre consumption (OR 0.77).(251) A systematic review of studies published since 2007 found a risk reduction for CRC between the highest and lowest fish consuming groups in all three case control studies identified (total n=6357 patients, OR 0.58-0.74) but mixed results in 6 prospective studies identified.(252) Finally, a meta-analysis of 41 studies published between 1990 and 2011 (total n=1,454,578 patients) found a significant reduction in CRC risk between the highest and lowest fish consumption groups in 19 case control studies (OR 0.83) and a slight reduction

in CRC risk between the highest and lowest fish consumption groups in 21 prospective cohort studies (OR 0.93).(253) The results of these more recent studies therefore continue to suggest a possible beneficial effect of ω-3 PUFA consumption, but the conflicting results and significant heterogeneity between the studies makes it difficult to draw any firm conclusions.

1.10.3 Translational studies of ω-3 PUFAs and CRC biomarkers.

The long natural history of colorectal carcinogenesis in humans precludes the use of CRC incidence as a primary endpoint in clinical intervention studies. Therefore many Investigators have measured the effect of ω-3 PUFA administration on putative mucosal biomarkers of future CRC risk, such as epithelial cell mitosis frequency in micro-dissected whole crypts or immunohistochemistry (IHC) for the Ki-67 'proliferation' antigen. The design of such studies and their main findings are summarised in Table 1.6. 8 studies of oral ω-3 PUFA supplementation in patients with previous 'sporadic' colorectal adenomas were identified (Table 1.6), in which colorectal mucosal biopsies were obtained at endoscopy before and after ω-3 PUFA supplementation. In 6 of 8 studies a 13-70% reduction in mucosal epithelial cell proliferation index (PI) was observed compared to the respective placebo group.(232, 254-258) One further study noted a more modest 16% reduction in PI after 28 days supplementation in healthy volunteers.(259) By contrast, two studies demonstrated no change in PI following administration of 2.4 g ω-3 PUFA daily for 12 weeks (260) or low-dose ω-3 PUFA (400 mg DHA + 100 mg EPA/day) for 1-2 years.(261) The latter study did demonstrate a 50% increase in apoptosis index (AI) and increased expression of the pro-apoptotic protein BAX. AI has been measured in only two other studies, which demonstrated a significant increase in AI after 3-6 months treatment with EPA 2g daily.(232, 257) In those studies measuring mucosal PUFA content, significant increases in mucosal DHA and EPA, together with a reduction in mucosal AA, were observed in all but one study (Table 1.6).

The development of colonic adenomas (polyps) is an alternative to mucosal biomarker studies as a surrogate for CRC risk. Such polyp prevention studies typically require a 3-5 yr intervention and follow-up period. However, patients with FAP have a heterozygous germline mutation in the *APC* gene. These patients develop multiple colorectal adenomas at a young age and prophylactic colectomy is advised in order to prevent CRC. Those who undergo total colectomy with ileorectal anastomosis rather than panproctocolectomy (total colectomy + removal of rectum) require regular endoscopic surveillance of the remaining rectum which remains at risk of developing polyps. Studies of potential chemopreventative agents in these patients allow polyp size and number to be used as endpoints over a much shorter period of time (6-12 months). A recent Phase III randomised, double-blind, placebo-controlled trial investigated treatment with EPA in the free fatty acid form (EPA-FFA) 2g daily for 6 months in patients (n=58) with FAP who had previously undergone colectomy and ileorectal anastomosis (Table 1.6).(262) Rectal polyp multiplicity and size were measured by blinded video-endoscopic assessment of a tattooed area at baseline and at 6 months. There was a 22.4% reduction in polyp number in the EPA group compared with placebo (p=0.01), a similar magnitude reduction to that seen with the selective COX-2 inhibitor celecoxib.(131) In keeping with previous studies, a significant increase in mucosal EPA levels was observed. The demonstration of chemopreventative efficacy of EPA-FFA in FAP patients has led to funding of a randomised, placebo-controlled trial of EPA-FFA in patients who have had “sporadic” colorectal adenomas removed and who require further colonoscopic surveillance (www.eme.ac.uk/projectfiles/0910025info.pdf).

Only one other polyp prevention study was identified (Table 1.6).(263) In this small study, 5 patients who had previously undergone colectomy for FAP were given 2.2g DHA + 0.6 g EPA daily for 1-2 years.(263) No significant change in polyp number was observed. One patient developed proximal CRC, a second patient developed

lung cancer and a third patient developed endometrial cancer prior to termination of the study. The published report does not make it clear whether this study was terminated prematurely and gives no indication of the planned sample size for the trial.

Study	Design	N=	ω-3 PUFA dose	Treatment Duration	Primary outcome	Tissue PUFA content	Side effects (at highest given dose)	Results
Mucosal biomarker studies								
Anti 1992 (255)	R, DB, PC 'sporadic' adenoma	24	7.7g FO_1 daily	12 wk	PI	↑EPA & ↓AA	Not reported. No dropouts in FO grp.	62% ↓PI
Bartoli 1993 (256)	R, DB, PC 'sporadic' adenoma	40	2.5-7.7g FO_1 daily	30 days	PI	Dose dependent ↑EPA/DHA & ↓AA	Not reported.	Dose dependent 40-70%↓ PI
Bartram 1993 (259)	DB crossover trial Healthy volunteer	12	4.4g FO_2 daily	4wk +4 wk	PI	ω-3 PUFA↔ ω-6 PUFA↓(NS)	Mild fish odour (9/12)	16%↓ PI & 35%↓ mucosal PGE_2
Anti 1994 (254)	R, DB, PC 'sporadic' adenoma	60	2.5-7.7g FO_1 daily	30 days	PI	Dose dependent ↑EPA/DHA & ↓AA	2/15 dropout, 5/15 fish odour, 1/7 diarrhoea	Dose independent 50-70%↓PI
Huang 1996 (258)	R, DB, PC Dukes A/B CRC or severely dysplastic polyp	27	7.2g FO_3 daily	6 months	PI	↑EPA/DHA & ↓AA	No SEs observed	71%↓PI (only in patients with high baseline PI)
Gee 1999 (260)	R, PC, single blind Awaiting CRC surgery	51	2.4g FO_4 daily	7-21 days pre- and 8-12 wk post- surgery	PI	↑EPA/DHA ↑ ω-3 : ω-6 ratio	1 dropout due to capsule intolerance (grp not stated)	No effect on PI at surgery or 12wk post-op
Cheng 2003 (261)	R, C, open label Previous CRC/adenoma	41	Dietary advice +- 500mg FO_5 daily	2 years	PI/AI	Not assessed	Not reported	PI↔, 50%↑AI, 50%↑ Bax, COX2 ↔
Courtney 2007 (264)	R, single blind 'sporadic' adenoma	30	EPA 2g daily as free fatty acid	3 months	PI/AI	↑EPA/DHA & ↓AA	1/15 dropout each grp 3/15 mild diarrhoea + 2/15 abdo pain	20%↓PI 7x↑ AI
West 2009 (232)	R, DB, PC 'sporadic' adenoma	152	EPA 1g/2g daily as free fatty acid	6 months	PI/AI	↑EPA/DHA & ↓AA	Not reported	13%↓PI 57%↑ AI (NS)

Study	Design	N=	ω-3 PUFA dose	Treatment Duration	Primary outcome	Tissue PUFA content	Side effects (at highest given dose)	Results
Polyp endpoint studies								
Akedo 1998 (263)	Open label FAP	5	Dietary advice & 2.8g FO_6 daily	1-2 years ?trial stopped early	Polyp no.	Not assessed	1x diarrhoea 1x itching 1x hypermenorrhoea	Polyp no. ↔ 1 x CRC, 1x lung Ca 1x endometrial Ca
West 2010 (262)	R, DB, PC FAP	58	EPA 2g as free fatty acid	6 months	No. rectal polyps	↑EPA DHA & AA↔	↑nausea EPA grp (31% vs 10%). Other SEs ↔	22%↓ polyp no. 30%↓ polyp size

Table 1.6. Clinical studies of ω-3 PUFA treatment on colorectal mucosa biomarkers and polyp number

Abbreviations: ↑ = increase, ↓= decrease, ↔ = no change, **abdo** = abdominal, **AI** = apoptosis index, **Ca** = cancer, **C**= controlled, **DB** = double blind, **FAP** = familial adenomatous polyposis, **FO**= fish oil (FO_1 = 54%EPA/46% DHA as ethyl esters; FO_2 = 48%EPA/44%DHA, as triglycerides; FO_3 = 55%EPA/30%DHA/15% other ω-3 PUFAs; FO_4=58% EPA/42%DHA; FO_5 = 20%EPA/80%DHA; FO_6 = 21%EPA/79%DHA), **grp** = group, **mo** = month, **NS** = not statistically significant, **PC** = placebo controlled, **PI** = proliferation index,**R** = randomized, **SE** = side effects, **wk** = weeks

1.11 Summary and context for the thesis

Recent advances in neoadjuvant chemotherapy and the surgical management of CRCLM have successfully increased the number of patients suitable for potentially curative surgery. However, we may now be at the limit of our definition of resectable disease and advances in adjuvant chemotherapeutic agents such as the new monoclonal antibody therapies have demonstrated only very modest survival advantages in patients with CRCLM. At the same time, the use of COX-2 inhibitors and other NSAIDs for the chemoprevention of CRC has been limited by the recognition of cardiovascular and gastrointestinal side effects associated with long term use (although the balance of risk-benefit may favour the use of aspirin for prevention of CRC).(142) There is therefore an urgent need for novel, safe agents for the prevention and treatment of CRC and CRCLM. Accumulating experimental evidence suggests that ω-3 PUFAs such as EPA have anti-CRC activity, and clinical trial data for ω-3 PUFAs has recently been published from mucosal biomarker and polyp prevention studies. This thesis presents the first clinical trial of oral EPA supplementation for the treatment of patients with CRCLM, together with supplementary *in vitro* studies of the effect of EPA on angiogenesis.

Chapter 2: Aims and hypotheses to be tested

The primary aim of this research is to investigate the effect of EPA on biomarkers of growth and vascularity of human CRCLM in a Phase II randomised, double-blind, placebo-controlled trial of EPA in patients awaiting surgery for CRCLM. The following specific hypotheses will be tested.

Treatment with EPA:

1. Is safe and well tolerated in patients with CRCLM
2. Does not inhibit platelet aggregation, and does not cause an increased risk of bleeding during liver resection or other post-operative complications
3. Is associated with reduced tumour cell proliferation compared with placebo
4. Is associated with increased tumour cell apoptosis compared with placebo
5. Is associated with reduced tumour microvessel density compared with placebo
6. Leads to an increase in tumour EPA content
7. Is associated with a reduction in intra-tumoural PGE_2 and an increase in PGE_3 levels
8. Is associated with a reduction in urinary PGE-M levels
9. Causes a reduction in active transcription factor NFκB levels in peripheral blood mononuclear cells

A secondary aim of this research is to investigate the effect of EPA on human endothelial cells *in vitro*. The following specific hypotheses will be tested:

10. EPA inhibits angiogenesis *in vitro*

Chapter 3: The EMT Trial

3.1 Introduction

The EPA for Metastasis Treatment (EMT Trial) was a Phase II randomised, placebo-controlled, double-blind trial of the safety and efficacy of EPA 2g daily in patients awaiting surgery for CRCLM. It was the first clinical trial of the anti-neoplastic activity of an oral ω-3 PUFA in patients with CRCLM. The EMT Trial was sponsored by The University of Leeds, and was a collaboration between The University of Leeds and the Leeds Teaching Hospitals NHS Trust Department of Hepatobiliary Surgery, based at St James' University Hospital, Leeds, UK. The Trial was been adopted onto the National Institute of Health Research Clinical Research Network Portfolio.

(http://public.ukcrn.org.uk/Search/StudyDetail.aspx?StudyID=8946).

3.2 Regulatory approvals

The EMT Trial was granted regulatory approval from the NHS Research Ethics Committee (REC), the Medicines and Healthcare related products Regulatory Authority (MHRA) and the Leeds Teaching Hospitals NHS Trust Research and Development (R&D) office. These approvals were all obtained prior to commencement of trial recruitment (Table 3.1). The trial was registered on clincialtrials.gov, a publically accessible database of clinical trials, prior to commencing recruitment.

(http://clinicaltrials.gov/ct2/show/NCT01070355?term=EPA&rank=12).

	Reference number	Approval date
EUDRACT no.	2009-015903-22	07/09/2009
Leeds (West) Research Ethics Committee	09/H1307/94	20/10/2009
MHRA	16767/0240/001-0001	15/12/2009
Leeds NHS Trust R&D	GA09/9094	09/02/2010
Clinicaltrials.gov	NCT01070355	12/02/2010

Table 3.1. A summary of the regulatory approvals for The EMT Trial

3.3 Trial Steering Committee

An internal Trial Steering Committee (TSC) met quarterly to review trial progress, and received input from an external Data Monitor on a quarterly basis. The TSC included:

Prof Chris Twelves	Independent Chair (Academic Clinical Oncologist)
Prof Mark Hull	Professor of Molecular Gastroenterology
Mr Giles Toogood	Consultant Hepatobiliary & Transplant Surgeon
Mr Andrew Cockbain	Research Fellow
Mr Richard Maltby	Patient Representative

3.4 Data Monitoring Committee

The Trial was subject to review from the University of Leeds and Leeds Teaching Hospitals NHS Trust joint Data Monitoring Committee.

There have been some concerns about an anti-platelet activity and possible increased risk of bleeding with ω-3 PUFA consumption,(265-267) however, no significant bleeding episodes have been seen in over 4000 clinical trial patients undergoing invasive interventions or operations whilst taking ω-3 fish oil

supplementation alone or in combination with aspirin.(268) Similarly, other clinical trials have shown that ω-3 PUFAs are safe even when given with conventional anti-platelet or anticoagulant treatments.(269) Nevertheless, in this first trial of an oral ω-3 PUFA supplement in patients undergoing liver resection, the potential for a small risk of increased bleeding during surgery could not be excluded. Therefore, patient bleeding complications were proactively and prospectively recorded on a Peri-operative Outcome Form (POF), namely the number of units of blood components transfused and whether the patient required reoperation for bleeding. An independent Data Monitor, who was unblinded to patient allocation, reviewed POFs every 3 months and was asked specifically to feed back to the TSC if the number of "significant bleeding events" (defined as a packed red blood cell transfusion greater than 2 units or reoperation for bleeding) in the EPA group exceeded that in the placebo group by 5 at any time, so that the TSC could consider stopping the Trial.

3.5 Trial Subject Selection

3.5.1 Eligibility Criteria

Patients undergoing liver resection for treatment of CRC liver metastasis(es)

Inclusion Criteria

(a) Age greater than or equal to 18 years

(b) Either sex

(c) Liver resection deemed clinically appropriate for management of metastatic CRC liver disease

(d) Duration between decision to perform liver resection and surgery expected to be greater than 2 weeks

(e) Ability to give written informed consent and follow study protocol

(f) Telephone contact possible

Exclusion Criteria

(a) Neo-adjuvant chemotherapy for CRC liver metastasis

(b) Chemotherapy for any cancer in the previous 3 months

(c) Known bleeding diathesis or anticoagulation therapy

(d) Fish or seafood allergy

(e) Use of fish oil supplements (e.g. cod liver oil) and unwilling to stop for the duration of the study

(f) Pregnancy

(g) Use of non-aspirin non-steroidal anti-inflammatories (NSAIDs) or use of systemic steroids (i.e. oral or intravenous preparations)

(h) Renal impairment (serum creatinine >150µg/l)

(i) Active inflammatory disease (e.g. inflammatory bowel disease, rheumatoid arthritis)

3.5.2 Recruitment

Patients with suspected or confirmed CRCLM are referred to the Department of Hepatobiliary Surgery at St James's University Hospital by General Practitioners, Oncologists and Colorectal Consultants from across the Yorkshire region. A number of patients are also referred to the Department from outside the region. All referral letters were screened prior to each clinic to identify patients who may be eligible for participation in The EMT Trial. Patients were seen in the surgical outpatient clinic by a Consultant Hepatobiliary Surgeon, and a decision made whether surgical resection of their CRCLM was appropriate. At the same clinic appointment, all patients who were offered liver resection were then approached to discuss participation in the EMT Trial, unless pre-screening of their referral letter clearly identified that they meet one or more of the trial exclusion criteria. Patients were given a verbal explanation of the trial and a copy of the Patient Information Leaflet. This included detailed information about the rationale, design and personal implications of the study.

3.5.3 Consent

Patients referred to the Hepatobiliary department at St James' University Hospital have often travelled considerable distance to attend their outpatient clinic, sometimes taking 2-3hrs each way. It was decided that it would be both impractical and an undue burden on the patient to discuss the study on one visit and then return the following day after a cooling off period for a second appointment to sign the consent form (they would not routinely have a second appointment before being admitted a few weeks later for surgery). A pragmatic approach was therefore adopted. After approaching patients and discussing participation in the Trial, patients were given a period of time (at least 1 hour) to read the patient information sheet and consider participation. Since these patients usually attend outpatient clinics with their family members, this gave patients the opportunity to discuss the trial with their family. After 1 hour, or when patients returned to the outpatient department, they were asked if they had had enough time to consider the information provided and address any further questions. Only if patients were sure that they have had been given sufficient time to consider participation, and had the opportunity to discuss with their family members if desired, were they asked if they were willing to take part in the trial. This process was clearly documented in the patients' medical notes.

Patients who wished to participate in the Trial were then formally assessed for eligibility and invited to provide informed, written consent. The right of patients to refuse consent without giving reasons was explained and respected. Patients were informed that they could also withdraw from the study at any time without giving reasons and without prejudicing any further treatment. One copy of the consent was given to the patient, a second filed in the Trial Master File, a third filed in the hospital notes and a fourth sent to the Sponsor. The consenting process was clearly documented in the patient's medical notes.

3.6 Randomisation and blinding

Consented patients were randomised 1:1 EPA-FFA:placebo by Leeds Teaching Hospitals Trust Pharmacy in a random permutated block of 4 using random number tables. The blind was held by Leeds Teaching Hospitals NHS Trust Pharmacy. Unblinding only occurred after all patients have completed follow up, and all collected samples had been analysed. Unblinding before the end of the study was only permitted if deemed necessary by the Chief Investigator and the Sponsor in the event of a patient safety issue. This situation did not arise.

3.7 Trial Medication

EPA-FFA and placebo were manufactured in Germany on behalf of SLA Pharma (UK). SLA Pharma (UK) supplied EPA and placebo free of charge for The EMT Trial. EPA was presented as an enteric-coated soft blue gelatin capsule containing 500mg of 99% pure EPA in the free fatty acid form. Placebo capsules were identical in form, except for the replacement of EPA with the medium chain triglycerides capric and caprylic acid. These were the same preparations used in the Phase III polyp prevention study in patients with FAP.(262) EPA is unlicensed for the treatment of CRCLM. It was therefore considered an Investigative Medicinal Product (IMP) for the purpose of this trial, and approval for use in this trial was obtained from the MHRA. Supplies of EPA and placebo were repackaged, labelled, stored and dispensed by Leeds Teaching Hospitals NHS Trust Clinical Trials Pharmacy in accordance with Good Clinical Practice (GCP).

3.8 Trial design and patient throughput

The trial design is outlined in Figure 3.1 and patient flow through the trial is described below.

Study visit 1 (routine out-patient appointment)

Immediately after a decision was made to offer surgery for CRCLM, patients were seen for trial screening and enrolment. Randomisation occurred immediately after consent had been obtained. Blood and urine samples were taken, and participants asked to complete the EPIC food frequency questionnaire (FFQ; Appendix 1) in order to determine dietary ω-3 PUFA intake.(270) Participants received study medication at the same visit. Patients took EPA 2g, or placebo, daily (as two capsules twice daily with food) until the day before surgery (2-4 weeks). Patients were followed up by telephone every 2 weeks to monitor for adverse effects and maximise compliance.

Study visit 2 (admission for surgery)

Patients were admitted to hospital either the day before or on the day of their operation and asked again about symptoms or side effects from medication. Blood and urine sampling were repeated, and the FFQ repeated to determine whether there had been any change in dietary ω-3 PUFA intake during the intervention period. Patients were asked to return all study medication for pill counting. CRCLM tissue was collected from the resected liver specimen immediately after resection. Patients were then managed according to Leeds Hepatobiliary Unit protocol during the post-operative phase.

Study visit 3 (routine post-operative out-patient appointment)

At the first routine post-operative outpatient appointment, approximately 6 weeks following discharge, a final blood and urine sample were taken. This marked the end of participation in the Trial. Patients then continued routine follow-up under the care of their Consultant Surgeon.

STUDY FLOW CHART

Interventions performed on each study day are listed in BLOCK CAPITALS below

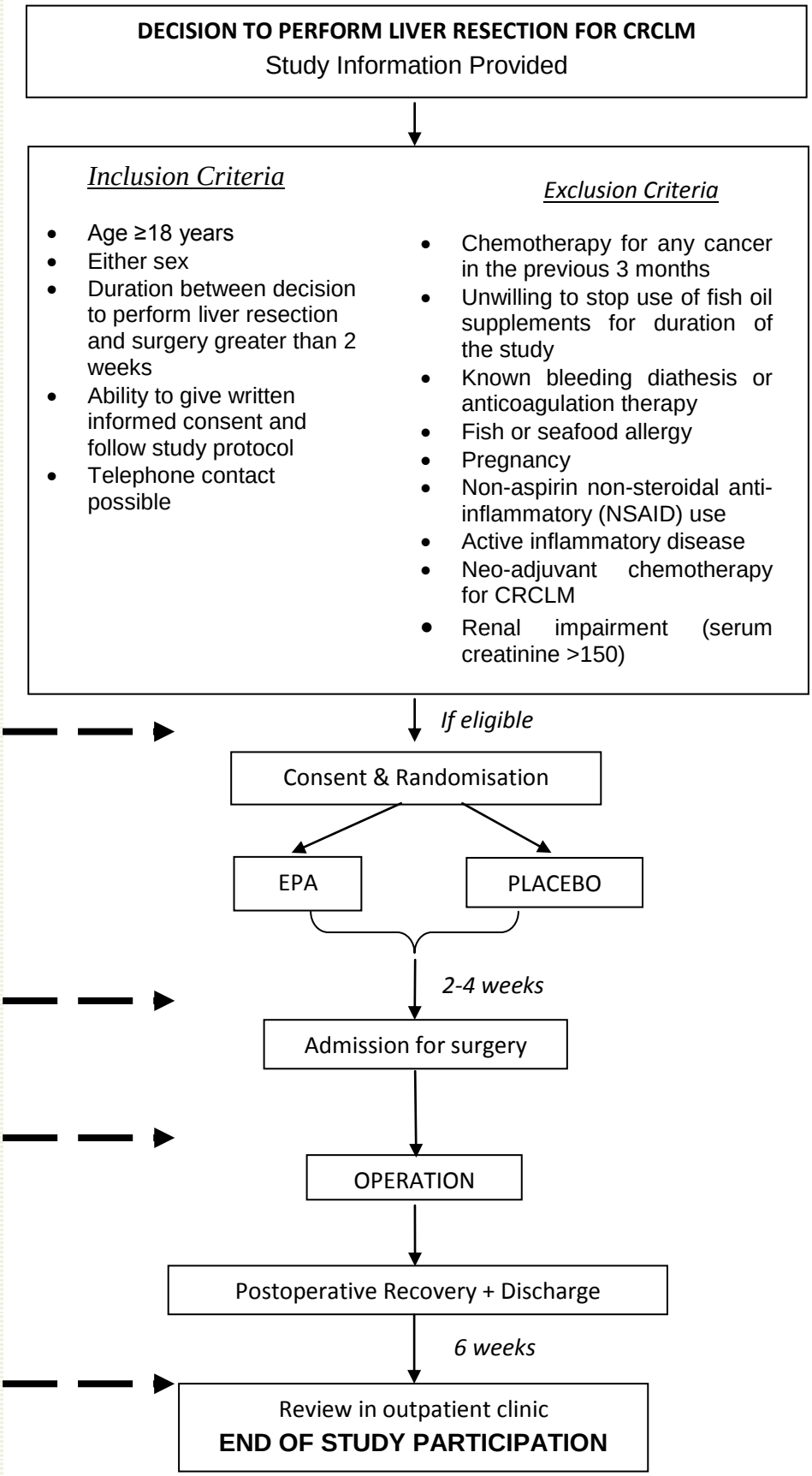


Figure 3.1. Flow chart of patient participation in The EMT Trial

3.9 Power calculation and sample size

There are no existing data on the effects of EPA on tumour cell proliferation in human CRCLM. In a previous double-blind RCT of CRCLM patients (271), the Ki-67 proliferation index (PI) in the placebo arm was 50 (mean) ± 22 (SD). From *in vitro* and *in vivo* studies of the effect of EPA on CRC cells, a 30% decrease in PI compared with placebo was predicted.(186, 199) Therefore a minimum of 35 patients in each of the 2 arms of the study (total 70 patients) was required to detect a 30% difference in PI between the groups with 80% power and a 5% 2-sided significance level. To allow for 20% drop out, a target recruitment of 44 patients in each arm (total 88 patients) was set.

The Leeds Hepatobiliary Unit performs approximately 200 liver resections for metastatic CRC per year, of which up to 20% receive neo-adjuvant chemotherapy (an exclusion criterion). Allowing for up to 45% ineligibility in the remaining 160 patients (either do not fulfil inclusion criteria or do not provide informed consent to enter the trial), recruitment of 88 eligible patients was predicted to take 1 calendar year. The recruitment rate was monitored monthly.

3.10 Design issues

Being unable to standardize the duration of study medication was an unavoidable consequence of ensuring that study participation did not delay patients' surgery. However, Leeds Hepatobiliary Unit data (unpublished) showed that the median time from decision to operate to surgery was 4 weeks and, importantly, the maximal increase in tissue and erythrocyte EPA content associated with oral EPA intake has been shown to occur over a similar time period.(272, 273) A variable pre-surgery treatment period was also a feature of the previous RCT of rofecoxib (271) when randomisation ensured well-balanced active and placebo groups. The variability in

treatment period was therefore considered to be an acceptable limitation in study design.

A second limitation of the study design is that there was no baseline (pre-treatment) tumour available for comparison with the post-treatment tumour samples. This is because pre-operative biopsy of CRCLMs is contra-indicated due to the risk of seeding tumour cells along the biopsy tract.(274, 275) Suspected CRCLMs are diagnosed radiologically and patients proceed to surgery based on this radiological diagnosis. Biopsy is reserved only for the small proportion of liver lesions which are of uncertain radiological diagnosis or of unknown origin, where tissue diagnosis may alter a patient's management. To obtain pre-treatment tumour samples in this trial would have been contrary to the accepted management of CRCLM, and unethical due to the risk of tumour seeding. The lack of pre-treatment tumour samples was therefore considered to be an unavoidable but acceptable limitation in study design.

3.11 Statistical analysis

For continuous variables, the difference in means (or medians where appropriate) between the EPA and placebo were compared, and significance tested by parametric (such as Student's t test) and non-parametric tests (such as the Mann-Whitney U test and Wilcoxon rank sum test). For outcomes that were measured serially, e.g. PGE-M, changes within a treatment group over time were compared using paired statistical tests, and differences between the two treatment groups at each time point were compared using unpaired statistical tests. Categorical data was compared using the χ^2 test. Due to the limitation of being unable to sample tumours at baseline, no comparison of tumour tissue pre- and post- treatment could be made. The effect of EPA on tumour tissue parameters was therefore evaluated by comparison of the EPA and placebo groups post-treatment only. In addition, the

effect of variability in the duration of treatment on each outcome measure was investigated using linear regression. Statistically significance was set at $p<0.05$ for all comparisons.

Chapter 4: Laboratory Methods

4.1 Sample collection and storage

4.1.1 Blood sampling

All patients were consented at recruitment into the trial for blood sampling on each of the three trial visits. On each occasion, venepuncture was performed aseptically using a 21G needle and vacuette® device. Venous blood was collected into the following sample tubes, and stored at either room temperature or 4^{o}C on crushed ice until further processing within 3 hrs:

- 2x 4ml EDTA coated vacuette® collection tubes (Greiner Bio-One, Kremsmunster, Austria) for separation of plasma and red cells. Stored on crushed ice.
- 1x Hirudin (25µg/ml) coated collection tube (Dynabyte, Munich, Germany) for platelet aggregation. Stored at room temperature.
- 4x 8ml BD Vacutainer® CPT™ collection tubes (BD, New Jersey, USA) for isolation of peripheral blood mononuclear cells. Stored at room temperature.

Separation of plasma and red cells: EDTA collection tubes were centrifuged at 700 x g for 10mins at 4 ^{o}C within 3 hours of venepuncture. A plastic Pasteur pipette was used to remove the plasma layer which was aliquoted into cryogenic vials. The buffy coat was discarded and the red blood cells aliquoted into cryogenic vials. Samples were stored at -80^{o}C for future analysis of i) fatty acid content of red cell membranes by gas chromatography – mass spectrometry (GC-MS) and ii) plasma PGE_2 and PGE_3 levels by liquid chromatography – tandem mass spectrometry (LC-MS/MS). These analyses have not been performed due to time and funding constraints. Samples remain stored for possible analysis at a later date.

4.1.2 Urine sampling

Patients were asked to provide a fresh urine sample in a sterile 30ml universal container at each study visit. Specimens were stored at 4^{o}C on crushed ice prior to aliquoting into cryogenic vials and storage at -80^{o}C for subsequent batch analysis of urinary PGE-M by LC-MS/MS.

4.1.3 Tumour sampling

Immediately after resection of the specimen containing the CRCLM, the specimen was passed out of the sterile surgical field and dissected with care not to disturb the resection margin (Figure 4.1). The tumour was palpated and a perpendicular incision made through the centre of the tumour. A wedge of tumour tissue incorporating normal liver margin and at least 1cm of tumour directed towards centre of the tumour was taken and placed in formalin (10% v/v). The orientation of the excised wedge from tumour margin to tumour centre was confirmed by making radial incisions in the remaining tumour, radiating from the deepest extent of excised wedge back out to the tumour margin in at least 2 opposing directions. A further 6-8 x 5mm cube-shaped samples of tumour were taken from an area 5mm from the macroscopic tumour margin, with care not to include any normal liver, and placed in a universal container for storage at 4^{o}C on crushed ice. The remaining resected liver specimen was placed in formalin (10% v/v) and sent to the pathology department for routine histopathological assessment, as per standard NHS protocols.

Tumour specimens were returned immediately to the laboratory. Two cube shaped tumour samples were mounted in Cryo-M-Bed (Bright Instruments, Huntington, UK) on corks, snap frozen in isopentane cooled in liquid nitrogen, and stored at -80^{o}C until subsequent analysis of tumour COX expression. The remaining cube-shaped tumour samples were placed in individual cryogenic vials, snap frozen in liquid

nitrogen, and stored at -80^{o}C until subsequent analysis of tumour prostaglandins and fatty acid content.

The wedge of liver was removed from formalin after 24 hours, washed twice in phosphate buffered saline (PBS) and stored in 70% (v/v) ethanol before processing in an automated tissue processor and embedding in paraffin. Blocks were stored at room temperature for future immunohistochemical analysis.

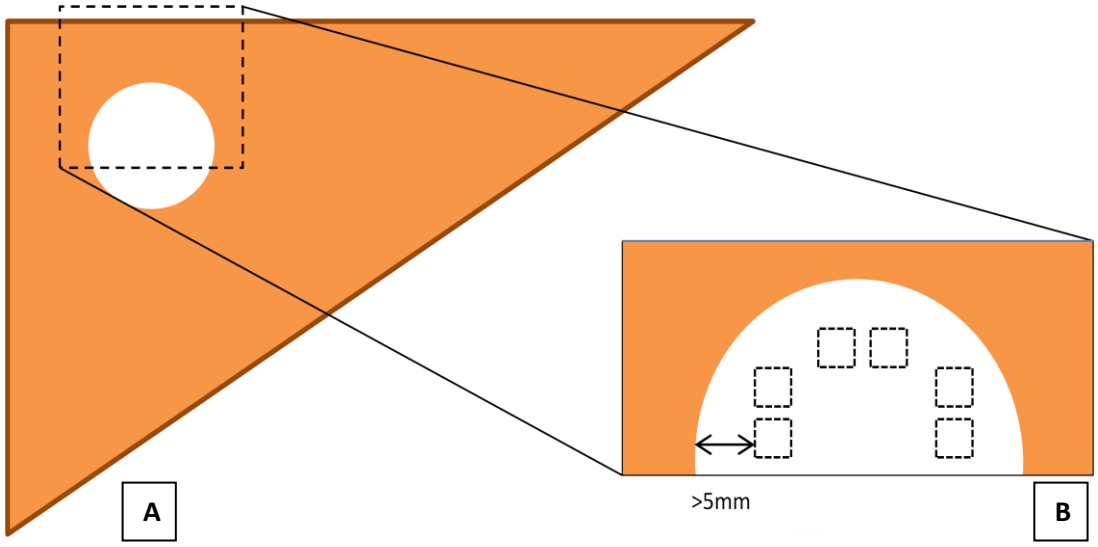


Figure 4.1. Tumour sampling technique. Dotted lines indicate area of sampling. The tumour was palpated within the liver parenchyma, and a perpendicular incision made through the liver capsule and liver parenchyma into the liver tumour. Having identified and incised the tumour with this first incision, the tumour was inspected and re-palpated to confirm that the tumour had been incised through its centre. Additional incisions radially out of the tumour were made when required to confirm that the centre of the tumour had been incised. **(A)** Having identified the centre of the tumour, a 2-3mm section orientated through the liver capsule, liver parenchyma and centre of the tumour was obtained for formalin fixation. **(B)** Secondly 6-8 cubes of tissue (at least 5x5x5mm) were taken from the remaining tumour, at least 5mm from the tumour edge, and snap frozen in liquid nitrogen.

4.2 Platelet aggregation studies

Overview:

There is no consensus regarding which *ex vivo* platelet function assay is best suited to measuring platelet function,(276-278) let alone which is best suited to measuring the effect of ω-3 PUFAs. Most work has been in the cardiovascular and anaesthetic fields looking at detection of 'aspirin resistance'. A summary of different platelet aggregation assays can be found in these two reviews.(279, 280) Whole blood platelet aggregation studies in this study were performed using the Multiplate® (Dynabyte, Munich, Germany) whole blood platelet aggregometer. Whole blood is added to a test cell containing two pairs of silver coated sensor wires. The addition of an agonist stimulates platelets to aggregate on the wires, causing an increase in electrical resistance measured across the wires (Figure 4.2). The change in resistance over time is a function of platelet aggregation, which when plotted and "aggregation" measured as the area under the curve.

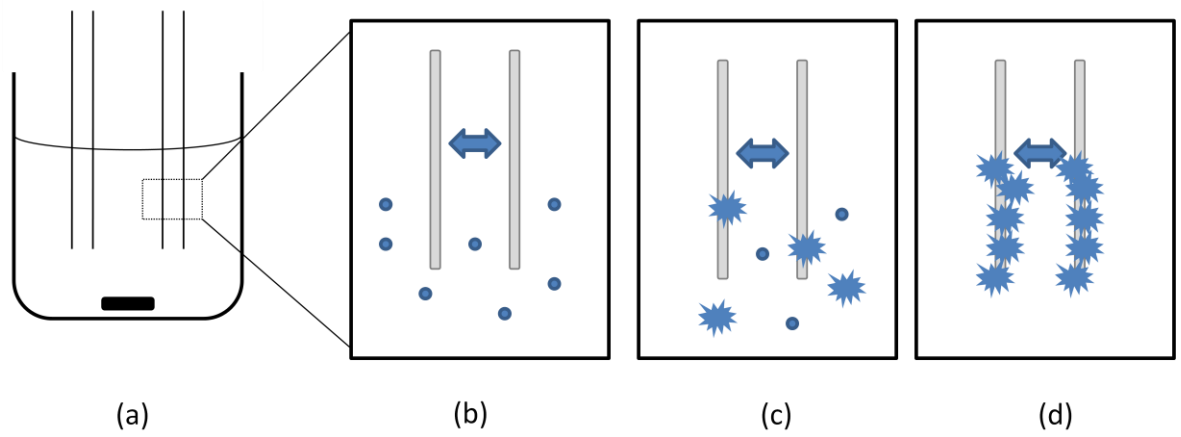


Figure 4.2 Schematic diagram of Multiplate® test cell. Each cell contains two pairs of sensor wires and a stirring magnet (a). Whole blood is added (b) and addition of agonist stimulates platelets (c), causing aggregation of platelets on sensor wires (d).

This has the advantage over the traditional gold-standard of light transmission aggregometry (LTA) of platelet-rich plasma in that it is less time-consuming, less time-sensitive, and does not require a highly-trained operator. Multiplate® has been shown to be as effective as LTA in detecting aspirin- and clopidogrel- induced inhibition of platelet aggregation.(281)

Similarly, there are a range of different agonists which can be used to stimulate platelet aggregation in both LTA and whole blood platelet aggregation assays. AA is typically used as the agonist when testing for the anti-platelet effect of aspirin. Acetylated COX does not metabolise AA to thromboxane A2 and therefore platelet aggregation is reduced. Other commonly used agonists are adenosine diphosphate (ADP) and collagen. ADP binds to the P2RY12 cell surface receptor, a G-protein coupled receptor which activates the glycoproteinIIb/IIIa complex and induces platelet binding to fibrinogen which is the major cofactor in platelet aggregation. Collagen stimulates platelet aggregation upstream of AA metabolism by COX, this being the first step in platelet activation *in vivo* when a platelet adheres to exposed subendothelial collagen after vessel wall injury. Amongst other effects, this results in liberation of AA from the cell membrane by phospholipase A2 and also stimulates the release of platelet endogenous ADP. A more comprehensive discussion of the different agonists can be found in these two reviews.(282, 283) In the absence of consensus on the precise mechanism by which EPA might inhibit platelet aggregation, or on the optimal agonist for detecting platelet inhibition by EPA, in this study Multiplate® whole blood platelet aggregation in response to AA, ADP, low dose collagen and high dose collagen was investigated.

Method: 0.3ml 0.9% saline + 0.3ml hirudin-anticoagulated whole blood were added to each of four test cells and left to incubate for 3 minutes at 37^{o}C with a PTFE coated stirring magnet. 20µl of each agonist (arachidonic acid 0.5mM,

adenosine diphosphate 6.5µM, collagen 3.2µg/ml, or collagen 0.64µg/ml) was added to respective test cells, and the electrical resistance across duplicate pairs of silver coated sensor wires was continuously measured for 6 minutes. Resistance is automatically transformed into arbitrary "aggregation units" and a plot of aggregation over time was generated, with maximal resistance, velocity and area under the curve recorded.

4.3 Peripheral blood mononuclear cell studies

Overview: Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and cultured for 24 hours with and without lipopolysaccharide (LPS) stimulation. Cell conditioned media was collected and frozen for future analysis of PGE_2/PGE_3 content. PBMCs were then either frozen for future COX RNA analysis, or further processed to obtain nuclear extract which was then frozen for subsequent batch analysis of nuclear NFκB activation.

4.3.1 PBMC culture

Blood collected in CPT™ tubes at each study visit was centrifuged within 3 hours of venepuncture at 1500 x g for 20mins to give a layer of mononuclear cells (Figure 4.3). The mononuclear cell layers were aspirated into two falcon tubes, washed with 15mls of Dulbecco's phosphate buffered saline (DPBS) and centrifuged at 300 x g for 15 mins. The wash step was repeated. The cells from each tube were re-suspended in 6 mls GlutaMAX + 10 % foetal calf serum (FCS) with or without 1µg/ml *Escherichia coli* serotype 026:B6 LPS (Sigma-Aldrich, MO, USA) respectively. After re-suspension in culture medium, a 20µl sample of LPS-stimulated and LPS unstimulated cells was taken, diluted 1:10 in DPBS and cells counted using a Neubauer chamber. The remaining cells were transferred to a 6 well culture plate (Figure 4.4) and incubated for 24hrs at 37^{o}C.

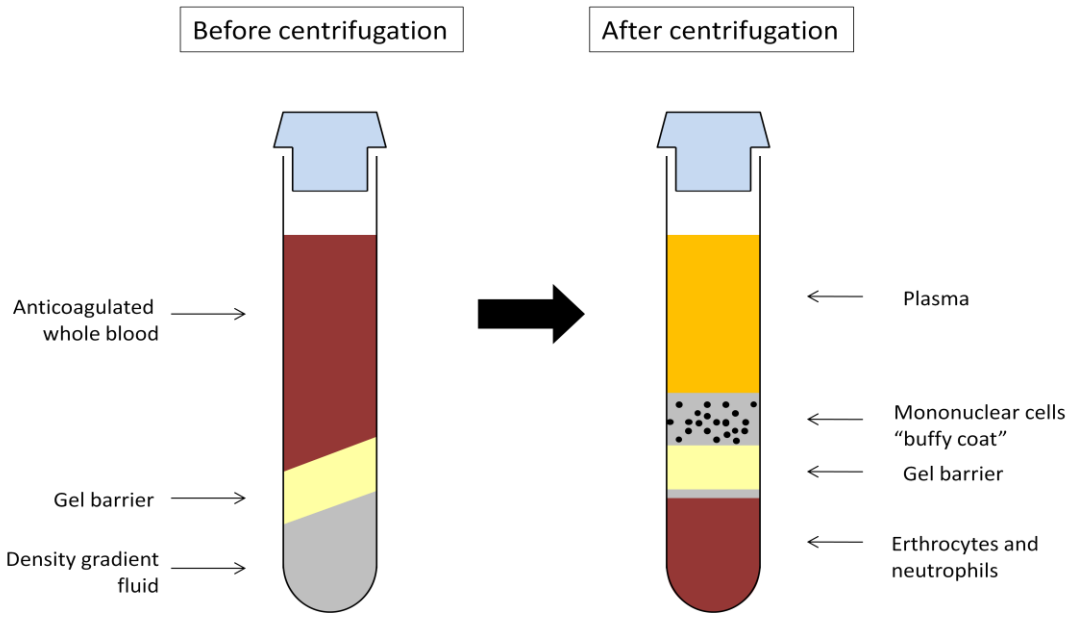


Figure 4.3. Separation of mononuclear cells after centrifugation of CPT™ tubes

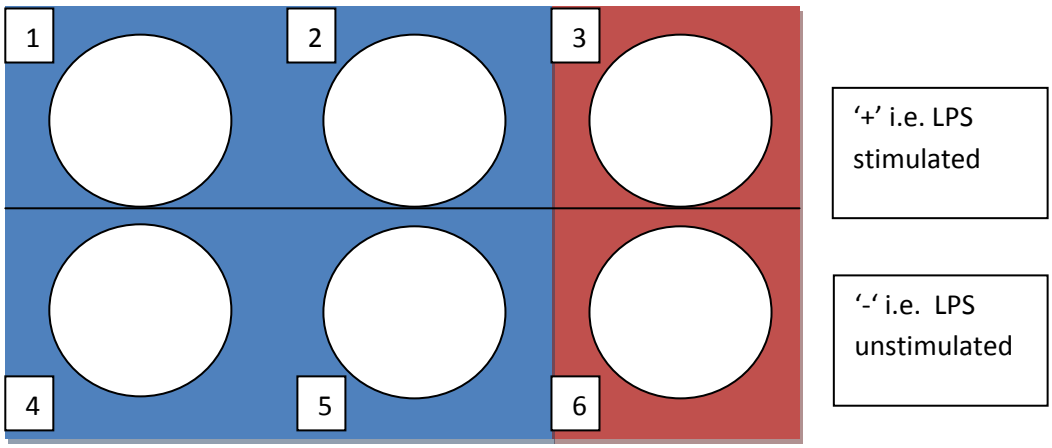


Figure 4.4. Layout of 6 well plate for culture of PBMCs. Following 24 hour culture, cells from wells 3 and 6 were harvested and frozen for future COX RNA analysis. Cells from wells 1,2,4 and 5 were processed further to obtain nuclear extract for the measurement of nuclear NFκB activation.

Culture medium from each well was aspirated into four 15µl falcon tubes as described below:

i) Wells 1+2 : LPS stimulated for NFκB nuclear expression

ii) Well 3 : LPS stimulated for COX RNA expression

iii) Wells 4+5 : LPS unstimulated for NFκB nuclear expression

iv) Well 6 : LPS unstimulated for COX RNA expression

Tubes were centrifuged at 300 x g at 4^{o}C for 10mins, and the culture medium aliquoted into cryogenic vials and stored at -80^{o}C until subsequent analysis of culture medium PGE_2 and PGE_3 content by LC-MS/MS. Cell pellets were left in the respective falcon tubes.

4.3.2 Harvest of PBMCs for COX expression analysis

Wells 3 and 6 were washed with 7mls DPBS, cells gently scraped using a cell scraper, and the cells aspirated back into their respective falcon tubes and centrifuged at 300 x g at 4^{o}C for 10mins. The supernatant was discarded and cells re-suspended in 1ml DPBS, transferred to a microcentrifuge tube and centrifuged at 300 x g at 4^{o}C for 5mins. The supernatant was discarded, cells re-suspended in 1ml TRIzol® (Invitrogen, Life Technologies, CA, USA) and stored at -80^{o}C. Analysis of COX RNA expression by real-time polymerase chain reaction (RT-PCR) was not performed due to time and funding constraints. Samples remain stored for analysis at a later date.

4.3.3 Isolation of PBMC nuclear extract

PBMC nuclear extract was obtained using the Active Motif Nuclear Extract Kit (Active Motif, CA, USA). Wells 1,2,4 and 5 were washed with 7mls of phosphate buffered saline - phosphatase inhibitor solution (PBS-PI), cells gently scraped using a cell scraper, and aspirated back into their respective falcon tubes. Addition of phosphatase inhibitor protects proteins from dephosphorylation. Tubes were

centrifuged at 300 x g at 4^{o}C for 10mins, the supernatant discarded, and cells re-suspended in 1ml PBS-PI. Cells were transferred to microcentrifuge tubes and centrifuged at 300g at 4^{o}C for 5mins. The supernatant was discarded and cells re-suspended in 1ml hypotonic buffer solution and incubated on ice for 15mins. Hypotonic buffer causes cell swelling, making the cell membrane more fragile.

50µl detergent was added to each microcentrifuge tube and vortexed gently. Detergent causes leakage of cytoplasmic proteins into the supernatant. Tubes were centrifuged at 14,000 x g at 4^{o}C for 30secs, and the supernatant (cytoplasmic fraction) aspirated and stored in cryogenic vials at -80^{o}C.

The nuclear pellet was re-suspended in 100µl of complete lysis buffer, vortexed for 10 seconds, and incubated on ice for 30mins on rocking platform at 150rpm. This causes lysis of nuclei, and nuclear proteins are solubilised in the lysis buffer, which contains a protease inhibitor cocktail to protect proteins from proteolysis. Tubes were centrifuged at 14,000g for 10mins at 4^{o}C, and the supernatant (containing the solubilised nuclear proteins) aspirated and aliquoted into microcentrifuge tubes and stored at -80^{o}C until subsequent batch analysis of nuclear NFκB activation (see section 4.3.4).

4.3.4 Quantification of PBMC nuclear NFκB activation

Overview: Nuclear NFκB activation was quantified using the Active Motif TransAM™ NFκB p65 enzyme-linked immunosorbent assay (ELISA) (Active Motif, CA, USA). Each well of the supplied 96-well plate is pre-bound with a double-stranded oligonucleotide containing the NFκB consensus binding site. Activated NFκB in the PBMC nuclear extract samples binds to the oligonucleotide, and in doing so an epitope on the p65 NFκB subunit is exposed. A primary antibody directed against this epitope is added. The primary antibody therefore only binds to activated, DNA-bound NFκB. Binding of an anti-rabbit horseradish peroxidise

(HRP)-conjugated secondary antibody provides a colorimetric reaction which can be quantified by spectrophotometry. Nuclear extract from Jurkat cells (an immortalised T-lymphocyte cell line) was supplied with the assay for use as a positive control.

Protein concentration of samples: The protein concentration in each nuclear extract sample was determined using the Bio-Rad *DC* Protein Assay (Bio-Rad, CA, USA), a modification of the Lowry assay.(284, 285) Bovine serum albumin (Sigma-Aldrich, MO, USA) was prepared in DPBS to standard concentrations of 2mg/ml, 1mg/ml, 0.5mg/ml and 0.1mg/m. 5µl of each standard and sample were added in duplicate to separate wells of a 96-well plate. 25µl reagent A' and 200µl reagent B were added to each well and incubated at room temperature for 15 mins. Optical density was read at 750nm on an Opsys MR™ plate reader (Dynex Technologies, VA, USA). The mean of the duplicate samples and standards was calculated. A graph of OD vs. protein concentration was plotted for the standards, and a line of best fit generated. The protein concentration of each sample was calculated according to the equation for the line of best fit.

Binding of NFκB to its consensus sequence: For each nuclear extract sample, 5µg of nuclear extract protein diluted in 20µl of complete lysis buffer (CLB) were added in duplicate to the TransAM™ NFκB 96-well plate. 5µg Jurkat nuclear extract in 20µl CLB and 20µl CLB were added in duplicate as positive and negative controls respectively. The plate was left to incubate for 1hr at room temperature at 100rpm on a rocking platform. Wells were washed three times with 200µl wash buffer.

Binding of primary antibody: 100µl of NFκB antibody (1:1000 dilution in antibody binding buffer) was added to each well and the plate incubated for 1hr at room temperature. Wells were washed three times with 200µl wash buffer.

Binding of secondary antibody: 100µl of HRP-conjugated antibody (1:1000 dilution in antibody binding buffer) was added to each well and the plate incubated for 1hr at room temperature. Wells were washed four times with 200µl wash buffer.

Colorimetric reaction: 100µl developing solution was added to each well and incubated for 4.5 mins at room temperature. 100µl of stop solution was added, and OD measured at 450nm and 630nm read on an Opsys MR™ plate reader (Dynex Technologies, VA, USA).

Quantification of NFκB: Optical density at 630nm (reference OD) was subtracted from OD at 430nm. The mean corrected OD of each duplicate sample was calculated, and divided by the mean corrected OD of the positive control wells to give the nuclear NFκB activation of each sample relative to that of the Jurkat nuclear extract standard.

4.4 Urinary PGE-M analysis

Analysis of urinary PGE-M involves 3 stages, 1) solid phase extraction, 2) liquid chromatography, and 3) tandem mass spectrometry.

1) Solid phase extraction (SPE)

Overview: SPE is a process which separates substances dissolved or suspended in a liquid based on their physical and chemical properties. This involves passage of liquid (the mobile phase, in this case urine) through a solid (the stationary phase) for which the substances of interest (anylates, in this case PGE-M) have an affinity. In reversed phase SPE, substances are separated based on their polarity. The stationary phase is typically a cartridge containing a sorbent such as octadecyl-bonded silica gel, which is silica bound to the hydrocarbon octadecane ($CH_3(CH2)_{16}CH_3$). In analytical chemistry this is referred to as simply "C18". C18 binds non-polar substances (e.g. PGE-M) by weak non-polar or hydrophobic

interactions (Van der Waal forces), whilst polar substances do not bind and remain dissolved or suspended in the liquid (urine) and exit the cartridge. The non-polar analytes of interest can be eluted from the cartridge by selectively washing with different concentrations of non-polar solvents. In this case, heptane was used as a weak solvent to wash through any impurities bound by weak non-polar attractions, and ethyl acetate then washed through to elute PGE-M and other compounds bound by strong non-polar attractions.

Method: Urine samples were thawed to 20^{o}C. Standards for the calculation of a calibration curve were formed by the serial dilution of commercially available PGE-M (Caymen Chemicals, Tallinn, Estonia) to give a concentration range from 0-100ng/ml PGE-M. The volume of each calibrant was 1ml. 20µl of 1µg/ml deuterated PGE-M (d6-PGE-M) was added to each sample as an internal standard.

Varian Bond Elut C18 SPE columns were mounted on a 'VacMaster ' extraction manifold (Biotage AB) and primed with 1ml methanol followed by 1ml of distilled water (acidified to pH 3 with acetic acid). 100µl 10% acetic acid was added to all samples and standards prior to being loaded into the columns. Columns were then sequentially washed with 1ml dH_2O pH3 and 1ml heptane before eluting the bound PGE-M with 1ml ethyl acetate. The eluted solutions were evaporated to dryness in a Genevac EZ-2 centrifugal evaporator (Genevac Ltd, Ipswich, UK) at 37^{o}C.

2&3) LC-MS/MS

Overview: The next stage involves sequential liquid chromatography (LC) and tandem-mass spectrometry (MS/MS) to give a highly accurate and sensitive quantification of PGE-M levels in the eluted sample. Liquid chromatography physically separates PGE-M and structurally similar compounds from other compounds remaining in the sample. The sample is introduced into a stream of fluid, known as the mobile phase, passing through a separation column. The time

at which compounds emerge at the other end of the column (retention time) depends on their physical and chemical interactions with the stationary phase of the column. This stage is important prior to mass spectrometry because the retention time of compounds with the same mass as PGE-M, e.g. isomers, will be different because of the different chemical structure and therefore different physical and chemical properties. Mass spectrometry is unable to differentiate between compounds of the same mass, but by analysing only those compounds emerging from the LC column at a specific retention time, the specificity of quantifying the compound of interest is increased.(286, 287) Secondly, purification of the sample before it enters the mass spectrometer reduces the interactions between different chemicals. Such interactions can reduce the efficiency of ionisation within the spectrometer, which is a particular problem when attempting to detect compounds that are present only in small concentrations.(286, 287)

Whilst mass spectrometry is an analytical tool for measuring the mass of a compound, tandem mass spectrometry fragments the sample and uses multiple analyzers to detect the mass of the fragments produced. This allows the structure of the compound to be elucidated. In the case of quantifying PGE-M, using MS/MS allows fragments corresponding to the breakdown of PGE-M to be discriminated from the breakdown fragments of other compounds in the urine with the same LC retention time. The sample delivered from the LC is ionized to give the compounds charge. The compounds pass through the mass analyser in gaseous phase and are accelerated into a finely focused beam. A voltage gradient is applied across the analyser to cause deflection of the ionized compounds based on their mass/charge ratio. The analyser is pre-set to only allow compounds with a specific mass/charge ratio to exit the analyser, thereby acting as a mass filter. These selected compounds then enter a collision chamber where they are accelerated to high speed and bombarded with inert gas to cause fragmentation (collision induced

fragmentation). These fragments pass through a second analyser, again pre-set to only allow fragments with a specific mass/charge ratio to exit and reach the detector. In this way the MS/MS can be set to detect and measure fragments with a mass/charge ratio corresponding to the known fragmentation products of PGE-M.(81)

Measurement of urinary PGE-M by LC-MS/MS is a highly accurate and sensitive technique,(81, 288) but requires specific expertise and equipment which are not available in our laboratory. These analyses were therefore performed by our collaborators Dr Paul Loadman and Amanda Race at the Institute of Cancer Therapeutics at the University of Bradford.

Method: Liquid chromatography was performed using an Acquity Ultra Performance LC™ (UPLC™) 2.1 x 100 mm, 1.7µm particle size BEH C18 column attached to an Acquity UPLC™ System (Waters, Milford, USA). Mobile Phase A was 95% dH_2O, 5% acetronitrile, 0.1% acetic acid, and mobile phase B was 50% dH_2O, 50% acetronitrile, 0.1% acetic acid. Samples were removed from the centrifuge evaporator and reconstituted in 25µl of mobile phase (60:40 A:B). Analytes were separated by a 95%-5% gradient of mobile phase A over 15min at a flow rate of 0.3ml/min prior to delivery to a Waters Quattro Premier™ XE bench-top tandem quadrupole mass spectrometer operating in negative ion mode and multiple reaction monitoring (MRM) mode. Capillary voltage was set at 3.5V, cone voltage at 20V and collision voltage at 16V. MRM channels were set for PGE-M at 327.4 →291 and 327.4 →309 and for d6-PGE-M at 333.4 → 296.8 and 333.4 →315.5. At the known LC retention time for PGE-M, the relative abundance of these fragmentation products of PGE-M and d6-PGE-M was recorded.

Quantification of PGE-M level in each sample was performed by calculating the ratio of detected PGE-M fragments to d6-PGE-M fragments for each sample. Since

a known concentration of d6-PGE-M was added to each sample at the start, this controlled for variability in the efficiency of the SPE process between samples. The ratio of PGE-M:d6-PGE-M was plotted for the PGE-M standards to give a calibration curve, against which the PGE-M:d6-PGE-M ratio for each patient sample was plotted and the PGE-M concentration calculated.

Standardisation for urinary creatinine: Urine volume is influenced by whole body fluid and electrolyte homeostasis. The concentration of analyte in random urine samples will therefore exhibit both inter- and intra-subject variability based on, for example, urine volume, time of day and dehydration status. Urinary PGE-M was therefore corrected for urinary creatinine as a marker of urine concentration. Urinary creatinine was measured by The Leeds Teaching Hospitals NHS Trust chemical pathology department based on a 'Jaffe' colorimetric assay(289) using an AVIDA 1800 (Siemens) analyser. Urinary PGE-M concentration was divided by urinary creatinine (Cr) concentration to give a corrected urinary PGE-M level in ng/mg Cr.

4.5 Tumoural PGE_2/PGE_3 analysis

PGE_2 and PGE_3 levels in fresh frozen tumour tissue were analysed using LC-MS/MS by Dr Paul Loadman and Ms Amanda Race at the Institute of Cancer Therapeutics at the University of Bradford. The principles of LC-MS/MS have already been described in this chapter (section 4.4). The methodology is the same as for analysis of urinary PGE-M (section 4.4) with a few minor modifications as described below.

Sample preparation: Fresh frozen tumour samples were defrosted and weighed. 0.9% saline was added to each sample in a microcentrifuge tube to make a homogenate of 1 part tumour to 9 parts saline, with the tumour tissue homogenised using ten strokes of a hand pestle. The homogenised tissue was centrifuged at

10,000 x g for 5mins and the supernatant removed to a fresh microcentrifuge tube. Standards for the calculation of a calibration curve were formed by the serial dilution of commercially available PGE_2 and PGE_3 (Caymen Chemicals) to give a concentration range from 0-100ng/ml. The volume of each calibrant was 1ml. 20µl of 1µg/ml deuterated PGE_2 (d4-PGE_2) standard was added to each sample as an internal standard. 100µl 1% (v/v) acetic acid was added to all samples and standards.

SPE and LC-MS/MS: SPE and LC-MS/MS was then performed as described for urine samples in section 4.4, with the exception that the MRM channels for MS/MS detection of PGE_2 and PGE_3 fragments were set at 351.5 → 271, 315, 333 for PGE_2 and 349.5 → 269, 313, 331 for PGE_3. At the known LC retention time for PGE_2 and PGE_3, the relative abundance of these fragmentation products of PGE_2 and PGE_3 and d4-PGE_2 was recorded.

Quantification of PGE_2 levels in each sample was performed by calculating the ratio of detected PGE_2 fragments to d4-PGE_2 fragments for each sample. Since a known concentration of d4-PGE_2 was added to each sample at the start, this controlled for variability in the efficiency of the SPE process between samples. The ratio of PGE_2:d4-PGE_2 was plotted for the PGE_2 standards to give a calibration curve, against which the PGE_2:d4-PGE_2 ratio for each patient sample was plotted and the PGE_2 concentration calculated. PGE_3 levels were calculated in the same way using the ratio of detected PGE_3 fragments to d4-PGE_2 fragments for each sample, and plotting this against a calibration curve drawn from the PGE_3 standards.

4.6 Tumoural PUFA analysis

Measurement of tumour PUFA content was performed by gas-chromatography mass-spectrometry (GC-MS). This is a highly accurate and sensitive technique, but

requires specific expertise and equipment which are not available in our laboratory. These analyses were therefore performed by our collaborators Dr Andrea Belluzzi and Dr Alessandra Munarini at the Saint Orsola-Malpighi Hospital, University of Bologna.

Overview: The principles of GC-MS are the same as for LC-MS/MS, using chromatography to first separate compounds in a sample based on their chemical and physical properties, and then using mass-spectrometry to quantify the level of a specific compound emerging at a particular retention time. In contrast to LC-MS/MS, GC-MS uses a gas rather than a liquid as the mobile phase for chromatography. The MS stage is similar in both GC-MS and LC-MS/MS, with ionization and fragmentation of compounds entering the spectrometer at a specific chromatographic retention time. With the single MS setup of GC-MS, these fragments are separated based on their mass/charge ratio and the fragments of interest then detected and quantified, whereas the tandem MS setup of LC-MS/MS utilizes a second phase of fragmentation and separation before detection and quantification of the fragments of interest.

Method: This is a summary of the method used by Drs Belluzzi and Munarini, which has previously been reported.(209, 247)

Sample extraction: Tissue samples were homogenised by hand in a pestle and mortar in 0.25ml of PBS. Homogenates were transferred to a Sovirel extraction tube and extracted with 3ml of chloroform:methanol (2:1, vol/vol) by a modification of the Folch method.(290) The mixture was shaken for 30min, the centrifuged at 1.5 x g for 10min and the supernatant transferred to a clean tube. This was repeated three times. The supernatant was evaporated to dryness under nitrogen and the solid residue resuspended in 2ml KOH 0.5M in methanol. This was then heated at 80^{o}C for 10min. 2.5ml of 14% BF_3 in methanol was added and the

mixture again heated at 80^{o}C for 10min to transesterify the lipids. Lipid methylesters were then extracted by the addition of 3ml hexane and evaporation under nitrogen. The residue was re-dissolved in 100µl ciclohexane.

GC-MS: GC-MS analysis was carried out using an Agilent HP6890 GC with PTV injector linked to an HP4973 mass spectrometer. Chromatography was performed using SUPELCO SP™2330 columns with Helium as the carrier gas at 0.5ml/min at a constant pressure. The column temperature started at 100^{o}C for 1.25mins, then rose by 30^{o}C/min to 185^{o}C. It then rose at 0.5^{o}C/min to 205^{o}C. Total GC runtime was 32min. MS was performed in the electron ionization mode at 70eV, and set to detect fragments of mass/charge ratio in the range *m/z* 40 to *m/z* 550. Peak identification was confirmed by comparison to GC retention times and MS peaks of commercially available standards. Data for each PUFA are quoted as the percentage of the total fatty acid content of the tumour.

4.7 Immunohistochemistry

Fresh 4µm sections were cut from each paraffin-embedded tissue block, mounted on a glass slide and left to dry at 37^{o}C overnight. Sections were then stored at room temperature, and stained within two weeks of sectioning to minimise antigen degradation. All immunohistochemistry was performed in runs of 23 slides. Each run contained 1 slide to which the primary antibody step was omitted ("no primary"). For each antibody protocol, 3 representative sections from the first run were selected and consecutive sections from these tumours used in each subsequent run as internal controls. The same batch of antibody was used for all runs to minimise variability of staining between runs.

Stock solutions consisted of:

- Low pH buffer: 15ml of Antigen Unmasking Solution (Vector Laboratories, Peterborough, UK) in 1.5L distilled H_2O
- Tris Buffered Saline (TBS): 60ml 2.5M NaCl + 20ml 1M Tris HCl (pH7.4) in 1l distilled H_2O
- Tris Buffered Saline Tween-20 (TBST): 1.2ml 10% (v/v) Tween-20 in 1L TBS
- DAB solution: 20µl of 3,3'-diaminobenzidine (DAB) chromogen substrate in 1ml of pH7.5 substrate buffer

4.7.1 Tumour proliferation (Ki-67)

Staining: Sections were dewaxed and rehydrated in serial xylene and ethanol (both x3), and rinsed under running water for 5mins. Antigen retrieval was performed using a pressure cooker. Briefly, 1.5L of low pH buffer was brought to the boil in a stainless steel pressure cooker, the sections added and the lid locked shut. Sections were heated for 2mins once full pressure had been reached, then removed and cooled immediately in running water for 5mins. Endogenous peroxidase activity was blocked using 0.3%(v/v) H_2O_2 for 10mins, then the sections were rinsed twice in TBS for 5 mins each.

Slides were placed on a humidified slide chamber and 100µl of antibody diluent solution (Zymed Laboratories, San Francisco, USA) added to each slide to block non-specific binding sites and immediately drained off. Mouse monoclonal anti-human Ki-67 antibody (MIB1 clone) (Dako, Glostrup, Denmark) was used as the primary antibody at 1:50 dilution in antibody diluent solution. 100µl of primary antibody was added to each slide and left to incubate for 60mins at room temperature. Slides were then rinsed twice in TBST and once in TBS, each for 5mins.

The Dako EnVision™ kit (Dako, Glostrup, Denmark) was used for the following steps. 100µl of EnVision™ secondary antibody (HRP-labeled polymer bound to goat anti-mouse immunoglobulin) was added to each slide and left to incubate for 30mins at room temperature. Slides were then rinsed twice in TBST and once in TBS, each for 5mins.

100µl of DAB solution was added to each slide and incubated for 10mins before rinsing slides in running water for 5mins. Slides were counterstained with haematoxylin, dehydrated in serial ethanol and xylene (both x3), and coverslips mounted using DePeX mounting medium. Slides were left to dry at room temperature overnight.

Scoring: A single high powered (x20) field of view was selected on each slide and photographed using a Nikon Eclipse E1000 microscope with NIS Elements software (Nikon Instruments Europe, Amsterdam, The Netherlands). The field of view was systematically selected according to a pre-determined method to minimise selection bias (Figure 4.5). The slide was placed on the stage and scanned at low power. The edge of the slide containing a tumour-liver interface was identified at low power. Where tumour-liver interface was present at more than one edge of the slide, the pre-determined hierarchy of right edge>left edge>top edge>bottom edge was used to select the edge of interest. The midpoint of the tumour-liver interface along this edge of the slide was identified at high power. The high powered field of view was moved incrementally towards the centre of the slide to select and photograph the first high powered field of view of viable tumour without capsule or normal liver. The edge rather than the centre of the tumour was chosen as the area of interest because this would a) likely capture the most highly proliferating area of tumour at the advancing edge, b) minimize the risk of selecting central necrotic areas of tumour, and c) minimise any potential discrepancy in proliferation between large tumours with necrotic centers and small tumours without central necrosis.

Photographs were analysed in Image J, a Java-based image processing and analysis software (http://rsbweb.nih.gov/ij/), using the cell counter plugin. All positively stained (brown) and negatively stained (blue) tumour cells were counted. The proliferation index was taken as the percentage of positively stained cells out of the total cell count, with a minimum count of 500 cells. If less than 500 cells were present, a second high powered field of view, immediately adjacent to the first, was selected and the cell counts combined.

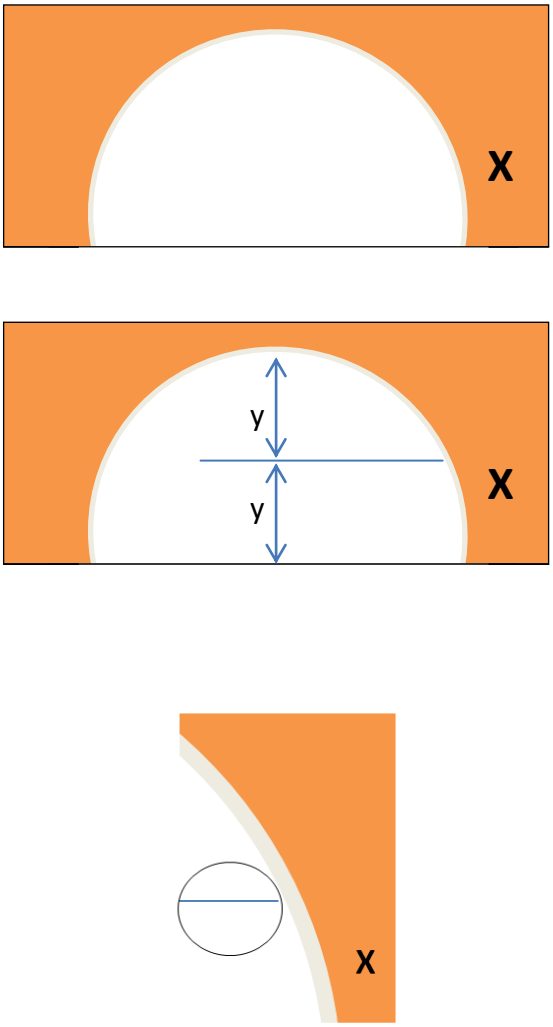


Figure 4.5. Protocol for selection of a high-powered field of view for measurement of Ki-67 proliferation index. a) The section was viewed under low power, and the edge of the slide containing liver-tumour interface was selected (X). Where more than one edge of the slide contains liver-tumour interface, the hierarchy right edge > left edge > top edge > bottom edge was used. b) the midpoint of tumour along this edge of the slide was located (y=y). c) The first high powered field of view at this mid-point which excluded capsule and normal liver was selected and photographed (circled). Images were opened in Image J for manual cell counts.

4.7.2 Tumour microvessel density (CD31)

Staining: Sections were stained using the same protocol as for Ki-67, with the following exceptions:

1. No antibody retrieval step was performed

2. The primary antibody used was mouse monoclonal anti-human CD31 antibody clone JC70A (Dako, Glostrup, Denmark) at 1:40 concentration diluted in antibody diluent, with overnight incubation at 4^{o}C.

Scoring: There are a number of methods for the quantification of tumour microvessel density on immunohistochemical stained sections, based upon the counting of microvessels in a microscopic field view.(291) These typically involve the identification of the most highly stained vascular regions of the section at low magnification, so called vascular "hotspots", followed by manual counting of the number of microvessels in these hotspots in one or more high powered fields of view. Clearly there is subjectivity in the identification of hotspots that introduces intra-observer variability and selection bias. Frequently, a single vessel will move in and out of the plane of section requiring the observer to decide whether to count two adjacent stained structures as a single vessel or two separate vessels. One option to reduce this variability is to use a Chalkley graticule, a random grid of points which is overlaid on the image. The Chalkley count is the number of points that hit a stained vascular structure, giving a relative vascular area rather than a true vessel count.(291) Alternatively, automated counting with image analysis algorithms permits a more objective vessel count and can be used to count across the whole section, overcoming the highly subjective identification of vascular hotspots. The drawback is the time and cost of the specialized equipment needed to firstly scan the whole slide at high magnification and secondly to analyse the scanned image.

Genentech (San Fransisco, USA) are a large biotechnology company who developed the anti-angiogenic drug bevacizumab (Avastin®). I am grateful to Dr Adrian Jubb, Pathologist at Genentech, California, USA, who used to work in the Section of Pathology and Tumour Biology at Leeds Institute of Molecular Medicine, for offering us access to the image analysis software used by Genetech to evaluate microvessel density. Stained tumour sections were scanned at x20 magnification using an Aperio ScanScope® slide scanner and digitally transferred to Genentech for image analysis. Below is a summary of the image analysis algorithm, which has previously been reported.(292, 293)

Scanned slides were analysed as 24bit RGB images using Matlab software (vR2010b by Mathworks, Natick, MA, USA). Areas of tumour were marked up by hand by Dr Jubb, to exclude areas of white space and normal liver. A segmentation algorithm was then applied to identify areas of viable tumour. Cells were identified as either tumour or non-tumour based on the size, shape and density of haematoxylin staining. Non-tumour areas were identified by the density of non-tumour cells versus tumour cells. Non-tumour areas were excluded from analysis. Brown DAB staining in each viable tumour region was isolated using a blue normalisation algorithm in addition to RGB colour.(294) Vessel lumina were then identified by a segmentation algorithm as white areas surrounded by DAB staining. Noise was removed based on size and shape criteria. Vessel number and perimeter were reported, as well as vessel area as a percentage of total tumour area. After analysis was complete, the output for each slide was checked by Dr Jubb to ensure that the results were consistent with the original unprocessed images and to remove any artefacts or aberrantly identified vessels or tumour/non-tumour areas.

4.7.3 Tumour apoptosis (M30)

Staining: Sections were stained using the same protocol as for Ki-67, with the following exceptions:

1. Antigen retrieval was performed by heating for 10mins in a microwave oven at full power with slides submersed in 750ml of low pH buffer in a glass bowl. Slides were then left to cool for 20mins in the glass bowl before proceeding with the protocol as for Ki-67 staining.

2. The primary antibody used was mouse monoclonal anti-human neo-cytokeratin 18 antibody (M30CytoDEATH; Roche, Mannheim, Germany) at 1:50 concentration diluted in antibody diluent, with 60mins incubation at room temperature.

Scoring: The M30 antibody recognises a neoepitope exposed by the activity of caspase-6 on the cytoskeletal protein cytokeratin-18 in epithelial cells. This neoepitope appears early in apoptosis, before DNA fragmentation occurs.(295) M30 staining is therefore an earlier marker of cell apoptosis than the TUNEL assay.(295, 296) The M30 epitope persists in late apoptosis, but is lost when cells become necrotic.

Apoptosis staining in human CRC tissue is more sparse than staining for microvessel density or proliferation. The difficulty in quantifying apoptosis is that the use of either a random or pre-determined field of view on a slide is likely to miss sparse apoptotic events. In addition, apoptosis does not cluster in "hotspots", so there would be significant operator-variability in selecting hotspots on a slide by slide basis. I therefore chose to score apoptosis across the whole slide using an automated image analysis algorithm. An automated image analysis algorithm was designed in collaboration with Mr Alex Wright and Dr Darren Treanor of the Section of Pathology and Tumour Biology at Leeds Institute of Molecular Medicine.

Stained tumour sections were scanned at x20 magnification using an Aperio ScanScope® slide scanner. Images were marked up by hand to select only areas of tumour, excluding normal liver and white space. A colour deconvolution programme was used to identify DAB staining, and individual DAB stained objects identified by thresholding. Thresholds for the intensity of staining were set at 0.5 standard deviations below the mean staining intensity. Size thresholds were used to exclude objects below 10microns and to remove noise and artifact. Total object count and area of staining as a percentage of total marked-up area were reported.

The algorithm was validated against a manual cell count by selecting 20 representative 2000x2000pixel regions of interest from three different slides, incorporating regions of low, medium and high staining. Manual image counting was performed on Image J using the cell counter plugin. All positively stained (brown) tumour cells were counted. The algorithm was then run on the same regions of interest. Manual apoptotic cell counts were plotted against the algorithm object count and algorithm % stained area, and correlation assessed with Spearman's rank correlation coefficient.

4.8 Human Umbilical Vein Endothelial Cell studies

Overview: The effects of EPA on angiogenesis have not been investigated fully. Human umbilical vein endothelial cells (HUVECs) have been widely used as an *in vitro* model of angiogenesis.(297, 298) I therefore performed a stand-alone series of assays investigating the effects of EPA on HUVEC proliferation, migration and tubule formation *in vitro* as a surrogate for endothelial cell behaviour and angiogenesis in tumours, but which would complement the microvessel density analysis of human CRCLMs in The EMT Trial.

Cell culture: Human umbilical vein endothelial cells (TCS Cellworks, Buckingham, UK) were cultured in T75 flasks in 15 ml of large vessel endothelial cell culture

medium (TCS Cellworks, Buckingham, UK) supplemented with endothelial cell growth supplements as supplied by the manufacturer (TCS Cellworks, Buckingham, UK) containing 2% foetal bovine serum, gentamicin, amphotericin B, epidermal growth factor, basic fibroblast growth factor, heparin and hydrocortisone (herein referred to as "plain culture medium"). The concentration of each of these supplements is not disclosed by the manufacturer. Cells were incubated at 37^{o}C with 5% CO_2 and culture medium was replaced every 48 hrs. Cells were split at 80% confluence and cells between passage 3 and 6 were used for all experiments.

Experimental culture medium: EPA-supplemented culture medium was created as follows. EPA-FFA was supplied in 500mg capsules by SLA Pharma AG. Three capsules were aspirated to dryness using a 21G needle and the aspirated content weighed. The aspirated weights were 424mg, 427mg and 426mg (mean 425.6mg per capsule). A stock of 0.1mM EPA solution was created by dissolving the aspirated EPA (425mg) in 14ml of 95% ethanol.

[EPA molecular weight = 302.451g and 0.425mg/302.451g ÷ 14ml/1000ml = 0.1mM]

10µl of 0.1mM EPA was added to 10ml of plain culture medium to give a 100µM EPA stock solution with an ethanol (carrier) concentration of 0.1% (v/v). This solution was further diluted with culture medium to provide a range of EPA-culture media with EPA concentrations from 0-100µM, with the maximum ethanol carrier concentration of 0.1% (v/v) at the 100µM EPA concentration.

Experimental media were made fresh from new EPA-FFA capsules for each experiment to minimise potential oxidation of EPA.

4.8.1 MTT proliferation assay

Acute EPA supplementation: HUVECs were grown in T75 culture flasks in plain culture medium. At 80% confluence, cells were trypsinised and centrifuged at 200 x g for 5mins. Cell pellets were resuspended in plain medium and cell density counted in a Neubauer chamber. Cells were seeded in 96-well plates at a density of 2000 cells/well in 100µl plain culture media. Six wells were seeded with plain culture media alone as a "no cell" control. After 24hrs incubation, the plain culture medium was aspirated and replaced with 100µl experimental media supplemented with EPA at concentrations ranging from 0µM to 100µM. Each EPA concentration was replicated in 6 wells. Six wells were replaced with culture medium + 0.1% (v/v) ethanol as a "carrier" control. Plates were seeded in triplicate and incubated for 24, 48 and 72hrs respectively. After incubation, the culture medium was aspirated and 50µl 1mg/ml Thiozolyl blue tetrazolium bromide (MTT) solution (Sigma-Aldrich, MO, USA) added to each well. The plate was left in the dark for 3hrs. Mitochondrial dehydrogenase in viable cells cleaves MTT to produce purple formazan crystals. After 3 hrs the MTT solution was aspirated and the purple crystals solubilised in 100µl propan-1-ol. The solution in each well was transferred to a fresh 96 well plate and optical density read at 570nm on a spectrophotometer. The mean optical density (OD) of the 6 wells for each EPA concentration was calculated and a graph of OD over time was plotted.

Further experiments were performed using the same method scaled up to a 48-well plate format, using $1x10^4$ cells/well in 0.5ml culture medium, 100µl of 1mg/ml MTT solution and 200µl of propan-1-ol. 100µl of the solubilised purple formazan crystals was then transferred to a fresh 96-well plate for measuring OD as above.

Chronic EPA supplementation: The effect of chronic EPA supplementation was studied by simultaneously growing batches of cells for two weeks in either plain culture medium or culture medium supplemented with 1µM EPA. Culture medium

was changed every 48hrs and cells trypsinised and split at 80% confluence. Cells were counted and re-suspended in plain or 1µM EPA-supplemented culture medium and seeded at $1x10^4$ cells per well with six replicates per condition on 3 separate plates and incubated for 24, 48 and 72hrs respectively. The media was not changed once cells had been seeded. An MTT assay was performed as described above.

The effects of VEGF and PGE_2 on cell proliferation were studied by the supplementation of either 10ng/ml VEGF or 1µM PGE_2 to the cell-conditioned media when cells were plated onto the 48 well plates. This was performed with six replicates for both the EPA-naive cells and chronic EPA-supplemented cells.

Concentration of PGE_2 and 6-keto-$PGF_{1\alpha}$ in HUVEC culture medium: To begin to explore the mechanistic basis of the effect of EPA on HUVEC proliferation, the PG levels in HUVEC cell conditioned medium were analysed. In the acute and chronic EPA supplementation assays, culture medium aspirated from each well prior to addition of MTT solution was combined for each of the replicate wells, and stored in 0.5ml cryovials at -80^oC until analysis.

Measurement of the concentration of PGE_2 in culture medium was performed using the same methodology as the analysis of urinary PGE-M (see section 4.4), with the exception that the MRM channels were set for MS/MS detection of PGE_2 as described in section 4.5.

However, PGI_2 rather than PGE_2 is considered the main prostaglandin product of AA metabolism in vascular endothelium and previous studies in HUVEC cultures have shown greater production of PGI_2 than PGE_2.(299-301) 6-keto-$PGF_{1\alpha}$ is a stable product of PGI_2 produced by its non-enzymatic hydration. Measurement of 6-keto-$PGF_{1\alpha}$ in HUVEC cell conditioned medium was performed using a competitive inhibition enzyme immunoassay (Enzo Life Sciences, Exeter, UK)

according to the manufacturer's instructions. In brief, the kit includes a microtitre plate to which a donkey anti-sheep antibody is pre-bound to each well. A polyclonal sheep antibody against 6-keto-$PGF_{1\alpha}$ is added which binds competitively to either 6-keto-$PGF_{1\alpha}$ in the sample or to an alkaline-phosphatase-6-keto-$PGF_{1\alpha}$ conjugate which is added to each well. The higher the concentration of 6-keto-$PGF_{1\alpha}$ in the sample, the lower the concentration of alkaline-phospatase-6-keto-$PGF_{1\alpha}$ conjugate that will competitively bind the sheep antibody and therefore bind to the well surface. Reagents are washed away to remove any unbound 6-keto-$PGF_{1\alpha}$. A substrate of p-nitrophenyl phosphate (pNpp) is added to each well. This is catalysed by alkaline-phosphatase conjugate bound to the plate and produces a colour change. The OD measured by spectrophotometry is inversely proportional to the original concentration of 6-keto-$PGF_{1\alpha}$ in the sample, which can be read off the optical density on the standard curve produced from the 6-keto-$PGF_{1\alpha}$ standards. The detailed methodology is described below.

The 6-keto-$PGF_{1\alpha}$ standard was serially diluted in fresh HUVEC culture medium to give concentrations of 50000, 10000, 2000, 400, 80, 16 and 3.2pg/ml. 100µl of HUVEC culture medium was added to the "non-specific-binding" (NSB) well and the no-competitive binding (Bo) well. 100µl of standard or sample was added to the remaining wells in duplicate. 50µl of assay buffer was added to the NSB well. 50µl of alkaline-phosphatase-6-keto-$PGF_{1\alpha}$ conjugate was added to each well. 50µl of polyclonal sheep antibody against 6-keto-$PGF_{1\alpha}$ was added to each well except the NSB well. The plate was incubated at room temperature on a plate shaker for 2hrs at 500rpm.

The contents of each well were washed with 400µl of wash solution for a total of three washes. 200µl of pNpp substrate was added to each well and incubated at room temperature for 45mins without shaking. 50µl of stop solution was added to

each well. Optical density was immediately measure on a spectrophotometer at 405nm, with correction at 570nm.

The OD for each sample and standard well was corrected for the average OD of the blank wells. The net OD for each standard and sample was calculated by subtracting the average NSB OD from the average OD of each standard or sample. The percentage binding of each standard as a percentage of the maximum binding wells (Bo) was calculated by dividing the net OD of each standard by the net OD of the Bo wells. For each standard, percentage binding was plotted against 6-keto-$PGF_{1\alpha}$ concentration to produce a standard curve. Percentage binding of the samples was plotted on this graph to calculate the concentration of 6-keto-$PGF_{1\alpha}$ in the samples.

The primary antibody in this kit is specific for 6-keto-$PGF_{1\alpha}$. The manufacture reports cross reactivity with other related prostaglandins at less than 3%.(302) They do not have data for cross-reactivity with the equivalent 3-series PGI_3 metabolite δ17-6-keto-$PGF_{1\alpha}$. One historic paper has reported less than 10% cross-reactivity between the two compounds with two different antibodies against 6-keto-$PGF_{1\alpha}$,(303) although this data is of limited value as the antibodies are different to those used in the Enzo Life Sciences kit. Nevertheless, cross-reactivity is likely to be small, and given that 3-series prostaglandins might be expected to be produced in smaller quantities than the respective 2-series prostaglandins by as much as an order of magnitude,(209) any cross-reactivity could reasonably be expected to have a negligible effect on the results of the assay.

4.8.2 Wound migration assay

HUVECs were seeded in a 6 well plate at a density of $2x10^5$ cells/well in 2ml plain culture medium and grown to confluence. Each well was pre-marked on the reverse with two horizontal reference lines using a fine permanent marker (Figure

4.6). Culture medium was replaced every 48hrs. At confluence, culture medium was aspirated and a vertical score made in each well with a yellow pipette tip. Medium was replaced with 2ml experimental medium supplemented with EPA at concentrations ranging from 10μM to 50μM. Photographs of each wound were taken immediately above and below the intersection of each reference line with the wound (4 photos per well) using an Olympus C7070 camera (Olympus, Essex, UK) attached to an Olympus CKX41 inverted light microscope (Olympus, Essex, UK) at x4 magnification (Figure 4.6). The plate was incubated at 37^{o}C and repeat photographs taken at regular intervals until the wounds had closed.

Scoring: Images were scored using TScratch, a freely available MATLAB (The Mathworks, Natick, MA, USA) based stand-alone programme designed by the Swiss Federal Institute of Technology (Zurich, Switzerland).(304) TScratch detects the edges of the wound using an edge detection algorithm, then calculates the percentage open area (i.e. the area of the wound) in each image. The detected open area is saved as an image overlay and available for review. Thresholds for edge detection can be user defined, and in these assays they were set at 0.36 arbitrary units which subjectively gave the best balance between sensitivity and specificity when the image overlays were reviewed. All overlay images were manually reviewed and occasional erroneously detected areas, corresponding to patches of poor HUVEC confluence within the image, were deleted prior to the final percentage wound area being calculated.

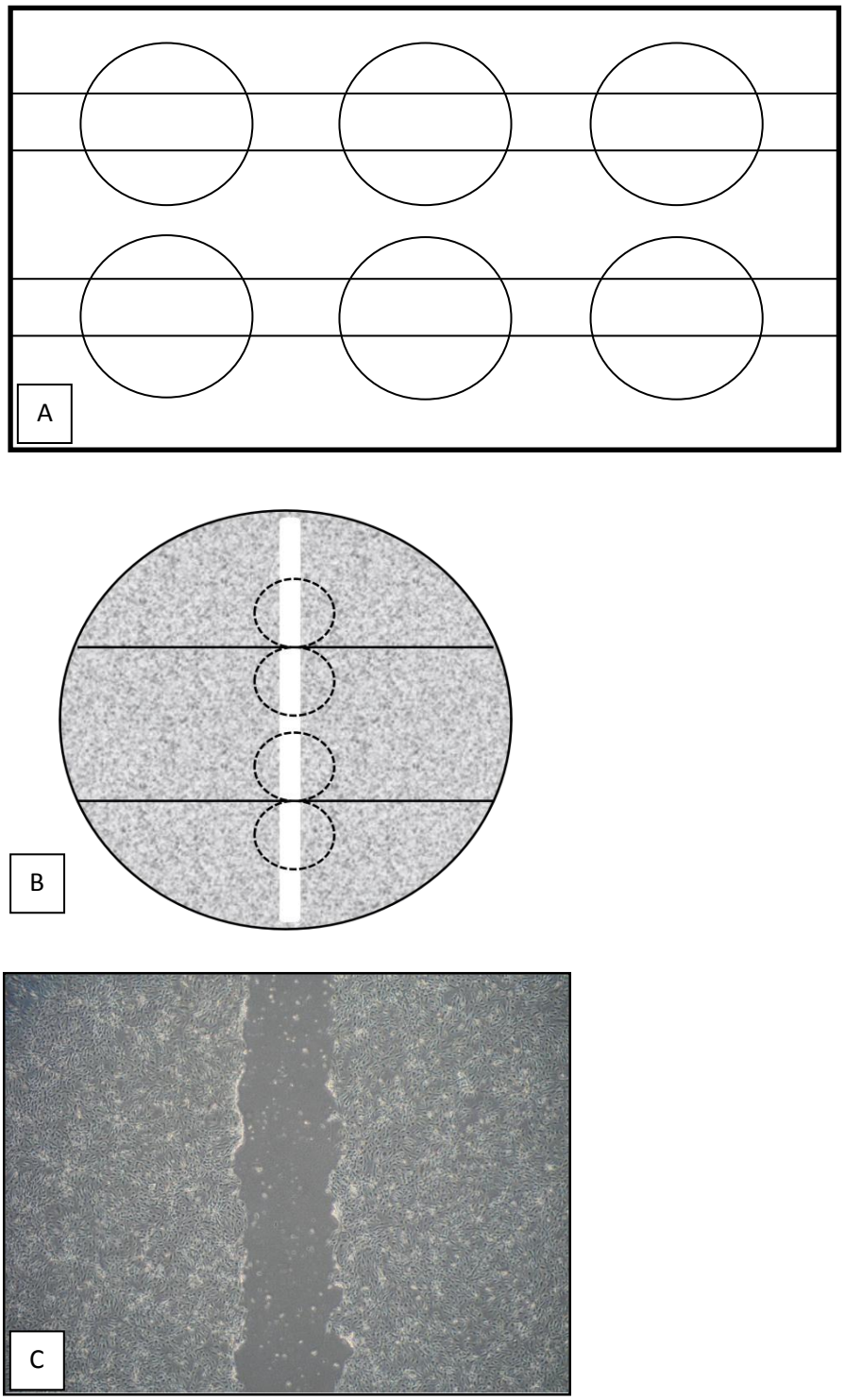


Figure 4.6. HUVEC wound migration assay.

a) Parallel reference lines were drawn across the back of each well. b) At confluence a scratch was made in the HUVEC monolayer, and photographs taken at x4 magnification, immediately above and below the intersection of the score with the reference lines, as indicated by the dotted lines. c) An example of a photographed wound.

4.8.3 Matrigel tubule formation assay

Matrigel™ (BD Biosciences, Oxford, UK) is a gelatinous reconstituted basement protein mixture extracted from the Engelbreth-Holm-Swarm mouse sarcoma.(305) It contains laminin, collagen, enactin, EGF, IGF-1, bFGF and other growth factors.(305) HUVECs seeded on a layer of Matrigel form a network of tube-like structures which are the closest *in vitro* representation of capillary microvessels. The effect of EPA on HUVECs in a Matrigel assay therefore evaluates the effect on the more complex process of tube formation, rather than the more simplistic assays of proliferation or migration described above.

Assay optimisation: BD Matrigel Basement Membrane High Concentration (BD Biosciences, Oxford, UK) was defrosted on ice overnight. Cold pipette tips, cryovials and PBS were used to dilute the liquid Matrigel with PBS into 0.5ml aliquots at 50%, 33%, 20%, 10% and 5% (v/v) Matrigel concentrations. The remaining Matrigel was immediately re-frozen. 0.5ml of each Matrigel concentration was pipetted into a single well of a cold 6-well plate and spread evenly across the well. These steps were performed on ice with cold equipment and reagents because Matrigel rapidly forms a gel above 10^{o}C. The 6 well plate was then placed at 37^{o}C in an incubator for 30mins to allow the Matrigel to solidify.

2x10^{5} HUVECs in 2ml plain culture medium were seeded in each well of the Matrigel-coated 6 well plate. The plates were reviewed regularly to assess the formation of tube-like structures. Based on the results of this, a 33% Matrigel concentration was chosen for the HUVEC-Matrigel assays. The remaining Matrigel was therefore diluted to 33% concentration and aliquoted into 0.5ml aliquots as described above and immediately refrozen. The original Matrigel protein concentration was certified at 19.0mg/ml by the manufacturer based on the Lowry method.(285) The 33% dilution therefore represents a 6.4mg/ml protein concentration.

Acute EPA supplementation assay: Wells of a 6-well plate were coated with 0.5ml of 33% Matrigel as described above. HUVECs were cultured in plain culture media, trypsinised, centrifuged, resuspended and counted as previously described. EPA-supplemented culture media was prepared as previously described. $2x10^5$ cells in 2ml of culture media supplemented with EPA to a concentration of 0-30µM EPA were added to each well. The plate was incubated at 37^oC in a cell incubator. Plates were reviewed every 2hrs for the formation of tube-like structures, and 4 non-overlapping representative photos taken of each well at each time point using an Olympus C7070 camera (Olympus, Essex, UK) attached to an Olympus CKX41 inverted light microscope (Olympus, Essex, UK) at x4 magnification.

Chronic EPA supplementation assay: HUVECs were grown for two weeks in either plain culture media or 1µM supplemented culture media as previously described. $2x10^5$ cells in 2ml of plain culture media were seeded onto each well of a Matrigel-coated 6 well plate, 3 wells for the EPA-naive cells and 3 wells for the chronic-EPA supplemented cells. Plates were incubated, reviewed and photographed as described above.

Scoring: Images were analysed using a web-based image analysis service, WimTube (Wimasis GmbH, Munich, Germany). Briefly, their algorithm involves filtering, segmenting, object recognition and data processing steps. The tube-like structures identified are highlighted on an overlay image, and the metrics outputted are number of tube-like structures, mean tube length, number of branching points and number of complete rings formed. This method has been validated against the alternative Angiosys system (TCS Cellworks, Buckingham, England) which requires the user to perform image processing in Adobe Photoshop followed by manual thresholding and skeletonisation steps in Angiosys.(306) Angiosys then calculates the same metrics as Wimasis, with the exception of number of complete rings

formed. Similar results have been obtained from either system, with the advantage that the Wimasis system required less user processing time.(306)

Chapter 5: Results - The EMT Trial

5.1 Patient recruitment and baseline characteristics

Between April 2010 and July 2011, 203 patients in the Leeds Hepatobiliary Unit outpatient clinics were screened for eligibility for The EMT Trial. A total of 102 patients did not fulfil the trial inclusion criteria, over half of these because of chemotherapy use in the last 3 months (Table 5.1 and Figure 5.1). Other reasons for ineligibility are detailed in Table 5.1. A further four patients were not approached for The EMT Trial because they were being considered for inclusion in other concurrent trials, and five patients were eligible but not approached because of time constraints in the outpatient clinic. Of the 92 eligible patients who were approached, the trial was well received with only four patients declining to participate. Target recruitment of 88 patients (see power calculation, Chapter 3.9) was achieved in 15 months (Figure 5.2).

Reason for ineligibility (n=102)	N= (%)
Chemotherapy within 3 months	57 (55.9%)
Primary CRC in situ, or surgery within 6 weeks	11 (10.8%)
Anticoagulation therapy	10 (9.8%)
Inoperable disease	9 (8.8%)
Regular NSAID use	4 (3.9%)
Uncertain CRCLM diagnosis	4 (3.9%)
CRCLM <9mm on cross-sectional imaging	4 (3.9%)
Active Crohn's disease	1 (1.0%)
Presence of other primary cancers	1 (1.0%)
Active participant in another trial	1 (1.0%)

Table 5.1. Patients screened but ineligible for inclusion in the EMT Trial

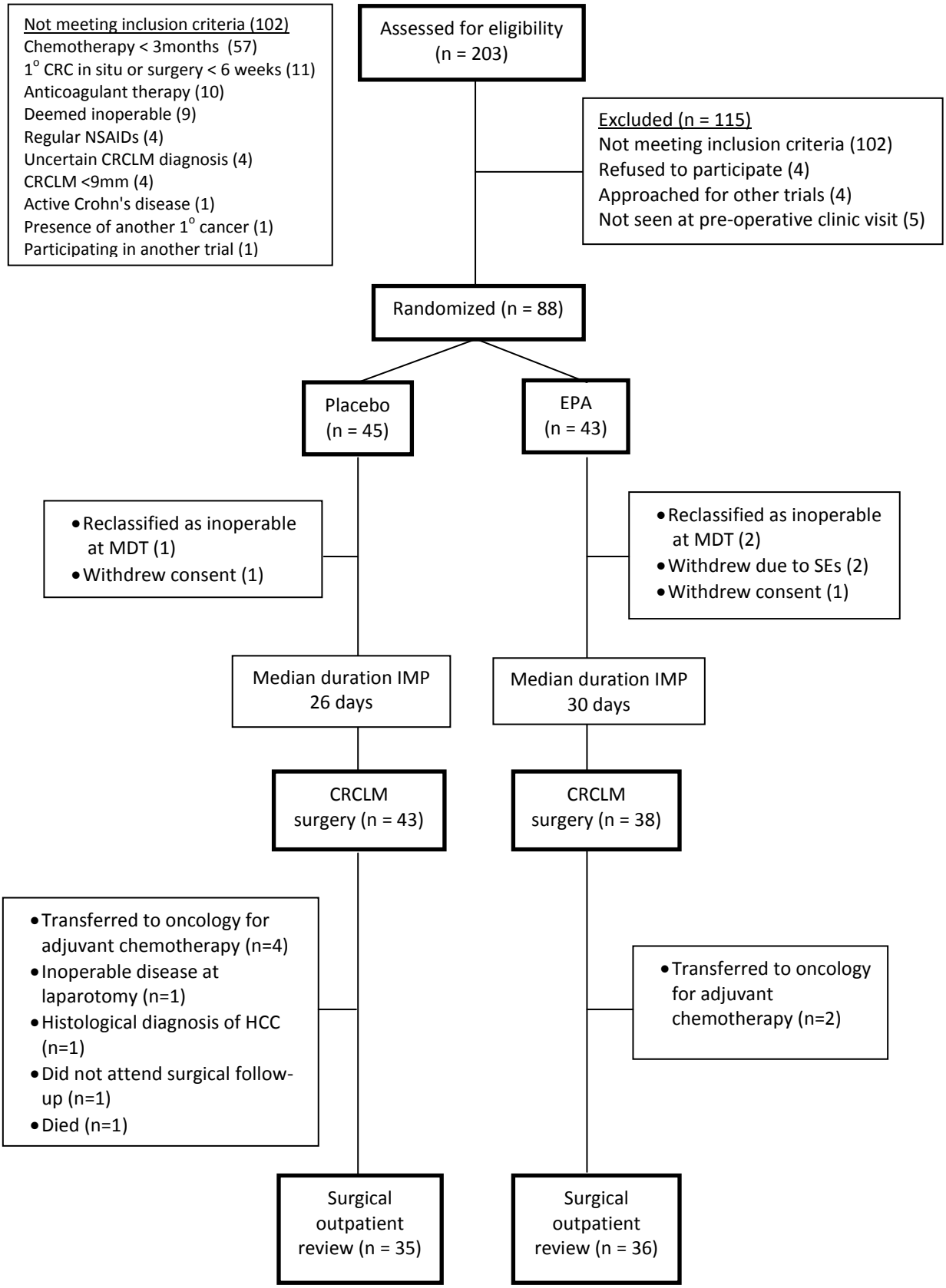


Figure 5.1. CONSORT diagram

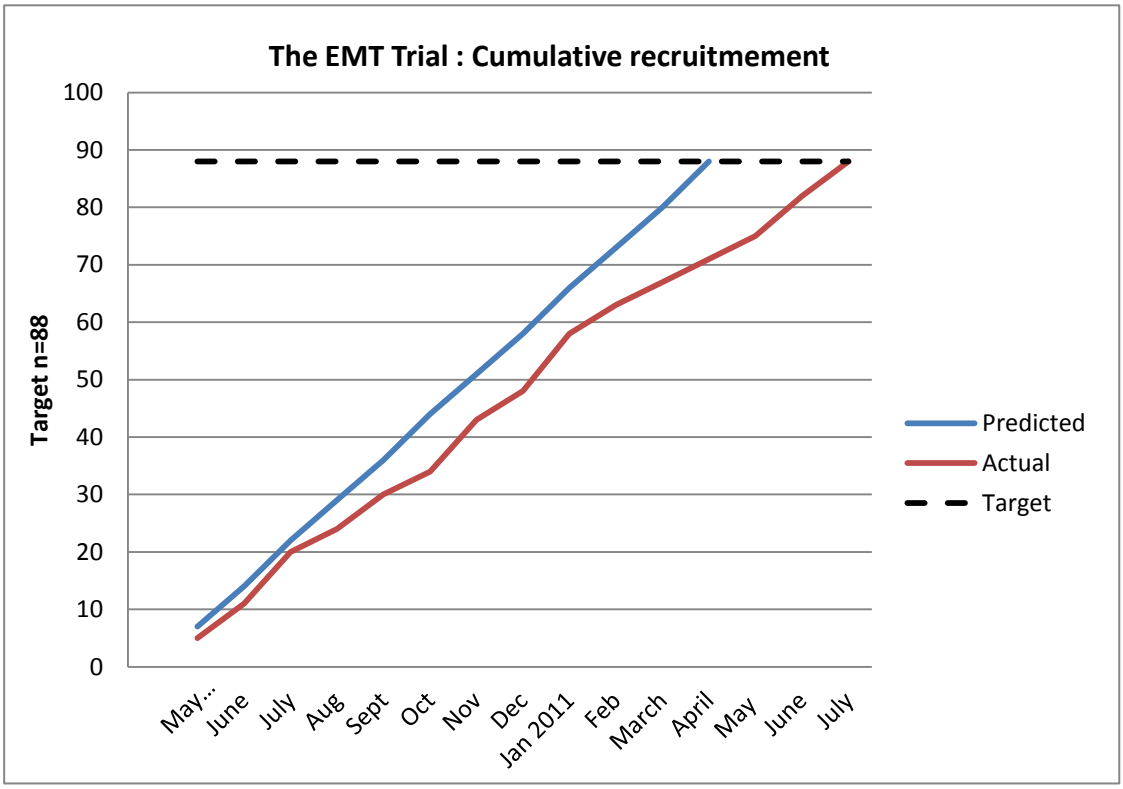


Figure 5.2. Graph of cumulative recruitment into The EMT Trial

Forty three patients were randomised to the EPA arm and 45 patients to the placebo arm of the trial (Figure 5.1). The two groups were well matched at baseline (Table 5.2) with no significant difference in age, stage of primary bowel cancer, node positive primary disease, size or number of liver metastases, previous chemotherapy or interval between the end of chemotherapy and CRCLM resection. There was a non-significant higher proportion of females in the EPA group (39% vs. 21%). The placebo group had a higher proportion of synchronous disease (44% vs. 30%), and a shorter interval between CRC resection and presentation with CRCLM in those patients with metachronous disease (median 19 months vs. 24 months). Neither measure reached statistical significance. Similar proportions of patients in each group were taking antiplatelet agents. Five patients in the EPA group and nine patients in the placebo group were already taking regular fish oil supplements before enrolment in the trial. All of these patients stopped taking their own fish oil supplements for the duration of the trial.

	Placebo n=45	EPA n=43	p=
Patient characteristics			
Age (years)	71 (35-87)	68 (44-82)	0.97
Sex M:F (% male)	35:10 (78%)	26:17 (61%)	0.08
Aspirin	10 (22%)	10 (23%)	0.91
Clopidogrel	3 (7%)	1 (2%)	0.33
Previous fish oil	9 (20%)	5 (12%)	0.28
Primary bowel cancer characteristics			
Dukes stage A B C D	 2 (4%) 13 (29%) 10 (22%) 20 (44%)	 2 (5%) 11 (26%) 17 (40%) 13 (30%)	0.33
Node Positive	25 (56%)	26 (61%)	0.64
Synchronous CRCLM	20 (44%)	13 (30%)	0.17
Liver metastasis characteristics			
Interval between primary CRC surgery and presentation with CRCLM (metachronous disease only)	19 months (3-80) n= 30	24 months (6-91) n= 32	0.16
Number of patients presenting for redo liver resection	11 (24%)	6 (14%)	0.21
Number of metastases	2 (1-5)	1 (1-9)	0.44
Largest metastasis (cm)	2.6 (0.9-12)	3.1 (0.9-15)	0.15
Previous chemotherapy			
Adjuvant chemotherapy > 3months prior to CRLM resection	20 (44%)	20 (47%)	0.85
Interval between end of chemotherapy and CRLM resection	12 months (5-73)	13 months (6-82)	0.86

Table 5.2. Comparison of patient and disease-specific characteristics at baseline. All data presented as n=(%) or median (range). Continuous variables were compared using Student t-test if data was normally distributed or Mann-Whitney U test if data was not normally-distributed. Categorical variables were compared using Chi-square test.

5.2 Withdrawals, duration of trial medication, and follow-up.

5.2.1 Withdrawals

Two patients in each group were withdrawn from the study following randomisation after their disease was reclassified as inoperable at the Leeds Hepatobiliary Multidisciplinary Team meeting. One patient in each group withdrew their consent after randomisation (Figure 5.1). One was a patient who decided a few days after randomisation that he wanted to start taking fish oil supplements and was not prepared to continue in the trial with a 50:50 chance of taking a placebo. He therefore withdrew his consent so that he could start taking commercially available fish oil supplements. The other was a patient who decided immediately after randomisation that he no longer wanted to take part in the trial. He did not receive any trial mediation. Two patients in the EPA group were withdrawn because of diarrhoea side effects, likely due to trial medication (see Section 5.3.4).

5.2.2 Patients proceeding to surgery

A total of 43 patients in the placebo group and 38 patients in the EPA group therefore proceeded to surgery. There was no difference in the duration of trial medication between the two groups (p=0.62). Median duration of trial medication was 26 days in the placebo group (range 15-73 days) and 30 days in the EPA group (range 12-65 days). At surgery, one patient in the placebo group was found to have inoperable disease and no tumour tissue was obtained. One patient in the EPA group had no identifiable tumour in the resected liver specimen, despite fine sectioning of the resected specimen. The specimen was subsequently confirmed as having two fibrotic lesions but no identifiable tumour on the formal histopathology report. One patient in the placebo group was diagnosed as having hepatocellular carcinoma not CRCLM after histological examination of the resected tumour and this patient was withdrawn from follow-up at this point. Tumour samples from this HCC, and all preceding blood and urinary samples for this patient were excluded

from all analyses. Samples of CRCLM for FFPE and subsequent immunohistochemical analysis, including the primary endpoint of Ki-67 proliferation index, were therefore obtained from 41 patients in the placebo group and 37 patients in the EPA group.

For two patients in the placebo group there was insufficient tumour to take fresh frozen tissue samples as well as for formalin-fixation. FFPE samples were therefore prioritised in order to maximise the number of samples available for primary endpoint analysis. This included one patient who had suspected lymph node involvement at the time of surgery which was confirmed on intra-operative fresh frozen histological examination. At the time of confirmation, only a limited metastasectomy had been performed and tumour samples could only be taken for FFPE. Similarly, the other patient had a very small tumour from which only a sample for FFPE could be obtained. Two further patients in the placebo group had samples taken only for FFPE because their surgery was brought forward without our knowledge. By the time these two patients had been identified, their tumour had already been fixed in formalin in the hospital's histopathology department. Formalin fixed tumour samples were taken and paraffin embedded in the same manner as all other samples, but it was not possible to obtain fresh tumour tissue. Fresh frozen tumour samples were therefore obtained from 37 patients in the placebo group and 37 patients in the EPA group.

5.2.3 Loss to follow-up

It is standard practice for patients to be reviewed 6 weeks after discharge from hospital in the surgical outpatient clinic to assess their recovery prior to referring patients to their local oncology centre for consideration of adjuvant chemotherapy. However, 6 patients (4 placebo group, 2 EPA group) who lived outside the Yorkshire region were not followed-up post-operatively in the surgical outpatient clinic and instead referred by their Consultant Surgeon directly to their local

oncology unit in order to minimise travelling. These 6 patients therefore did not complete the final trial visit. One further patient in the placebo group did not attend the final study visit, and another patient in the placebo group died before the final study visit. Follow-up was therefore completed by 35 patients in the placebo group and 36 patients in the EPA group (Figure 5.1).

5.3 Compliance, safety and tolerability

5.3.1 Compliance

Compliance was assessed by counting of capsules returned by the patient when they were admitted for their liver resection. With the caveat that patients may not have returned all of their unused medication, mean compliance in the EPA group was 91% (range 50% - 100%) and in the placebo group 94% (range 43% - 100%).

5.3.2 Suspected unexpected serious adverse reactions (SUSARs)

There were no SUSARs recorded during this trial.

5.3.3 Serious adverse events (SAEs)

There were no SAEs reported whilst patients were taking study medication. Seven SAEs were reported in the six week post-operative follow-up period. These are detailed in Table 5.3. Those SAEs that occurred in the post-operative period, including one death (1.1%), one pulmonary embolism (1.1%) and four patients requiring drainage of a subphrenic collection (4.5%) are all in keeping with the expected morbidity of liver resection surgery. None of these were attributed to study medication and none required unblinding of the trial.

Patient ID	Arm	SAE category	SAE description	Time between surgery & SAE
055	Placebo	Death	Elderly gentleman requiring a more extensive resection than anticipated developed intra-abdominal sepsis, multi-organ failure and died.	31 days
058	Placebo	Life-threatening event	Readmitted with pulmonary embolism	21 days
026	EPA	Readmission	Readmission for drainage of subphrenic collection	21 days
046	EPA	Readmission	Readmission for drainage of subphrenic collection	16 days
086	EPA	Readmission	Readmission for drainage of subphrenic collection	31 days
060	Placebo	Intervention causing increased length of stay	Drainage of subphrenic collection	9 days
084	Placebo	Readmission	Planned re-admission for resection of known lung metastases	49 days

Table 5.3. Summary of SAEs reported during the trial

5.3.4 Adverse events (AEs)

Adverse events were classified as pre-operative or post-operative and graded in severity as either mild, moderate or severe. AEs were graded *mild* if no intervention was required, the patient did not require hospitalisation or the AE did not prolong inpatient stay. AEs were graded *moderate* if intervention was required (e.g. dose reduction of IMP) but the patient did not did not require hospitalisation or the AE did not prolong inpatient stay. AEs were graded *severe* if the patient required hospitalisation, required re-operation, or required transfer to a higher level of care (e.g. from ward care to High Dependency Unit, HDU). Based on this grading, all severe AEs qualify as SAEs and have been included in section 5.3.2.

A total of 16 pre-operative AEs were documented (Table 5.4). These were all gastrointestinal symptoms, and were all considered likely attributable to study medication. There was a greater proportion of patients in the EPA group reporting diarrhoea (19% vs. 7%) but this did not reach statistical significance. Of the eight patients reporting diarrhoea in the EPA group, two patients were withdrawn and two patients' symptoms were effectively controlled with a dose reduction from 2g to 1g daily. Of the three patients reporting diarrhoea in the placebo group, one required a reduction in the dose of trial medication. These AEs were all graded as moderate severity. All other reported diarrhoea was mild in severity. There was a significant increase in patients reporting upper gastrointestinal upset (abdominal pain, nausea, dyspepsia) in the EPA group compared to the placebo group (12% vs. 0%, p=0.01). None of these patients required dose reduction or withdrawal from the study.

Adverse Event	**Placebo (n=45)**	**EPA (n=43)**	**p=**
Preoperative			
Diarrhoea • *Mild* • *Moderate*	3 (7%) *2* *1*	8 (19%) *4* *4*	0.09
Upper GI upset (mild)	0	5 (12%)	0.01
Postoperative			
Encephalopathy (moderate)	1	1	
Intra-abdominal collection (mild)	2	1	
Bleeding (mild)	0	1	
Wound infection • *no antibiotics (mild)* • *required antibiotics (moderate*)	4 *2* *2*	1 *1* *0*	
Lower respiratory tract infection (moderate)	4	0	
Urinary tract infection (moderate)	0	3	

Table 5.4. Summary of AEs reported during the trial

A total of 18 post-operative AEs were documented in The EMT Trial (Table 5.4), all of which were considered to be in keeping with the expected complications of liver resection surgery, with none of these AEs considered attributable to study medication. Combining the postoperative SAEs and AEs, morbidity was 33% in the placebo group and 23% in the EPA group, and the overall operative mortality (30-day mortality) was 1.1%. This is in keeping with current outcome data for liver resection from the Leeds Hepatobiliary Unit which shows an operative mortality rate of 1.9%, and morbidity of 15.9-30%,(307, 308) of which postoperative collection was the most common complication occurring in approximately 7% of patients (307) as compared to in 8% of patients in The EMT Trial.

5.5 Surgical outcomes

Extent of liver resection was defined according to the International Hepato-pancreatico-biliary Association (IHPBA) 2000 Brisbane classification.(309) This defines liver resection as minor (<3 segments resected), major (3-4 segments resected), or extended (5 or more segments resected). Approximately two-thirds of patients underwent minor liver resection and one-quarter of patients underwent a major liver resection. There was no significant difference in the extent of resections performed between the two groups (Table 5.5).

Length of hospital stay is a surrogate marker of surgical quality and complications. There was no significant difference in either the length of HDU stay or total length of hospital stay between the two groups (Table 5.5). There was no significant difference in the requirement for transfusion of packed red cells between the two groups (Table 5.5).

Surgical outcomes	Placebo n=43	EPA = 38	p=
Extent of resection • Minor • Major • Extended • Inoperable	 30 (70%) 9 (21%) 3 (7%) 1 (2%)	 25 (66%) 10 (26%) 3 (8%) 0 (0%)	0.75
Packed red cell transfusion	4 (9%)	3 (8%)	0.82
HDU stay (days) *median + IQR*	1 (1-3)	2 (0-2.25)	0.95
Total hospital stay (days) *median + IQR*	7 (5-10)	6.5(5-9)	0.72

Table 5.5. Summary of surgical outcome measures

5.6 Food Frequency Questionnaire

Food frequency questionnaires were analysed for all patients who proceeded to surgery (n=81). Baseline FFQs of those patients who were withdrawn prior to surgery (n=7) were not included in the analysis because no post-treatment outcome data was available for these patients. FFQs were completed by 72 of 81 patients (89%) at baseline, 59 of 81 patients (73%) post-treatment and 67 of 71 patients (94%) at six week follow-up (Figure 5.3).

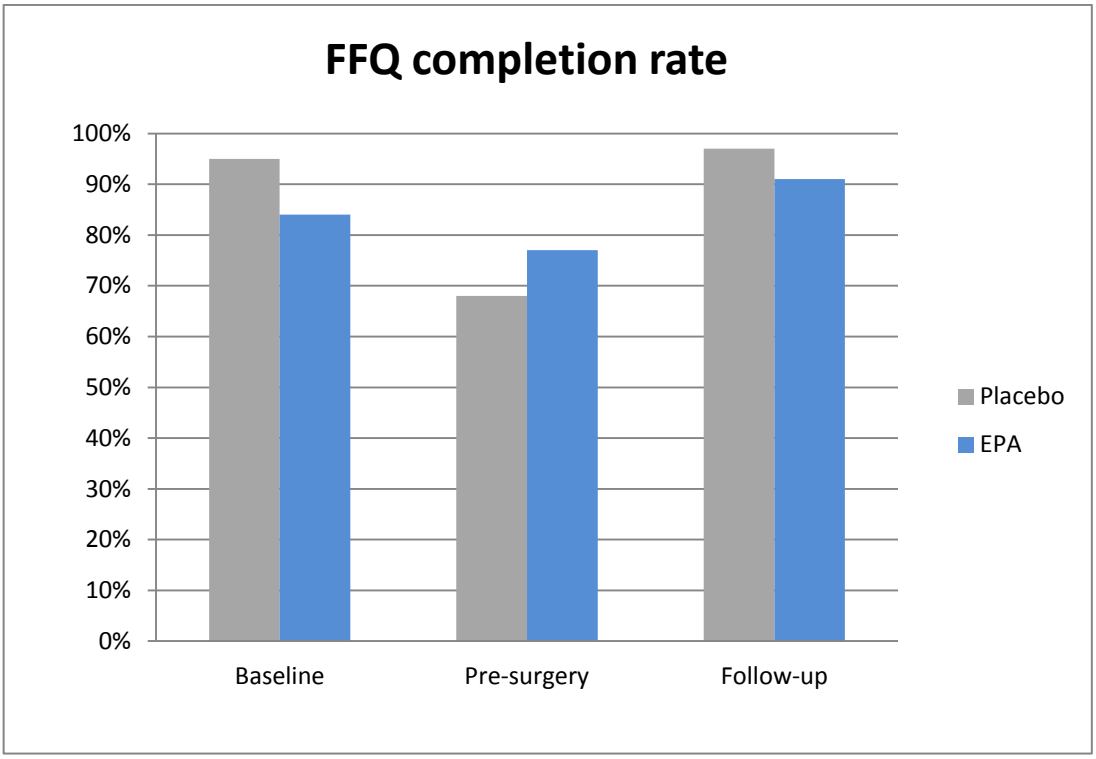
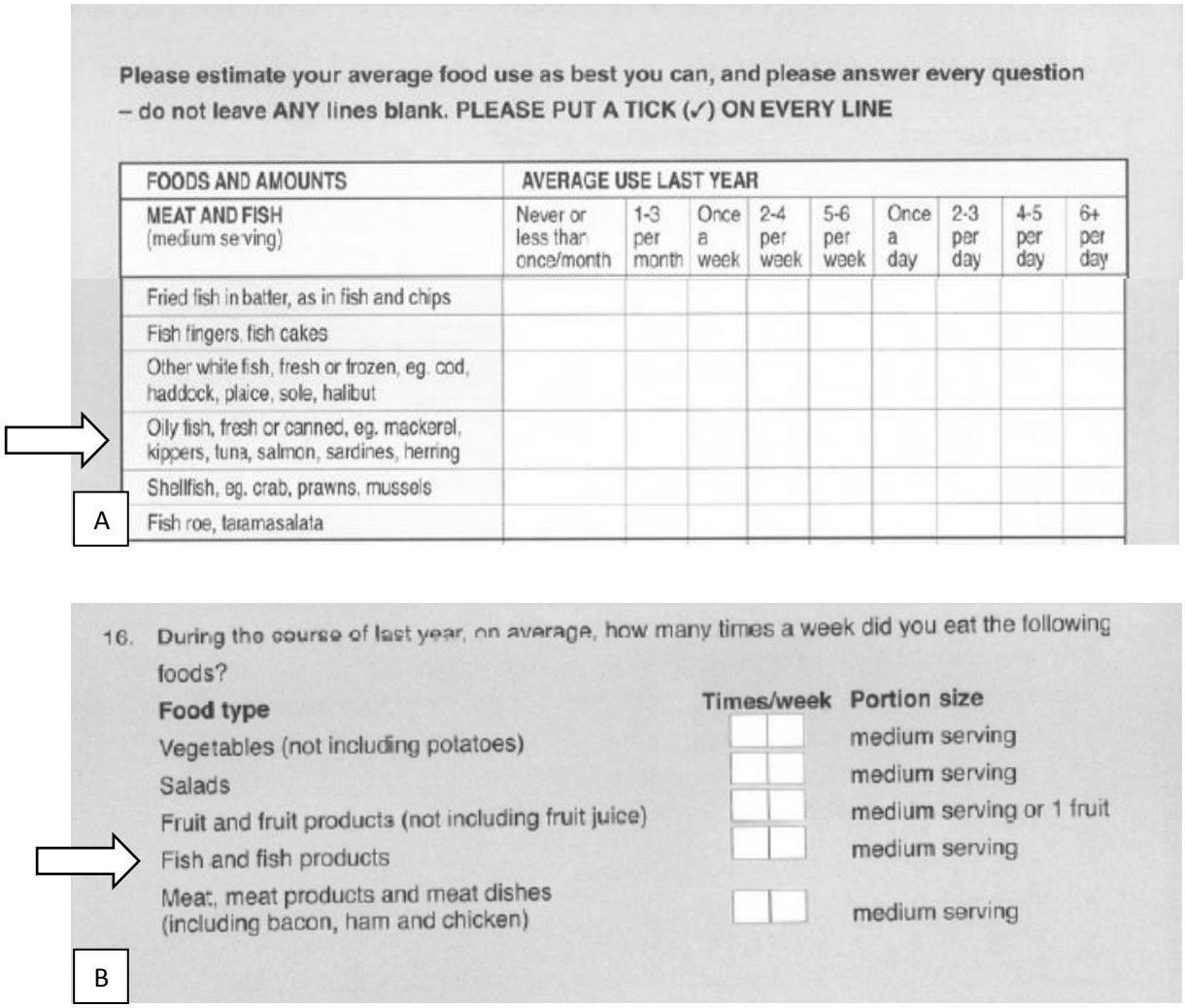


Figure 5.3. FFQ completion rate

Responses to the tick-box question concerning frequency of oily fish intake (Figure 5.4A) were recorded and analysed according to the original questionnaire categories. Patients' responses were analysed to compare oily fish consumption between the two groups at each time-point in the study, as well as comparing changes in individual patients' oily fish consumption during their participation in the study.



Please estimate your average food use as best you can, and please answer every question – do not leave ANY lines blank. PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, eg. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, eg. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, eg. crab, prawns, mussels									
Fish roe, taramasalata									

A

16. During the course of last year, on average, how many times a week did you eat the following foods?

Food type	Times/week	Portion size
Vegetables (not including potatoes)		medium serving
Salads		medium serving
Fruit and fruit products (not including fruit juice)		medium serving or 1 fruit
Fish and fish products		medium serving
Meat, meat products and meat dishes (including bacon, ham and chicken)		medium serving

B

Figure 5.4. Excerpt from the FFQ. Arrows indicate the questions relating to oily fish intake which were analysed for comparison between groups. Patients were asked to reflect on the last 1 month of food use, not 1 year as indicated on the questionnaire.

At baseline, the EPA group were non-significantly higher consumers of oily fish than the placebo group (Figure 5.5), with 61% of patients consuming 1 or more portions per week compared to only 44% of patients in the placebo group (χ^2 p=0.16). This difference was not seen when patients were asked again post-treatment and at follow-up, with 42-47% of patients in each group consuming 1 or more portion per week at these time points. When examining patients in the highest oily fish consumption category of 2-4 portions/week (Figure 5.5), the proportion of patients

in this category was similar between groups at baseline (17% placebo vs. 14% EPA, χ^2 p=0.74). This proportion fell in the placebo group and increased in the EPA group post-treatment (12.5% vs. 19.2%, X^2 p=0.48), and at follow-up this proportion rose in the placebo group and fell in the EPA group (19% vs. 9%, χ^2 p=0.20). Paired data was available for comparison of individual patients' change in oily fish intake between baseline and post-treatment for 26 of 43 patients in the placebo group (60%) and 24 of 38 patients in the EPA group (63%). In the placebo group 27% of patients report a higher category of fish intake post-treatment than at baseline, 27% report a lower category, and 46% reported the same category. In the EPA group, 25% reported a higher category, 35% reported a lower category and 42% reported the same category.

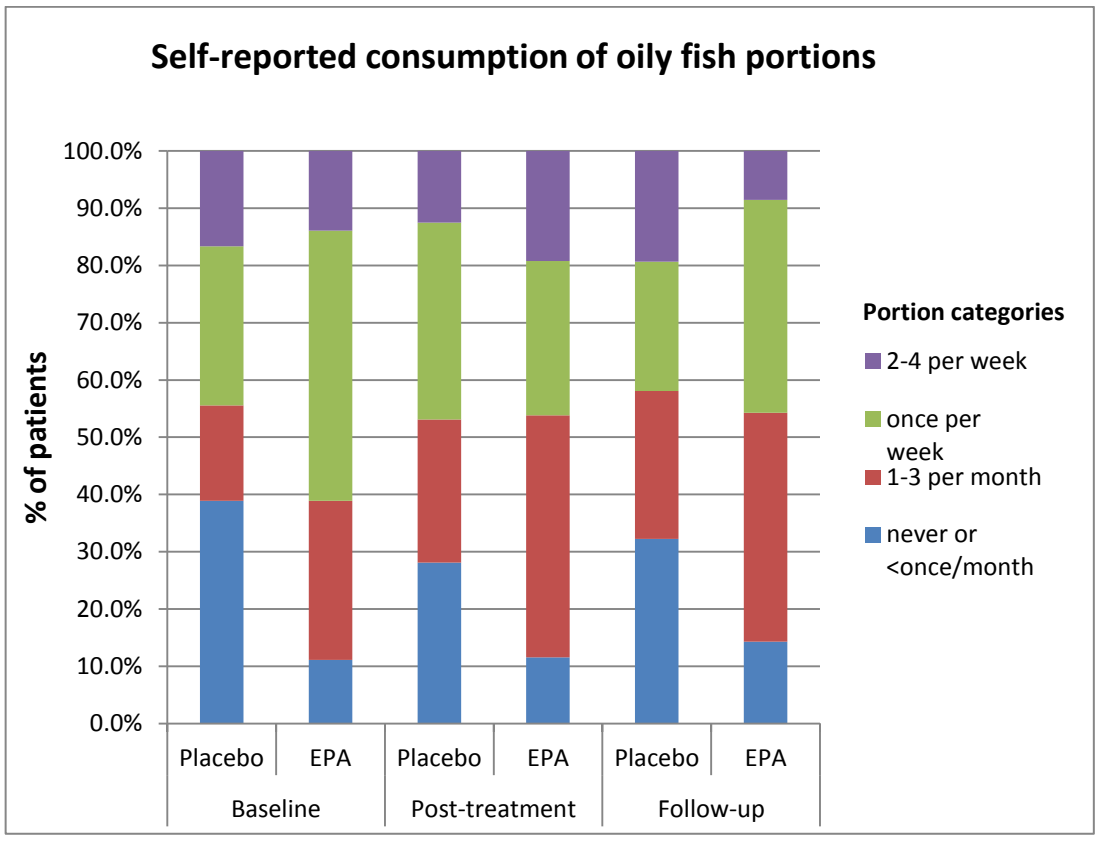


Figure 5.5. Patient-reported consumption of oily fish portions per week/month.

Responses to the question "how many times did you eat fish or fish products" (Figure 5.3B) were analysed as the actual numerical response (Figure 5.6). Consumption of oily fish portions remained constant across the duration of the study in the placebo group (Wilcoxon matched-pairs signed rank test comparing baseline to post-treatment and post-treatment to follow-up, p=0.21 and p=0.56 respectively). The EPA group reported a increase in the number of oily fish portions consumed post-treatment compared to baseline. This did not reach statistical significance when compared to baseline consumption in the EPA group (Wilcoxon matched-pairs signed rank test p=0.79), but was significantly higher than the oily fish consumption in the placebo group post-treatment (Mann-Whitney U test p=0.02).

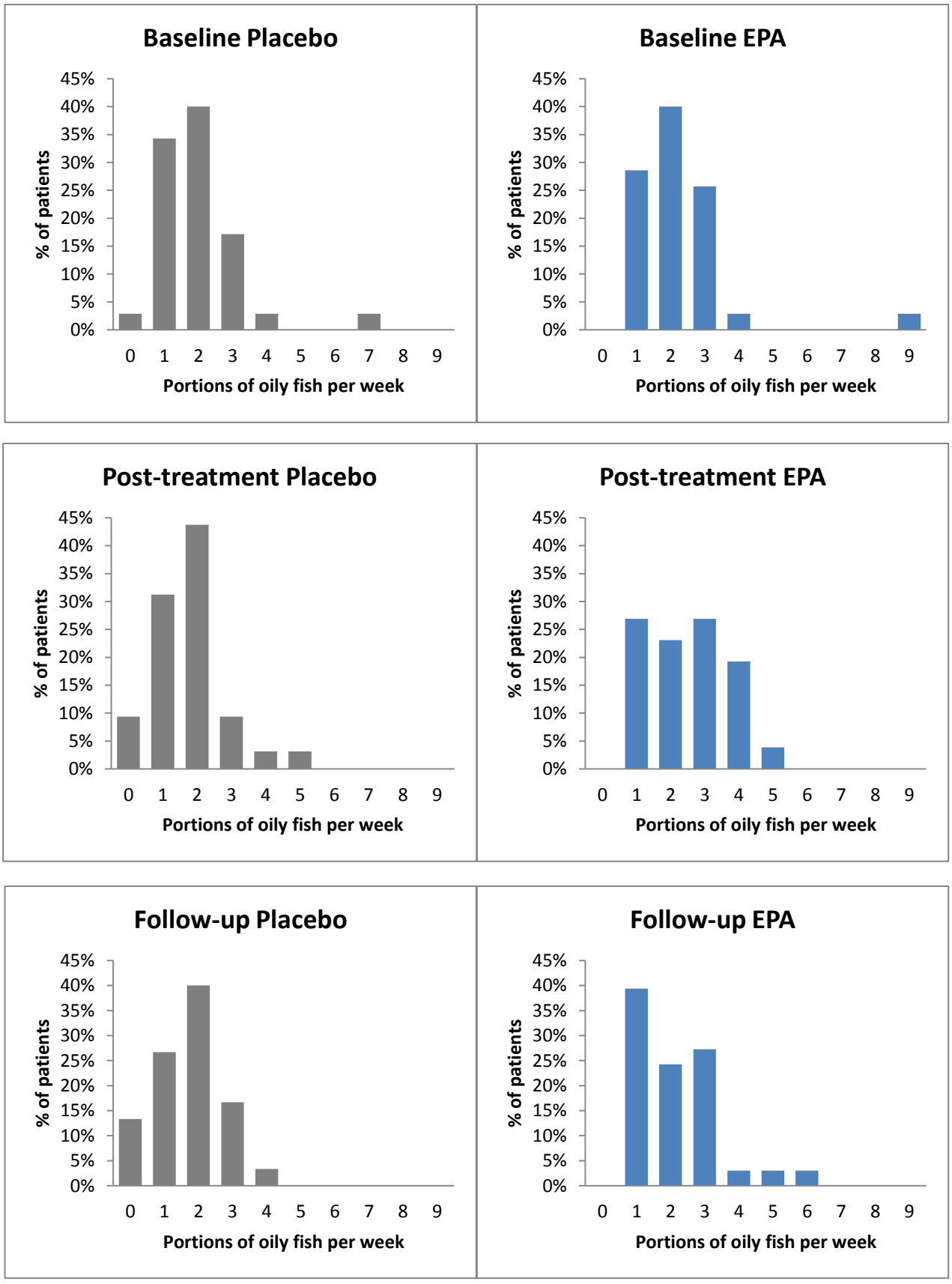


Figure 5.6. Self-reported number of oily fish portions consumed per week at each time point in the trial

5.7 Tumour PUFA levels

Tumour samples were available for analysis of PUFA content for 37 patients in the EPA group and 37 patients in the placebo group (as described in Section 5.2, fresh frozen tissue samples could not be obtained for all patients). Levels of individual PUFAs in each tumour were analysed and expressed as a percentage of the total fatty acid content of the tumour (Table 5.6). Mean PUFA levels were compared between groups (Figure 5.7), with statistical comparisons using unpaired student t-tests. Compared to the placebo group, tumours from the EPA group had 40% higher EPA levels ($p<0.01$), but no significant difference in AA levels. The AA:EPA ratio was reduced in the EPA group compared to the placebo group, predominantly due to a higher level of EPA rather than a lower level of AA in the EPA group. Tumours from the EPA group also had 41% higher levels of the ω-3 PUFA DPA ($p<0.01$) and 11% lower levels the ω-3 PUFA DHA ($p=0.05$).

There was no correlation between duration of EPA treatment and tumoural levels of AA (Pearson's correlation -0.073, $p=0.67$), EPA (correlation 0.031, $p=0.86$), DHA (correlation -0.053, $p=0.74$) or DPA (correlation 0.258, $p=0.12$).

Tumour PUFA	PLACEBO	EPA	% change compared to placebo	p=
EPA	1.30% (+-0.10)	1.82% (+-0.11)	+40%	<0.01*
DPA	1.25% (+-0.08)	1.76% (+-0.09)	+41%	<0.01*
DHA	2.89% (+-0.12)	2.56%(+-0.11)	-11%	0.05*
AA	12.82% (+-0.53)	12.03% (+-0.45)	-6%	0.26
AA:EPA ratio	11.35 (+-0.72)	7.58 (+-0.53)	-33%	<0.01*

Table 5.6. Tumour PUFA content. Individual tumour PUFA levels were analysed and expressed as a % of the total tumour fatty acid content for each tumour. Data represent mean (+-SEM)

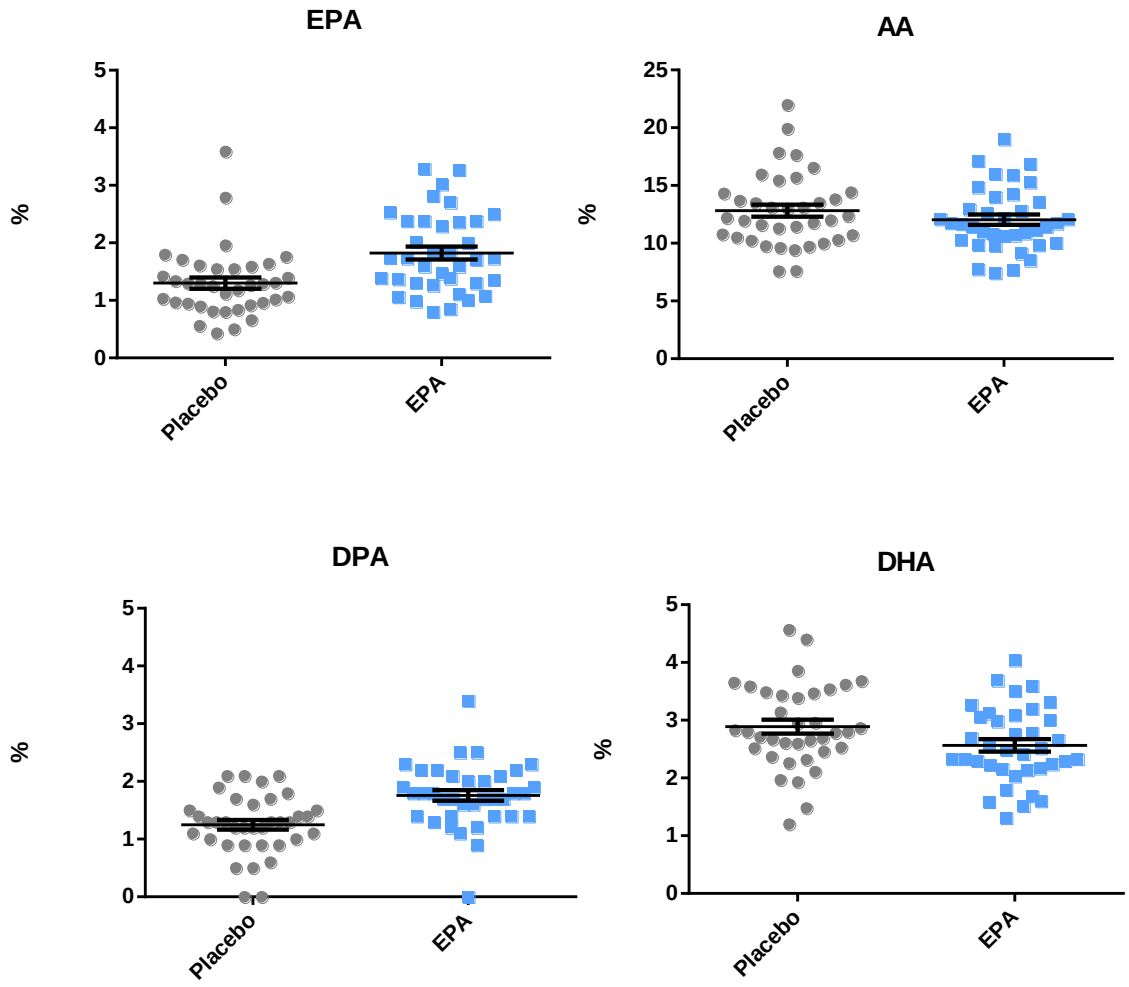


Figure 5.7. Scatter plots of PUFA levels in the tumours from each group. PUFA levels were analysed and expressed as a % of the total tumour fatty acid content for each tumour. Error bars represent mean+-SEM.

Those patients who were either taking fish oil supplements prior to enrolment in the trial, or who were in the highest category of oily fish consumption at baseline (2-4 portions oily fish per week) were identified and highlighted on the tumour PUFA graphs (Figure 5.8 A). This represented 12 patients in the placebo group and 7 patients in the EPA group. Those patients in the placebo group who fell into this category had significantly higher tumoural EPA content than those patients in the placebo group who did not fall into this category (unpaired t-test p=0.04). Re-analysis of tumour PUFA levels excluding these patients shows that the magnitude of EPA increase, AA decrease, and AA:EPA decrease in the EPA group are all more pronounced when these patients are excluded (Table 5.7).

Re-analysis of tumoural PUFA content excluding the 7 patients in the EPA group who were prior fish oil users or in the highest category of oily fish still demonstrated no correlation between duration of EPA treatment and tumour PUFA levels.

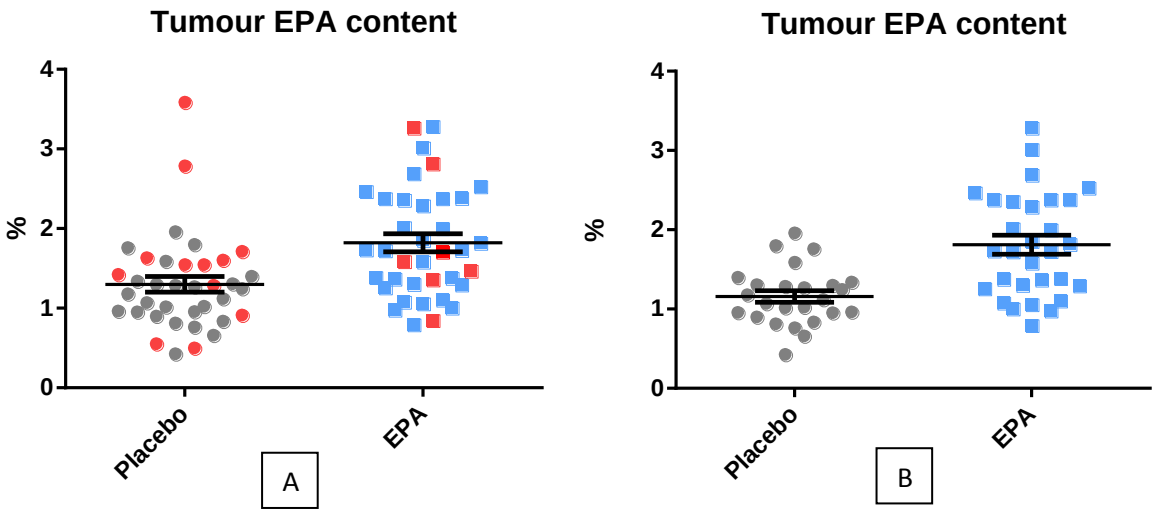


Figure 5.8. Scatter plots of tumoural EPA content. A) Tumours from patients who were pre-existing FO users or who reported the highest category of oily fish intake (2-4portions per week) are marked in red. B) Tumour EPA content after exclusion of those 12 patients in the placebo group and 7 patients in the EPA group highlighted in red in Figure A.

Tumour PUFA	PLACEBO	EPA	%change compared to placebo	p=
AA	13.45% (+-0.70)	12.31% (+-2.74)	-8.5%	0.19
EPA	1.16% (+-0.07)	1.81% (+-0.12)	+56.0%	<0.01
DPA	1.22% (+-0.09)	1.81% (+-0.10)	+48.4%	<0.01
DHA	2.77% (+-0.13)	2.54%(+-0.13)	-8.3%	0.20
AA:EPA ratio	12.42(+-0.78)	7.66 (+-0.57)	-38.3%	<0.01

Table 5.7. Subanalysis of tumour PUFA content. Mean (+-SEM) levels of individual PUFAs in the tumours from each group after exclusion of patients who were either taking fish oil supplements or in the highest category of oily fish consumption prior to enrolment in the trial. Individual tumour PUFA levels were analysed and expressed as a % of the total tumour fatty acid content for each tumour.

5.8 Tumour PGE_2 and PGE_3 levels

Tumour samples were available for analysis of PGE_2 and PGE_3 levels for 37 patients in the EPA group and 37 patients in the placebo group (see section 5.2). PGE_2 was detected in all tumour samples (Figure 5.9). Median (range) PGE_2 content of tumours was 6.62ng/g (0.44-33.40ng/g) in the EPA group and 5.87ng/g (1.89-447.00ng/g) in the placebo group. There was no significant difference in tumoural PGE_2 content between the two groups (MWU p=0.68). Due to the potential confounding factor that aspirin irreversibly acetylates COX, one of the pathways by which EPA is proposed to exert anti-CRC activity, subanalysis excluding patients who were taking concurrent aspirin was performed for all mechanistic analyses. This subanalysis showed no difference in tumoural PGE_2 content between the EPA and placebo groups (median 6.9 vs. 7.2ng/g respectively, MWU p=0.77).

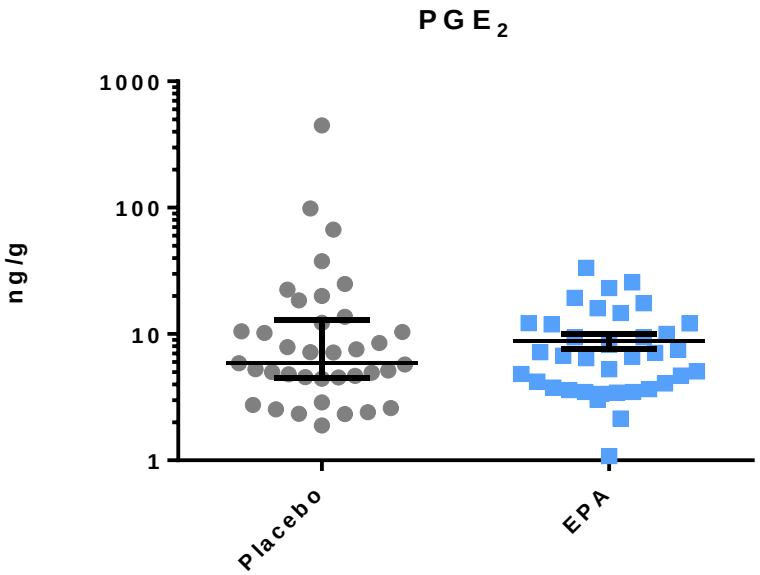


Figure 5.9. PGE_2 content of tumours. Error bars represent median+-IQR.

PGE_3 was detected in only one tumour sample; that with the highest level of PGE_2. PGE_3 level in this tumour was 21.10ng/g. If present, PGE_3 in all other tumour samples was present at concentrations below the limit of detection (<10ng/g).

There was no correlation between duration of EPA supplementation and tumoural PGE_2 level (Pearson correlation -0.18, p=0.28). There was a weak inverse relationship between tumoural EPA level and tumoural PGE_2 (Figure 5.10; correlation -0.30, p=0.01), but not between tumoural AA level and tumoural PGE_2 (correlation -0.11, p=0.36).

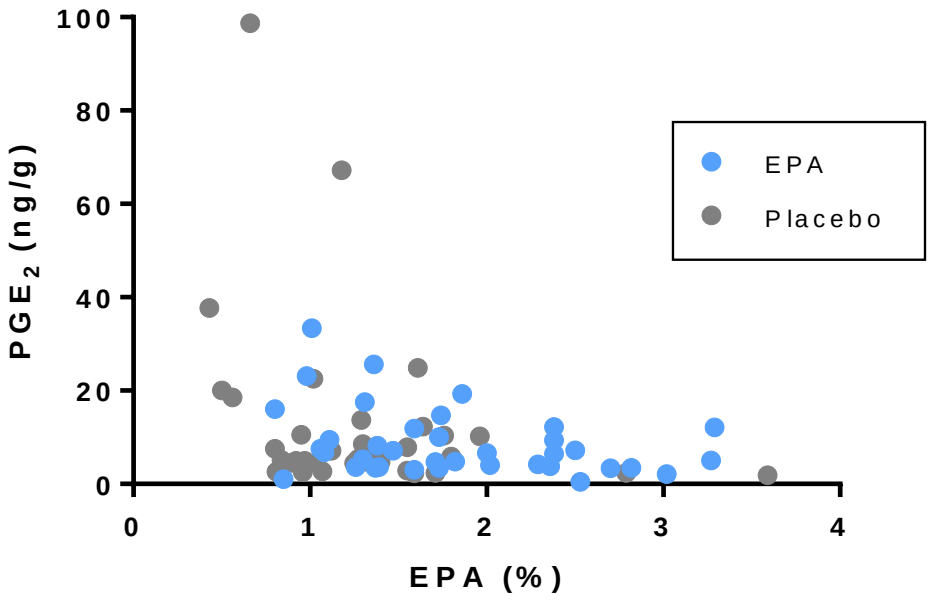


Figure 5.10. Correlation between tumoural PGE_2 level and tumoural EPA content

5.9 Ki-67 Proliferation index

Immunohistochemistry for Ki-67 was performed on 78 tumour samples (EPA n=37, placebo n=41) in five batches. Three sections, with a range of staining intensity, were chosen from the first batch and sequential sections from these tumours used as internal controls for each subsequent batch. Clearly identifiable distinct morphological regions of these internal controls were identified and photographed in each batch and scored for PI. Subjectively, there was good consistency of staining with a similar pattern and intensity of staining to the naked eye across the five batches for each internal control (Figure 5.11). Objectively there was low variance in the PI when calculated for each internal control across the five batches (Figure 5.11).

Regions of interest (ROI) in the sample sections were then identified and scored as described in Chapter 4.7.1. The Ki-67 antigen is a nuclear protein expressed in all proliferating cells during late G1, S, M and G2 phases of the cell cycle. Figures 5.11 and 5.12 show that the pattern of Ki-67 staining is, as expected, localised to cell nuclei with no staining of acellular, mucinous or stromal regions of tumour. Figure 5.12 shows three representative ROIs, one from a typical cellular tumour with dense cellular staining, one from a tumour with a greater amount of stroma and more patchy areas of dense nuclear staining, and the other from a mucinous tumour with very sparse nuclear staining. Mucinous tumours were found in 11 patients (13.6%), six in the EPA group (16.2%) and 5 in the placebo group (12.2%). These mucinous tumours with sparse cellular areas typically required more than one ROI to be photographed and scored to satisfy the requirement of counting >500 cells. One patient in the placebo group had a very mucinous tumour with no identifiable tumour cells in the section. It was not possible to score this section for Ki-67 PI. Ki-67 PI was therefore scored for 40 patients in the placebo group and 37 patients in the EPA group.

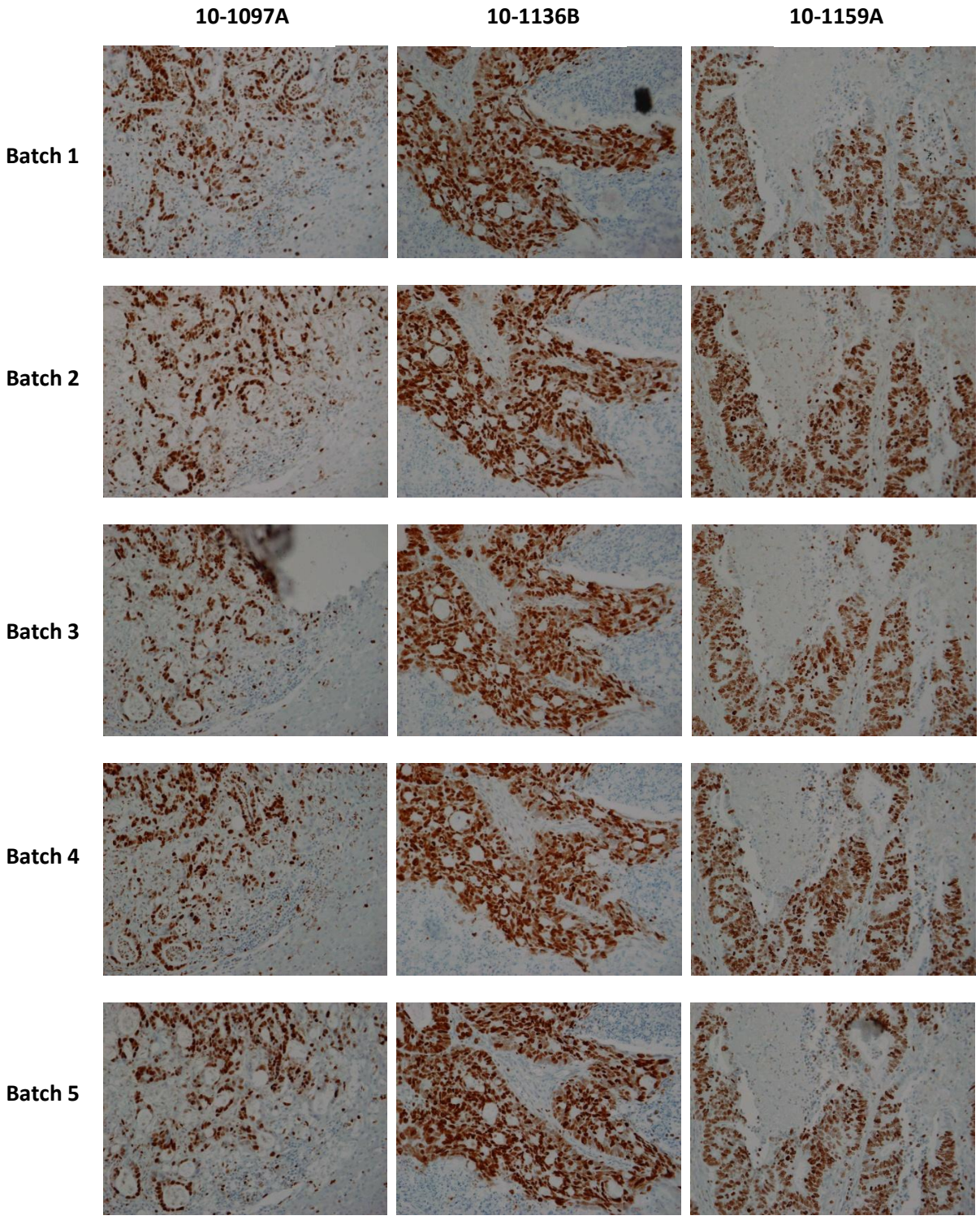


Figure 5.11. Internal control sections in each batch of Ki-67 staining.

This Figure and legend is continued overleaf.

Internal control ID	Mean PI	SEM	Range	Coefficient of variance
10-1097A	77.6%	0.7	75.9 - 79.1	0.02%
10-1136B	93.7%	0.7	92.5 - 96.1	0.02%
10-1159A	83.0%	0.8	81.1 - 85.8	0.02%

Figure 5.11. Internal control sections in each batch of Ki-67 staining. Three tumours with a range of Ki-67 staining were selected for use as internal controls. Adjacent sections of these tumours were used as internal controls in the five batches of Ki567 staining. A distinctive ROI was identified in each internal control, and this ROI scored for proliferation index (PI) as a comparison of staining across the 5 batches. The photographs of these ROIs demonstrates the consistency of staining of each internal control across the 5 different batches of Ki-67 IHC. The table shows the mean PI and variance for each internal control.

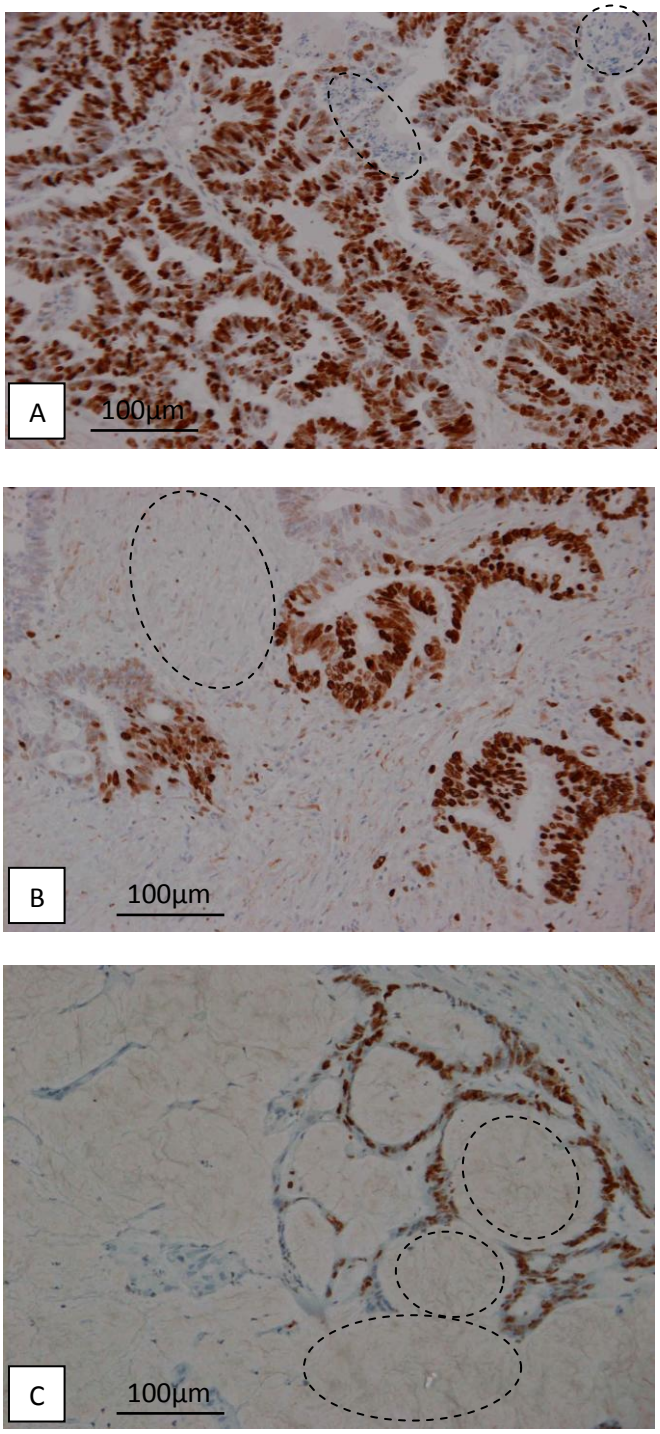


Figure 5.12. Examples of tumour staining for Ki-67. These regions of interest show tumours with (A) a predominantly cellular architecture, (B) larger amounts of stroma, and (C) a predominantly mucinous architecture. Note that regions of non-tumour cells were not counted in the calculation of PI. Examples of these regions are marked in the dotted areas representing in (A) inflammatory infiltrate, in (B) stroma, and in (C) mucin.

The PIs for each group are illustrated in Figure 5.13. The mean PI in the placebo group was 72.96% +- 2.36 SEM (range 33.51% – 96.20%). The mean PI in the EPA group was 70.70% +- 2.65 SEM (range 32.77% - 90.92%). There was no significant difference in PI between the two groups (p=0.68).

The variable duration of EPA supplementation in this study was a potential confounder for the Ki-67 proliferation analysis. I therefore performed a linear regression analysis to investigate the effect of EPA treatment duration on tumour Ki-67 PI. Duration of EPA supplementation was not associated with the Ki-67 PI (Figure 5.14, p=0.75). It was also hypothesised that EPA might affect tumour proliferation by increasing tumour EPA content and driving a reduction in tumour PGE_2 levels. I therefore performed linear regression analysis to investigate for any association between these variables. Tumour Ki-67 PI was not associated with either tumour EPA content (p=0.95) or with tumour PGE_2 levels (p=0.64). Subanalysis performed after exclusion of patients who were taking concurrent aspirin also showed no difference in Ki-67 PI content between the EPA and placebo groups (p=0.86).

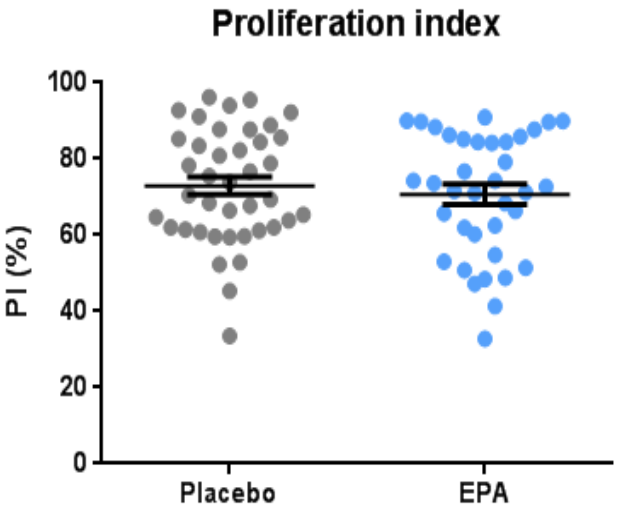


Figure 5.13. Ki-67 proliferation index. Error bars represent mean+- SEM.

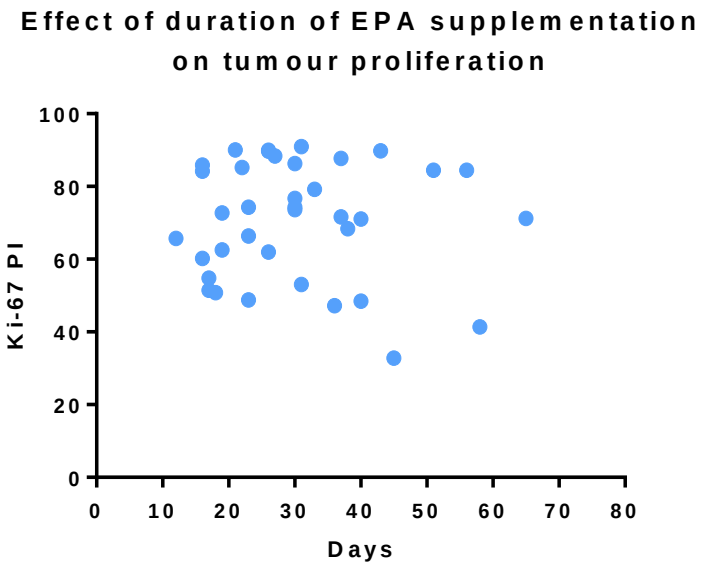


Figure 5.14. Relationship between the duration of EPA supplementation and tumour Ki-67 proliferation index.

5.10 neo-CK18 Apoptosis index

Validation of the automated image analysis algorithm (Chapter 4.7.3) was performed on a total of 20 representative ROIs taken from 3 separate tumour sections. The image analysis algorithm recorded both the number of positive pixels and the number of "objects" i.e. the number of discrete, separate areas of positive pixels. From this, the percentage positive pixels (positive pixels / total pixels x 100) and an object count standardised to tumour size (number of objects per million pixels) were calculated. Note that these measures are not referred to as an apoptosis index, since neither measure is a true cell count of positive and negatively stained cells.

After reviewing the markup images from the automated image analysis (examples shown in Figure 5.15), it was clear that the areas of DAB staining detected by the algorithm included both discrete apoptotic epithelial cells in areas of glandular epithelium and extruded neo-CK18 positive cells and cell debris in more necrotic areas of tumour (Figure 5.15). By contrast, areas of light brown non-specific staining in acellular areas of the tumour were not detected. I decided that it was justifiable to include both of these detected regions in a measure of apoptosis, since counting only discrete apoptotic cells would exclude from the analysis those tumours with larger areas of necrotic apoptotic debris. This would risk excluding large areas of staining which may be the result of pro-apoptotic activity of EPA. Secondly, to the naked eye the sections of low, medium and high intensity of apoptosis staining seemed to correlate with the distribution of low, medium and high detection on the algorithm markups. I therefore proceeded to perform a formal comparison of manual AI scoring of the ROIs against each of the two measures of apoptosis obtained from the automated image analysis.

There was a strong positive correlation (Figure 5.16) between the manual AI and the algorithm “object count” (Spearman $r=0.81$, $p<0.01$), and between the manual

AI and the algorithm “% positive pixels” (Spearman r=0.80, p<0.01). Because a) the two measures of apoptosis obtained from the automated image analysis provide a slightly different means of quantifying apoptosis, b) neither of them represent a true apoptosis index, and c) they both correlated well with the manual cell count for AI, I decided to use both measures when comparing apoptosis between the EPA and placebo groups. Furthermore, although the measures of apoptosis of the automated analysis are not directly comparable to AI scores in other studies, the manual AI scores of 0-7% in the tumour sections are comparable with the hotspot AI scores (0.07-8.44%) in the similarly designed rofecoxib study by Fenwick *et al*,(271) and with AI scores in various other studies of CRCLM by Marshall *et al* (2-8%),(310) Howells *et al* (3% in control arm),(311) Backus *et al* (13% in control arm)(312) and Tatebe *et al* (4.8-8.2%).(313) Therefore any changes in apoptosis scoring found on automated analysis could be considered representative of changes in AI scores in other studies, even though these different methods cannot be directly compared.

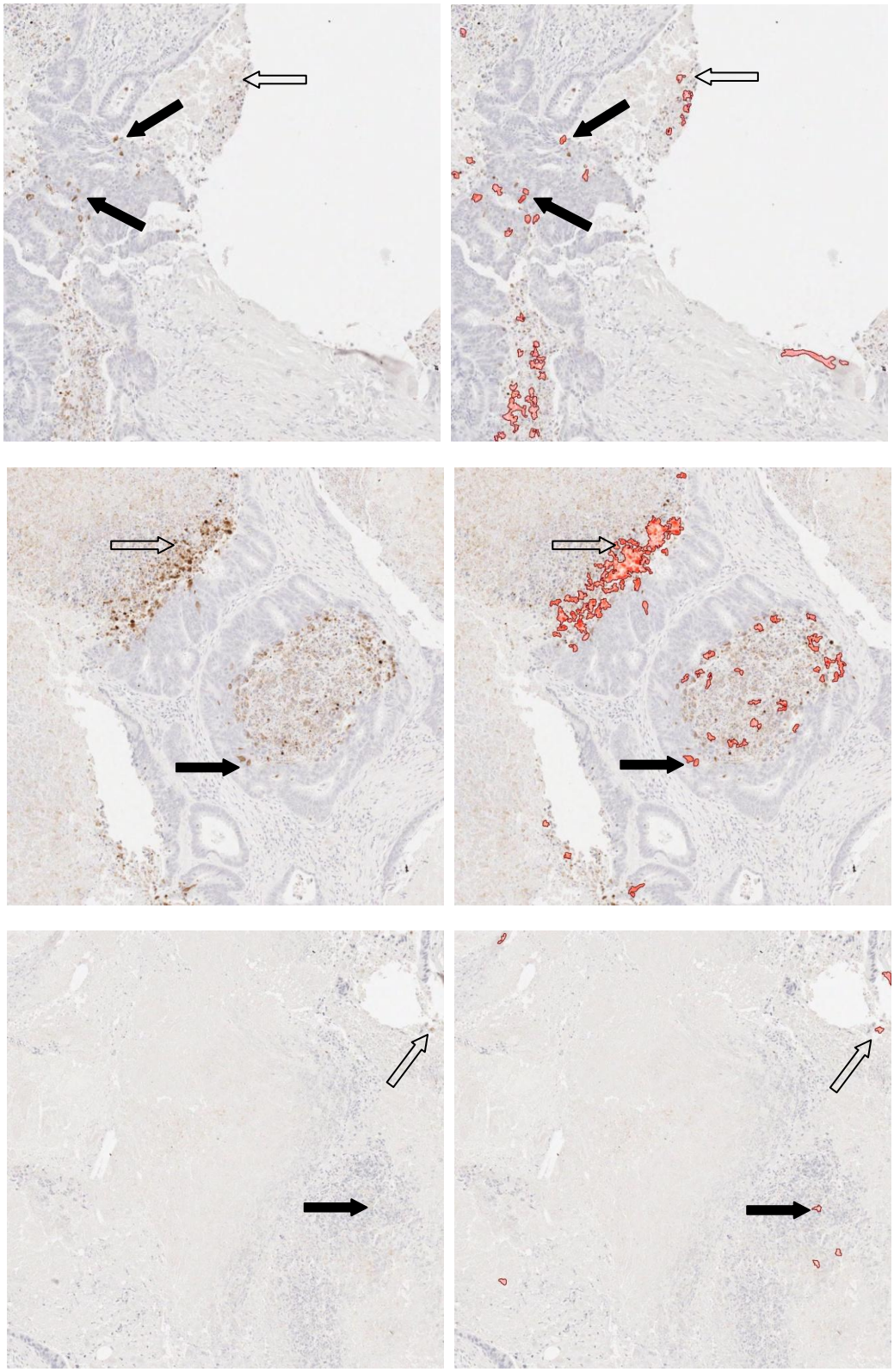
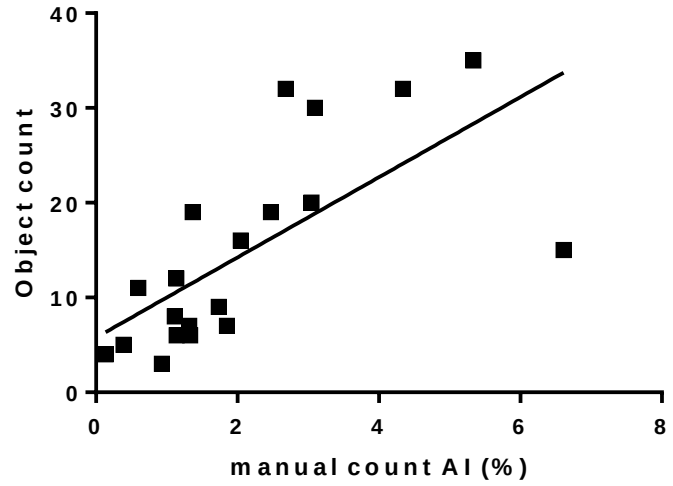
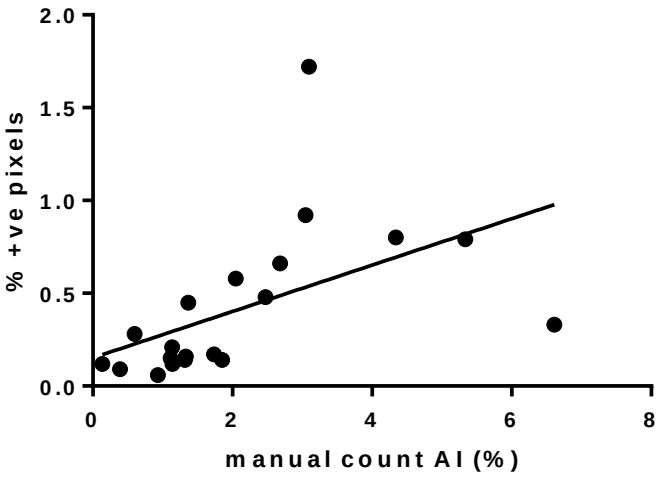


Figure 5.15. Representative sections of neo-CK18 staining. The neo-CK18 stained sections are shown (left) together with the corresponding automated image analysis mark-ups (right). Areas of apoptosis detected by the algorithm are highlighted in red. Note that the algorithm has detected both apoptotic epithelial cells in glandular epithelium (solid black arrows) as well as positive cells or cell debris in necrotic areas of tumour (open arrows).



Spearman r = 0.8080
P < 0.0001



Spearman r = 0.8006
P <0.0001

Figure 5.16. Correlation between manual and automated scoring of neo CK-18 staining. Lines represent line of best fit.

Immunohistochemistry for neo-CK 18 was performed on 78 tumour samples (EPA n=37, placebo n=41) in 5 batches. Three sections, with a range of staining intensity, were chosen from the first batch and sequential sections from these tumours used as internal controls for each subsequent batch.

Subjectively the consistency of staining across the five batches was good, with a similar pattern and intensity of staining to the naked eye across the five batches for each internal control (Figure 5.17). However, there was wide variation in both apoptotic object count and apoptotic area (% positive pixels) across the five batches for each of the internal controls (Table 5.9). The high coefficient of variance in the internal controls was more likely due to the very low count of stained apoptotic bodies across a large area, with small variations in apoptotic count resulting in a large coefficient of variance, rather than due to poor reproducibility of the assay.

Internal control ID	Object count per million pixels			
	Mean	SEM	Range	Coefficient of variance
11-0266	4.26	1.21	1.44 - 8.33	65.6%
11-0271	5.05	1.05	1.54 - 6.46	46.3%
11-0586	13.91	1.24	10.70 - 15.90	19.9%

Internal control ID	% positive pixels			
	Mean	SEM	Range	Coefficient of variance
11-0266	0.0028	0.0011	0.0004 - 0.0063	85.9%
11-0271	0.0017	0.0004	0.0004 - 0.0029	54.0%
11-0586	0.0102	0.0010	0.0080 - 0.0133	21.6%

Table 5.8. Measures of apoptosis for the internal control sections stained for neo-CK18 in each batch.

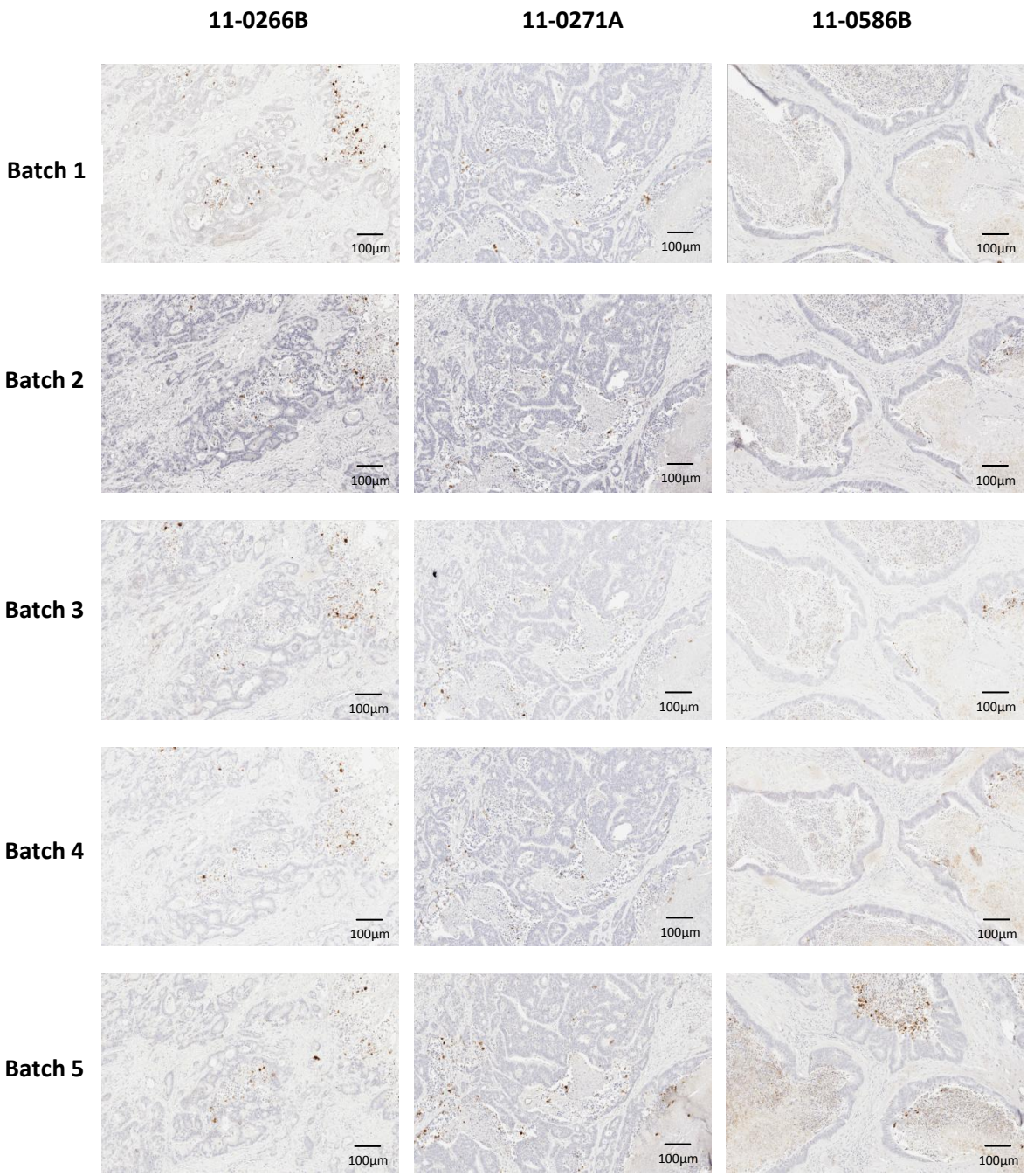


Figure 5.17. Internal control sections in each batch of neo-CK 18 staining. Three tumours with a range of neo-CK 18 staining were selected for use as internal controls. Adjacent sections of these tumours were used as internal controls in the five batches of neo-CK 18 staining. A distinctive ROI was identified in each internal control, photographed, and presented here as an example of the consistency of staining between batches. Note that the measures of apoptosis were scored for the whole of each slide, not just the representative ROIs in this figure.

Apoptosis was scored for all tumour samples. The measures of apoptosis for each group are illustrated in Figure 5.18. The median object count per million pixels was 4.98 (interquartile range, IQR 2.43 - 10.52) in the EPA group and 3.83 (IQR 2.31 - 8.23) in the placebo group (MWU p=0.56). The median % positive pixels was 0.0028 (+-0.0004 SEM) in the EPA group and 0.0020 (+- 0.0004 SEM) in the placebo group (MWU p=0.53). There was no significant difference in the measures of apoptosis between the two groups. Subanalysis performed after exclusion of patients who were taking concurrent aspirin also showed no difference in object count or % positive pixels between the EPA and placebo groups (p=1.00 and p=0.76 respectively).

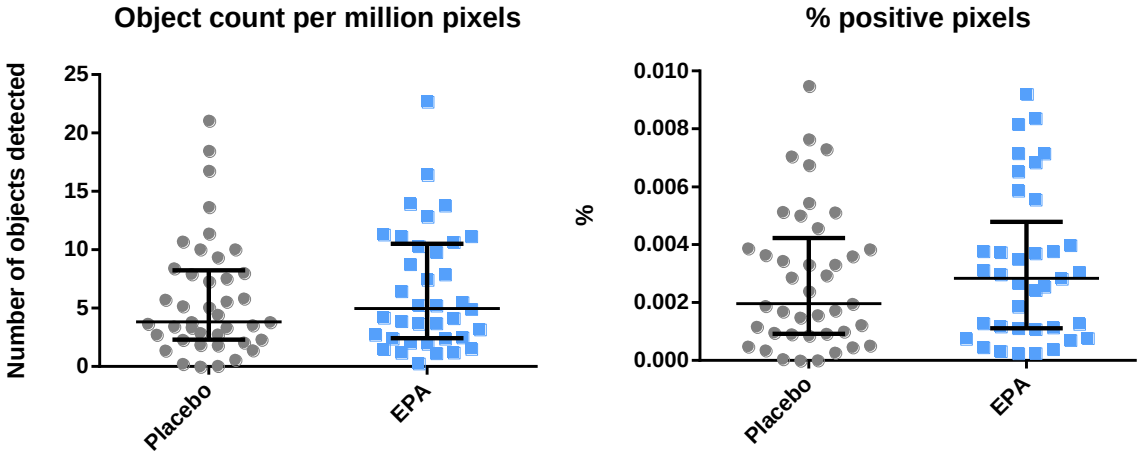


Figure 5.18. Measures of neo-CK 18 positive apoptosis detected by automated image analysis. Two measures of apoptosis, the standardised object count and percentage positive pixels were recorded for each tumour. Error bars represent median +/- IQR.

5.11 CD31 Microvessel density

Immunohistochemistry for CD31 was performed on 78 tumour samples (EPA n=37, placebo n=41) in 5 batches. Examples of the automated CD31-stained vessel detection are shown in Figure 5.19 and Figure 5.20.

Three sections, with a range of staining intensity, were chosen from the first batch and sequential sections from these tumours used as internal controls for each subsequent batch. Subjectively there was good consistency of staining across the five batches (Figure 5.21) although there was wide variation in both tumour vessel density and percentage vascular area across the five batches for each of the internal controls (Table 5.10).

Internal control ID	Vessel density (vessels per million square microns)			
	Mean	SEM	Range	Coefficient of variance
11-0587	157.46	18.21	105.49 - 198.45	25.9
11-0261B	116.33	27.37	33.62 - 204.69	52.6
11-0263B	26.52	4.89	18.40 - 45.61	41.1

Internal control ID	% vascular area			
	Mean	SEM	Range	Coefficient of variance
11-0587	0.29	0.04	0.17 - 0.37	29.8
11-0261B	0.38	0.10	0.09 - 0.66	55.9
11-0263B	0.04	0.02	0.03 - 0.07	43.2

Table 5.9. Measures of tumour vascularity for the internal control sections stained for CD31 in each batch.

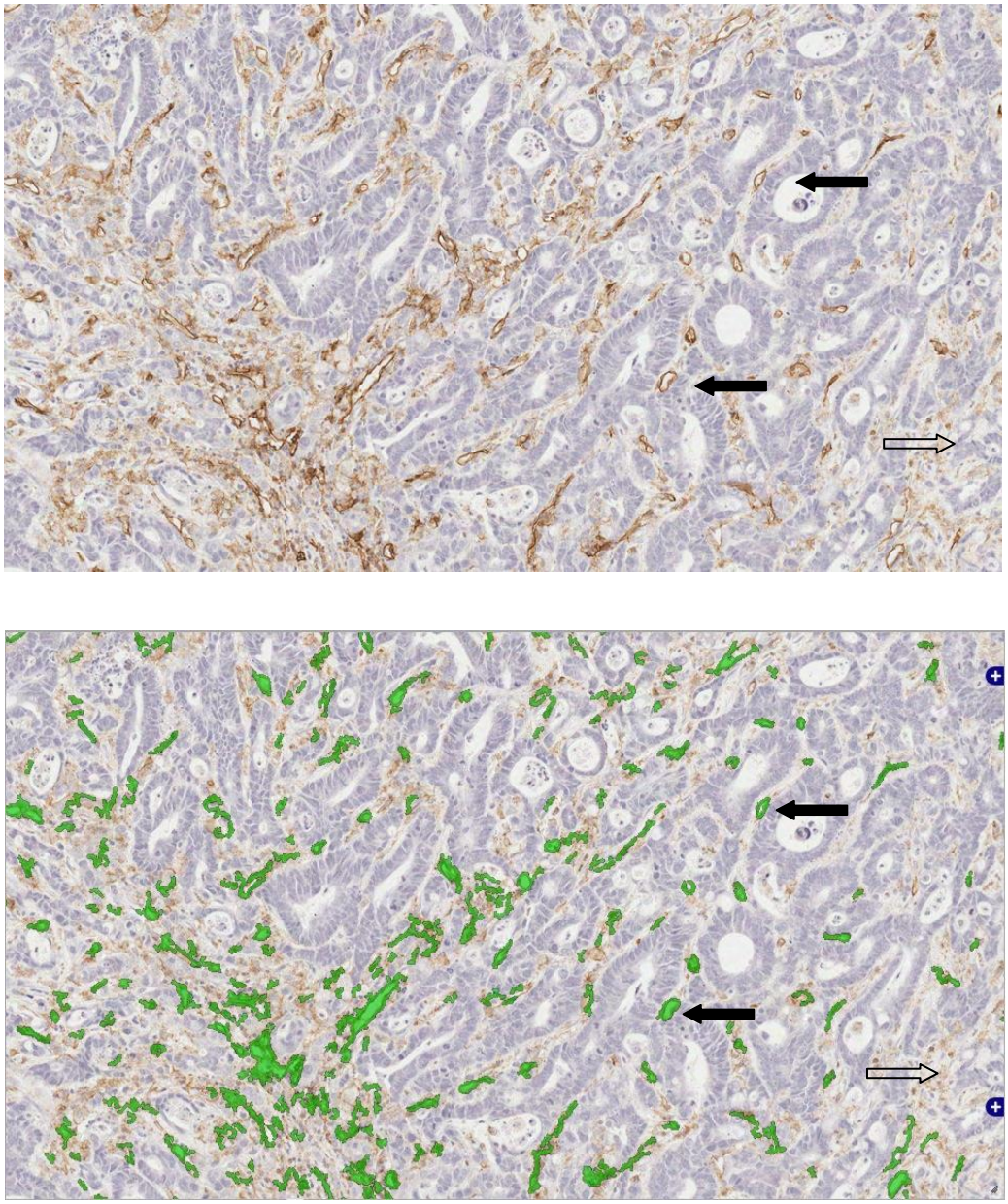


Figure 5.19. Representative sections of immunohistochemistry for CD31 tumour vascularity. CD31-stained endothelial vessels are visible in brown, and those vessels detected by the automated image analysis algorithm are highlighted in green on the corresponding markup image (examples of corresponding vessels marked with solid black arrows). Note that the algorithm does not highlight areas of non-specific DAB staining as endothelial cells (examples marked with open arrows).

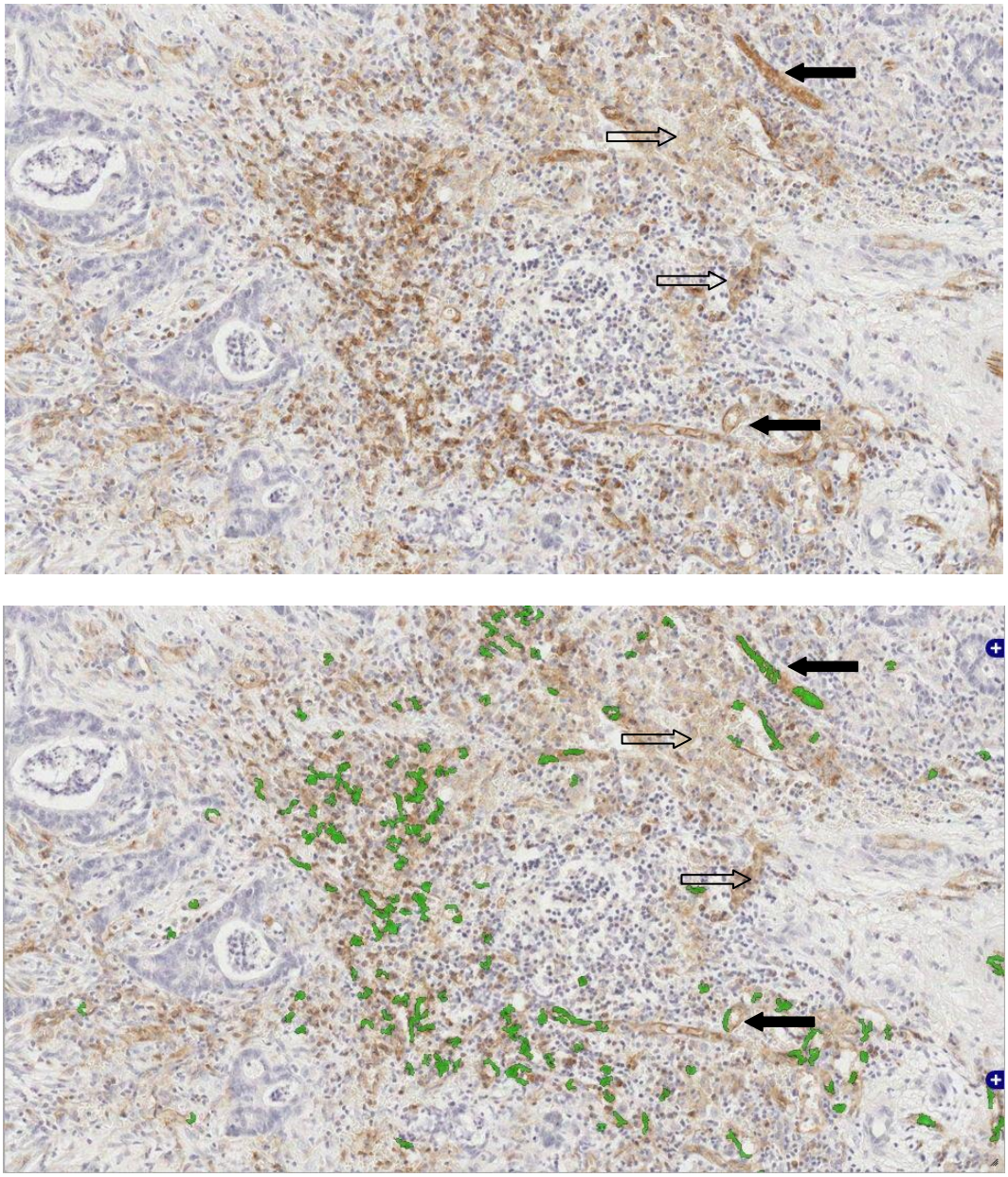


Figure 5.20. Further representative sections of immunohistochemistry for CD31 tumour vascularity. CD31-stained endothelial vessels are visible in brown, and those vessels detected by the automated image analysis algorithm are highlighted in green on the corresponding markup image (examples of corresponding vessels marked with solid black arrows). Note that the algorithm does not highlight areas of non-specific DAB staining as endothelial cells (examples marked with open arrows), which are more extensive in this section than in Figure 5.18.

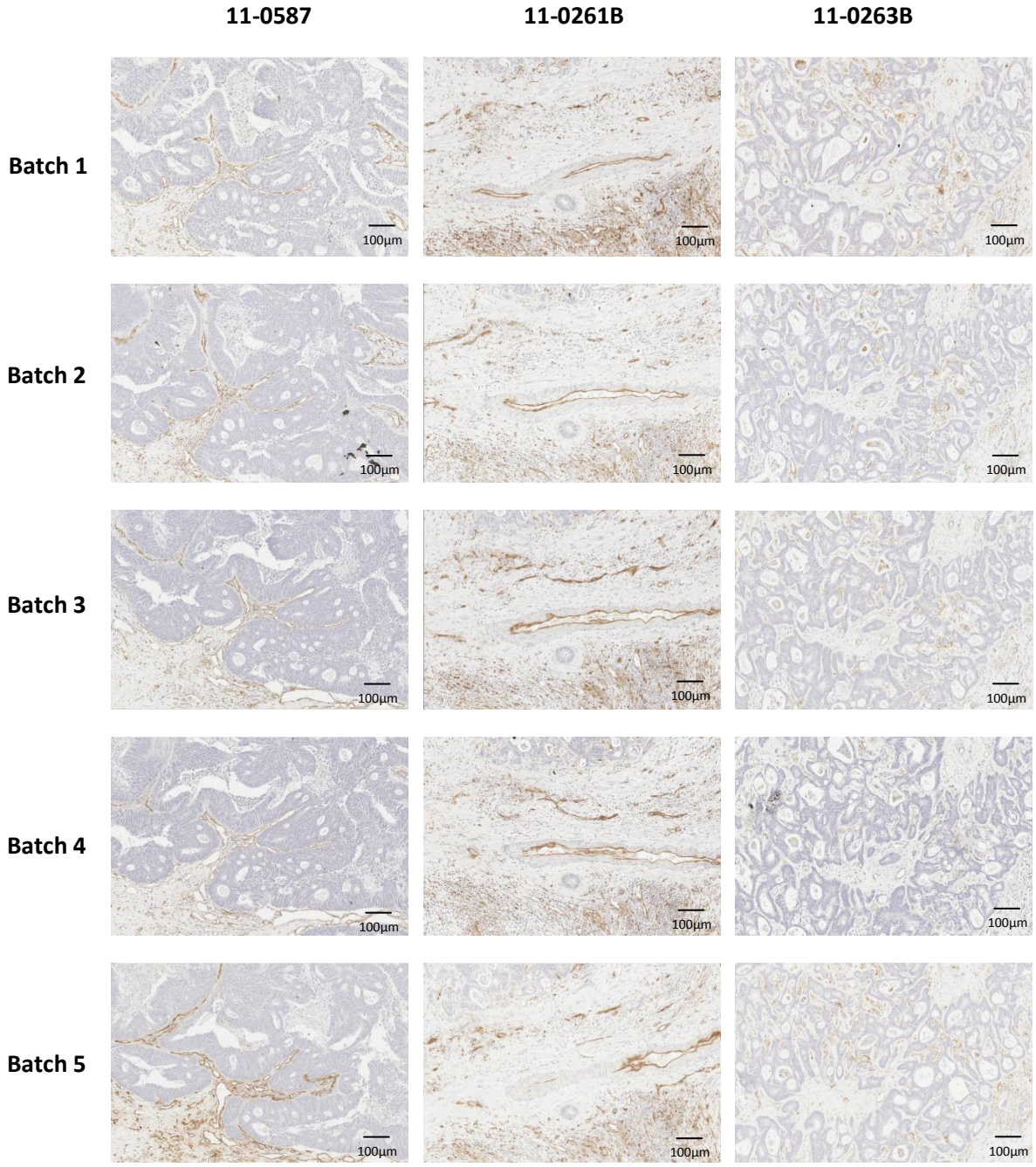


Figure 5.21. Internal control sections in each batch of CD31 staining. Three tumours with a range of CD31 staining were selected for use as internal controls. Adjacent sections of these tumours were used as internal controls in the five batches of CD31 staining. A distinctive ROI was identified in each internal control, photographed, and presented here as an example of the consistency of staining between batches. Note that the measures of tumour vascularity were scored for the whole of each slide, not just the representative ROIs in this figure.

In the EPA group, one section was scanned out of focus and was unable to be scored, and in the placebo group two tumour sections were erroneously scanned in duplicate in place of two other tumour sections. MVD was therefore scored for 36 tumours in the EPA group and 39 tumours in the placebo group. The measures of tumour vascularity for each group were log transformed to normalise the distribution, then summary statistics were converted back to the original scale for presentation in numerical and graphical form (Figure 5.22). The geometric mean vessel density in the EPA group was 18.54 vessels per million square microns compared to 19.50 vessels per million square microns for the placebo group (p=0.87), and the geometric mean % vascular area was 0.030% in the EPA group compared to 0.034% in the placebo group (p=0.74). There was no correlation between indices of tumour vascularity and either duration of treatment or tumoural PUFA content. Subanalysis performed after exclusion of patients who were taking concurrent aspirin also showed no difference in vessel density or % vascular area between the EPA and placebo groups (p=0.66 and p=0.76 respectively).

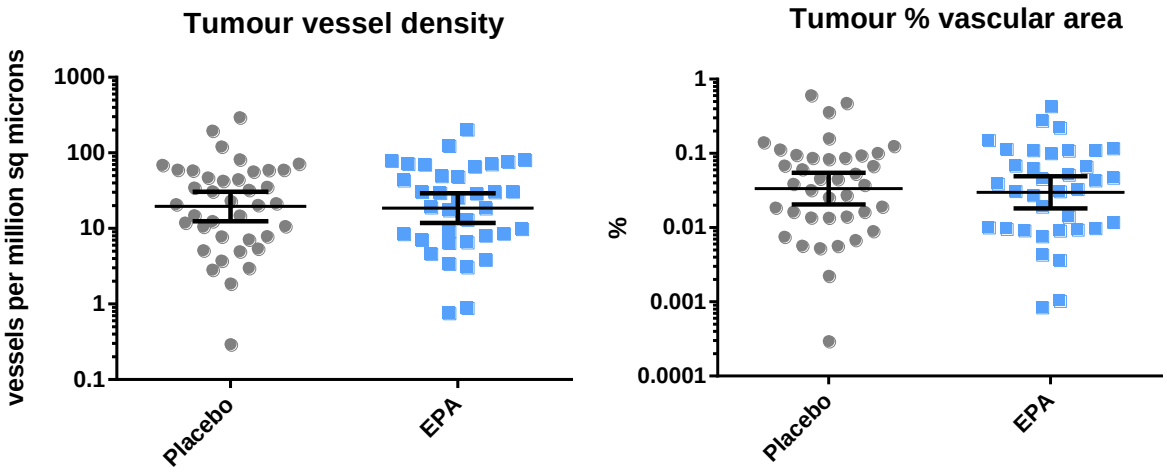


Figure 5.22. Tumour vessel density and % vascular area. Error bars represent geometric mean and 95% confidence interval.

Subanalysis was performed excluding those patients who were either pre-existing FO users or who were in the highest oily fish consumption category at baseline. These patients are highlighted in red in Figure 5.23A. When these patients were excluded (Figure 5.23B) and the data re-analysed there was a trend to reduced tumour vascularity in the EPA group compared to placebo. Geometric mean tumour vascular area was 48% lower in the EPA group lower compared to placebo (0.027% vs. 0.052%, p=0.07), and geometric mean tumour vessel density was 43% lower in the EPA group compared to placebo (16.87 vs. 29.58, p=0.09).

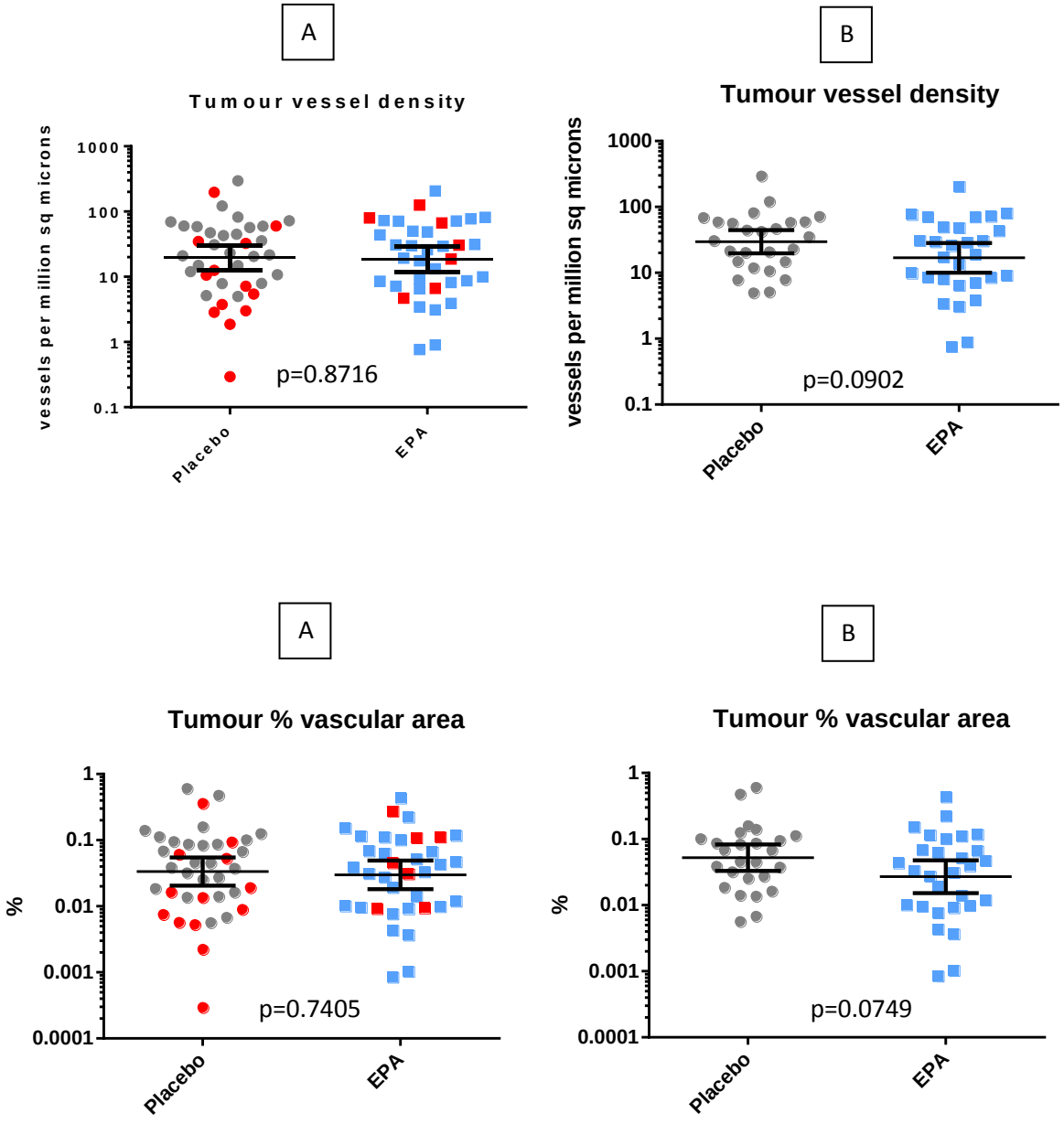


Figure 5.23. Subanalysis of tumour vascularity. A) Patients who were already taking ω-3 PUFA supplements prior to inclusion in The EMT Trial, or who reported the highest category of oily fish consumption at baseline, are highlighted in red. B) Sub-analysis comparing tumour vascularity in the EPA and placebo groups was then performed after excluding these patients. Error bars represent geometric mean and 95% confidence interval.

5.12 Urinary PGE-M

Urine samples were not analysed from the seven patients withdrawn from the study before undergoing surgery and the one patient who had a histological diagnosis of HCC. Baseline urine samples were therefore analysed for all 80 patients with CRCLM who underwent surgery (EPA n=38, placebo n=42). Pre-operative urine samples were not obtainable from the two patients in the placebo group whose surgery was brought forward without my knowledge. Pre-operatively urine samples were therefore analysed for 78 patients (EPA n=38, placebo n=40). One patient in the EPA group was unable to provide a final urine sample at the final study visit, and final urine samples were not obtainable from the seven patients in the placebo group and two patients in the EPA group who were discharged from the Hepatobiliary Unit's care after recovering from their surgery and did not attend the final study visit. Follow-up urine samples were therefore analysed for 68 patients (EPA n=35, placebo n=33).

Urinary creatinine levels were below the limit of detection (<1.5mmol/l) in four samples in the placebo group and a technical problem with the MS/MS apparatus prevented detection of PGE-M in one sample in the EPA group. PGE-M standardised to urinary creatinine could therefore not be calculated for these samples. Ten samples in the EPA group and 7 in the placebo group had PGE-M levels below the limit of detection (LOD, <10ng/g), and were therefore recorded as this LOD value for the purpose of analysis. Data was log transformed to normalise the distribution for analysis, then summary statistics were converted back to the original scale for presentation in tabular and graphical form.

EPA	Baseline n=38	Post-treatment n=37	Follow-up n=34	[1]p= (37 pairs)	[2]p= (33 pairs)
Geometric mean	13.58	9.83	14.96	0.17	**0.02**
95% CI	10.21 - 18.07	6.82 - 14.16	10.00 - 22.39		
Placebo	**Baseline n=39**	**Post-treatment n=40**	**Follow-up n=33**	**[1]p= (37 pairs)**	**[2]p= (31 pairs)**
Geometric mean	15.92	20.37	15.52	0.10	0.14
95% CI	11.75 - 21.63	16.41 - 25.29	11.32 - 21.28		
[3]p=	0.44	**<0.01**	0.89		

Table 5.10. Urinary PGE-M. Geometric mean and 95% confidence interval (CI) for PGE-M in ng/mg creatinine at baseline, post-treatment (i.e. completion of study medication), and at the six weeks follow-up appointment (off study medication). [1] Paired t-test comparing paired patient data at baseline and post-treatment. [2] Paired t-test comparing paired patient data at post-treatment and follow-up. [3] Unpaired t-test comparing EPA vs. placebo groups at each time point.

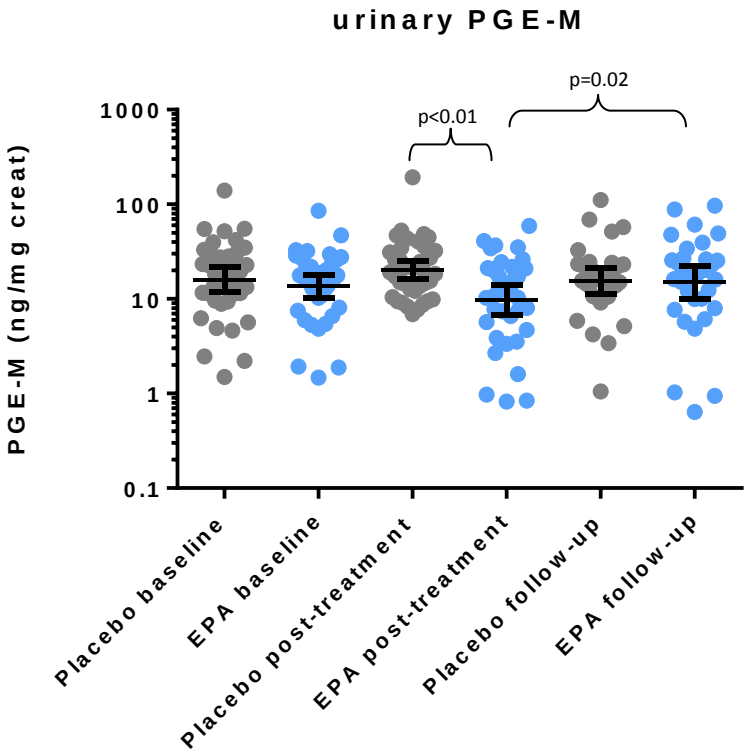


Figure 5.24. Scatterplot of urinary PGE-M. Data given in ng/mg creatinine at baseline, post-treatment (i.e. completion of study medication), and at the six weeks follow-up appointment (off study medication). Error bars represent geometric mean and 95% confidence interval.

There was no significant difference in mean PGE-M between the two groups at baseline (Table 5.11 and Figure 5.24). Post-treatment PGE-M was 51.8% lower in the EPA group compared to placebo ($p<0.01$). This represented a 27.6% reduction in urinary PGE-M in the EPA group between baseline and post-treatment, and a 27.9% increase in urinary PGE-M in the placebo group between baseline and post-treatment. Urinary PGE-M levels rose again by 52.2% at follow-up in the EPA group ($p=0.02$), and fell by 18.2% in the placebo group ($p=0.14$). There was no significant difference in PGE-M between the two groups at the six weeks post-operative follow-up appointment ($p=0.89$), and no significant change in urinary PGE-M between baseline and follow-up in either group (EPA $p=0.61$, placebo $p=0.60$).

There was no correlation between the duration of treatment in the EPA group and the change in urinary PGE-M between baseline and post-treatment (Pearson correlation coefficient 0.059, $p=0.73$). Sub-analysis by gender revealed no significant differences between the two groups or between male and females (Figure 5.25). Sub-analysis excluding those patients who were already taking ω-3 PUFA supplements prior to enrolment in the Trial or who were high consumers of oily fish did not reveal any significant differences between the two groups. Subanalysis performed after exclusion of patients who were taking concurrent aspirin also showed no difference between the two groups.

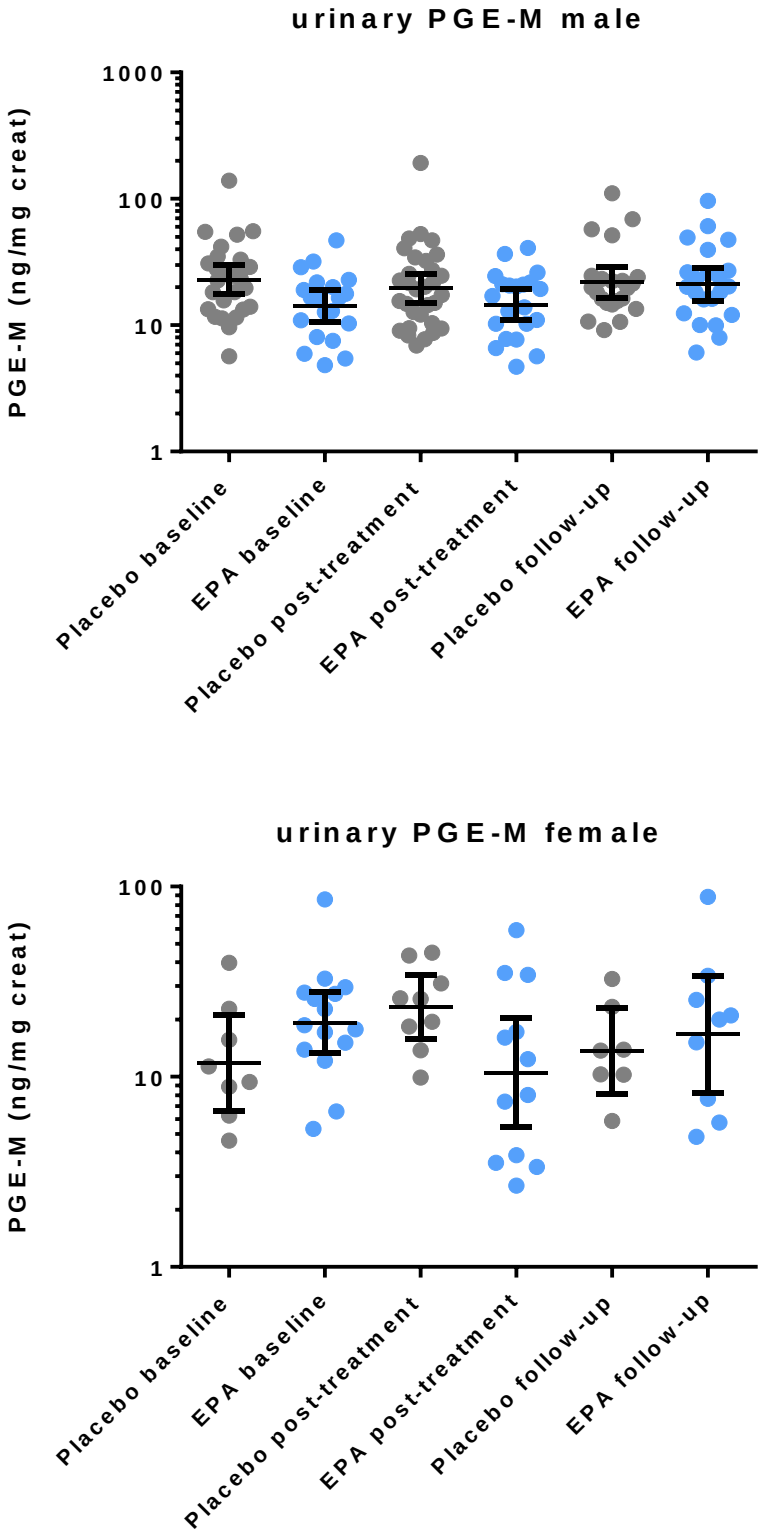


Figure 5.25. Scatterplots of urinary PGE-M stratified by sex. Error bars represent geometric mean and 95% confidence interval.

5.13 PBMC nuclear NFκB activation

Blood samples were not analysed from the seven patients withdrawn from the study before undergoing surgery and the one patient who had a histological diagnosis of HCC. Baseline blood samples were analysed for all 80 patients with CRCLM who underwent surgery (EPA n=38, placebo n=42). Post-treatment blood samples were not obtainable from the two patients in the placebo group whose surgery was brought forward without my knowledge. Post-treatment blood samples were therefore analysed for 78 patients (EPA n=38, placebo n=40). Follow-up blood samples were not obtainable from seven patients in the placebo group and two patients in the EPA group who did not attend the final study visit. Follow-up blood samples were analysed for 69 patients (EPA n=36, placebo n=33). Paired samples were available for 40 placebo and 38 EPA patients between baseline and post-treatment, and for 33 placebo and 36 EPA patients between post-treatment and follow-up. One spuriously high LPS-stimulated sample from the EPA group, suggestive of protein contamination in the sample, was excluded from analysis. There was insufficient sample left to repeat this analysis.

LPS stimulation of PBMCs consistently increased nuclear NFκB activation compared to un-stimulated PBMCs in both groups and at each time point (Figures 5.26 and 5.27). There was no significant difference between the two groups at any time point in either LPS-stimulated or LPS un-stimulated nuclear NFκB activation. Within the placebo group, there was no significant change in nuclear NFκB activation across the study period. In the EPA group there was a reduction in both LPS-stimulated and un-stimulated nuclear NFκB activation post-treatment compared to baseline (15.5% and 20.3% reduction, p=0.03 and p=0.07 respectively). At follow-up, both LPS-stimulated and un-stimulated nuclear NFκB activation increased back to baseline values (28% and 29.5% increase, p<0.01 and

p=0.02 respectively). Subanalysis performed after exclusion of patients who were taking concurrent aspirin also showed no difference between the two groups.

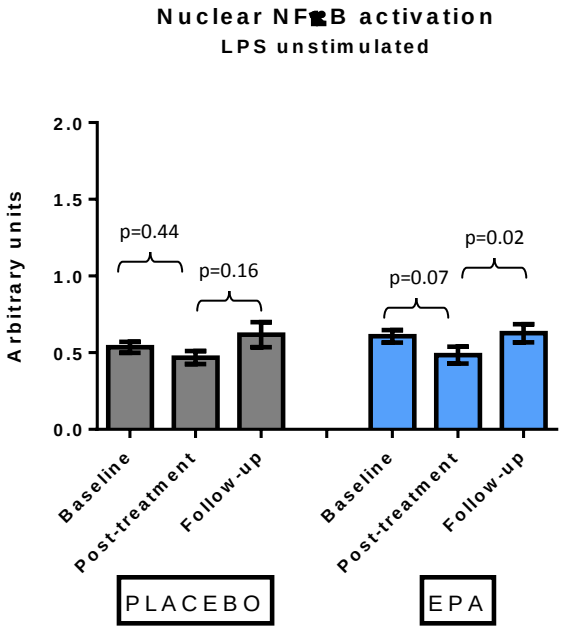


Figure 5.26. Barchart of LPS un-stimulated mean nuclear NFκB activation. Paired t-tests were used to compare change in nuclear NFκB activation over time within each group. All p-values not depicted were non-significant. Error bars represent mean +- SEM.

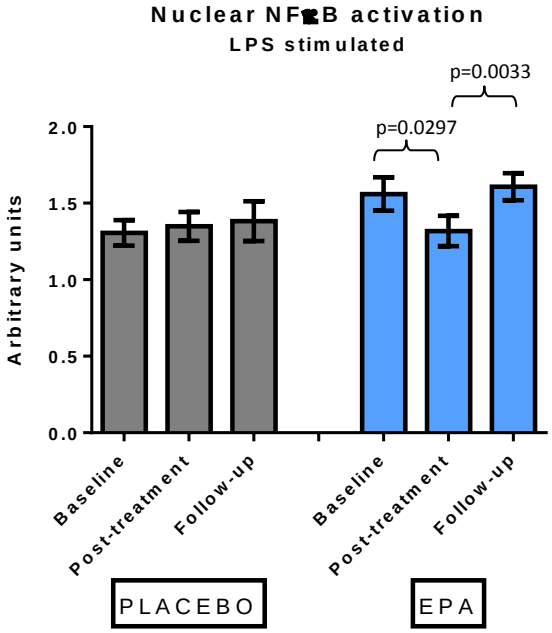


Figure 5.27. Barchart of LPS-stimulated mean nuclear NFκB activation. Paired t-tests were used to compare change in nuclear NFκB activation over time within each group. All p-values not depicted were non-significant. Error bars represent mean +- SEM.

5.14 Platelet aggregation

Platelet aggregation (arbitrary units, AU) at baseline was stratified based on aspirin, clopidogrel, or fish oil (FO) supplementation use at the time of entry into the EMT Trial to assess the sensitivity of the assay to the anti-platelet effect of these medication (Figure 5.26). Baseline blood samples were available for 15 aspirin users, 4 aspirin + FO users, 2 clopidogrel users, 1 clopidogrel + FO user, 9 FO users, and 54 patients who were naive to all of these. One patient withdrew consent after randomisation and prior to baseline blood sampling. One patient's blood sample was delayed in returning to the laboratory (greater than the 3 hours window for analysis recommended by the manufacturer) and had partially clotted. Another patient had poor venous access and blood sampling was technically difficult. This patient's sample had partially clotted on return to the laboratory. Both these partially clotted samples were discarded and not analysed.

Mean (+-SEM) platelet aggregation in aspirin users compared to naive individuals was 83% lower in response to AA stimulation (13.1+-2.4AU vs. 78.2+-3.8 AU, $p<0.01$), 36% lower in response to collagen stimulation (48.6+-5.3AU vs. 75.6+-3.3AU, $p<0.01$), and 62% lower in response to low dose collagen stimulation (22.6+-4.0AU vs. 59.1+-3.6AU, $p<0.01$). Stimulation with ADP had no effect on platelet aggregation in aspirin users compared to naive individuals. Platelet aggregation was further reduced in response to all agonists in the small number of patients taking aspirin and FO compared to aspirin alone, but this did not reach statistical significance (Figure 5.28A).

Because of the small number of patients in the clopidogrel (n=2) and clopidogrel +FO (n=1) groups, these patients were grouped together for the purpose of evaluating the assays sensitivity to the anti-platelet effect of clopidogrel. Mean (+-SEM) platelet aggregation in clopidogrel users compared to naive individuals was 58% lower in response to ADP stimulation (27.3+-3.2AU vs. 64.8+-3.9AU, $p=0.03$)

and non-significantly 35% lower in response to low dose collagen stimulation (38.7+-11.6AU vs. 59.1+-3.6AU, p=0.20). Stimulation with AA or collagen had no effect on platelet aggregation in clopidogrel users compared to naive individuals (Figure 5.28B).

There was no significant difference in platelet aggregation between FO users and naive individuals in response to either AA, ADP, collagen or low dose collagen stimulation (Figure 5.28C).

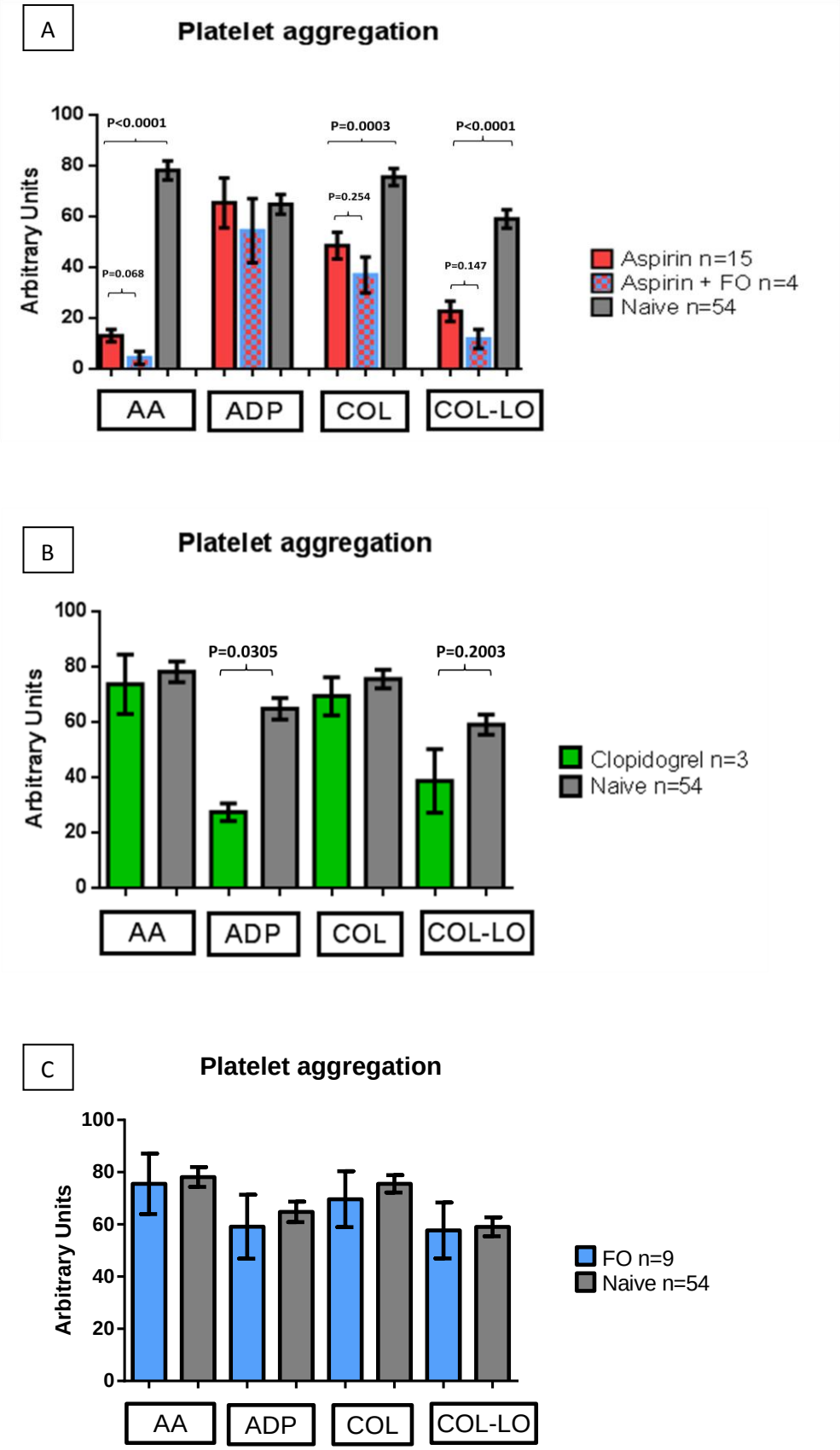


Figure 5.28. Baseline platelet aggregation (arbitrary units) in response to different agonists. Patients have been stratified by prior aspirin (A), clopidogel (B) and fish oil (FO) use (C) and compared to "naive" patients not taking these at baseline. Error bars represent mean +- SEM. AA=arachidonic acid, ADP=adenosine diphosphate, COL=collagen 3.2µg/ml, COL-LO = low-dose collagen 0.64µg/ml.

Blood samples were not analysed from the seven patients withdrawn from the study before undergoing surgery and the one patient who had a histological diagnosis of HCC. One patient in the placebo group had difficult venepuncture and his baseline blood sample partially clotted prior to analysis and was discarded. Baseline blood samples were therefore analysed for 79 patients (EPA n=38, placebo n=41). Post-treatment blood samples were not obtainable from the two patients in the placebo group whose surgery was brought forward without my knowledge. Post-treatment blood samples were therefore analysed for 78 patients (EPA n=38, placebo n=39). Follow-up blood samples were not obtainable from the seven patients in the placebo group and two patients in the EPA group who were discharged from the Hepatobiliary unit's care after recovering from their surgery and did not attend the final study visit. Follow-up blood samples were analysed for 68 patients (EPA n=36, placebo n=32).

There was no significant difference between the two groups in platelet aggregation in response to either AA, ADP, collagen or low dose collagen at any time point in the study (Figure 5.29, unpaired t-tests). There was a small reduction in platelet aggregation in response to low dose collagen stimulation in the EPA group compared to the placebo group post-treatment, but this did not reach statistical significance (p=0.08), and was in fact due to an increase in platelet aggregation in the placebo group compared to baseline rather than a reduction in platelet aggregation in the EPA group compared to baseline. Neither EPA nor placebo treatment was associated with a change in platelet aggregation in response to any of the four agonists between baseline and post-treatment (Figure 5.29, paired t-test analysis, all p=NS).

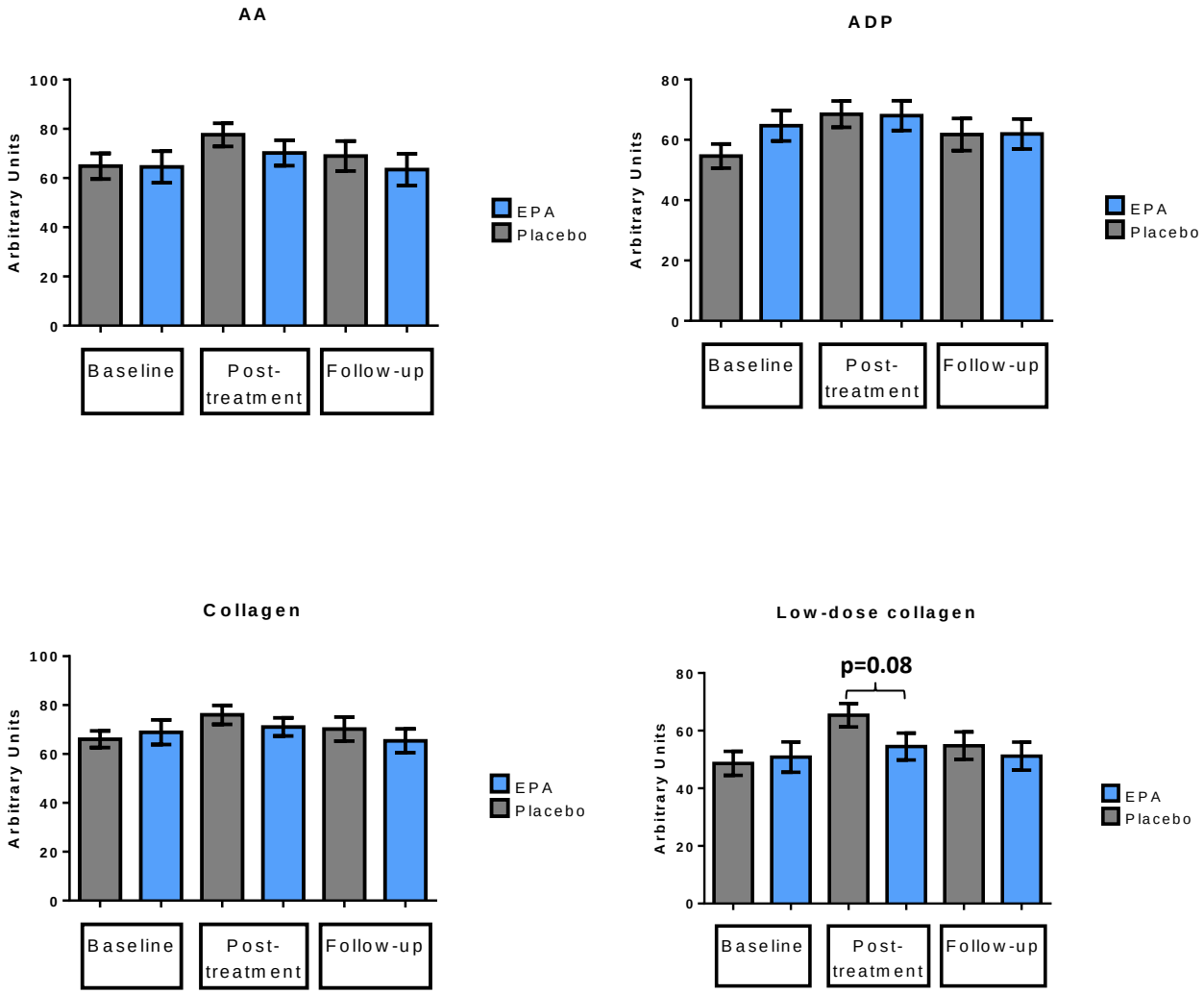


Figure 5.29. Platelet aggregation at baseline, post-treatment and follow-up. Each graph shows platelet aggregation in response to a different agonists. Error bars represent mean +- SEM. AA=arachidonic acid, ADP=adenosine diphosphate.

Although no antiplatelet effect was seen in the EPA group, because of historic concerns about the risk of bleeding with ω-3 PUFA supplementation and because many patients are already takin aspirin pre-operatively, I performed subgroup analysis to investigate the effect on of EPA and aspirin when taken in combination. The antiplatelet effect of aspirin alone at baseline has already been shown in Figure 5.28. There were seven patients in each group who were taking aspirin and who had paired baseline and pre-operative samples available for analysis. There was no significant difference in platelet aggregation at baseline between the aspirin consumers in each group (Figure 5.30 A). Both at baseline, and post-treatment (Figure 5.30 B), platelet aggregation was non-significantly lower in the EPA group compared to the placebo group. In both groups there was a significant increase in platelet aggregation between baseline and post-treatment (Figure 5.31 A and B) However, these results may have been confounded by patients stopping their aspririn pre-operatively before they came in to hospital. Whilst it is not the policy of the Hepatobilary Unit to stop aspirin pre-operatively, it is conceivable that either patients chose to stop their aspirin because of a perceived bleeding risk of major surgery, or they are advised to do so by their GPs or other health professionals for the same reason. Data was not collected on the date of last aspirin use, so this could not be investigated further, but on the basis of the available evidence EPA + aspirin did not impair platelet aggregation compared to aspirin alone.

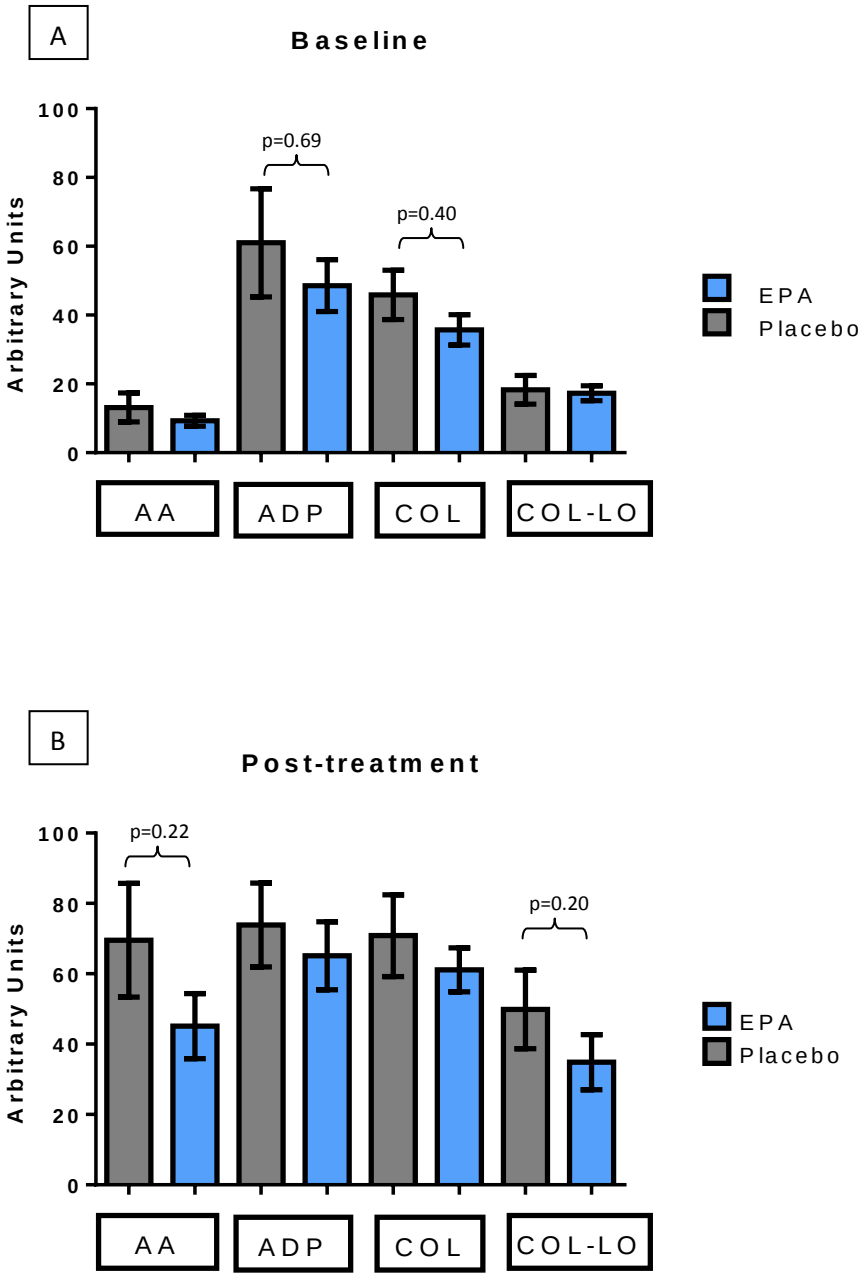


Figure 5.30 Subanalysis between groups of platelet aggregation in aspirin users. Seven patients in each group were taking aspirin during the study and had paired baseline and pre-operative samples available for analysis. Error bars represent mean +- SEM. Groups were compared at baseline (A) and pre-operative (B) using Mann Whitney U test. Selected p-values are presented, with all other p-values being non-significant. AA=arachidonic acid, ADP=adenosine diphosphate, COL=collagen 3.2µg/ml, COL-LO = low-dose collagen 0.64µg/ml.

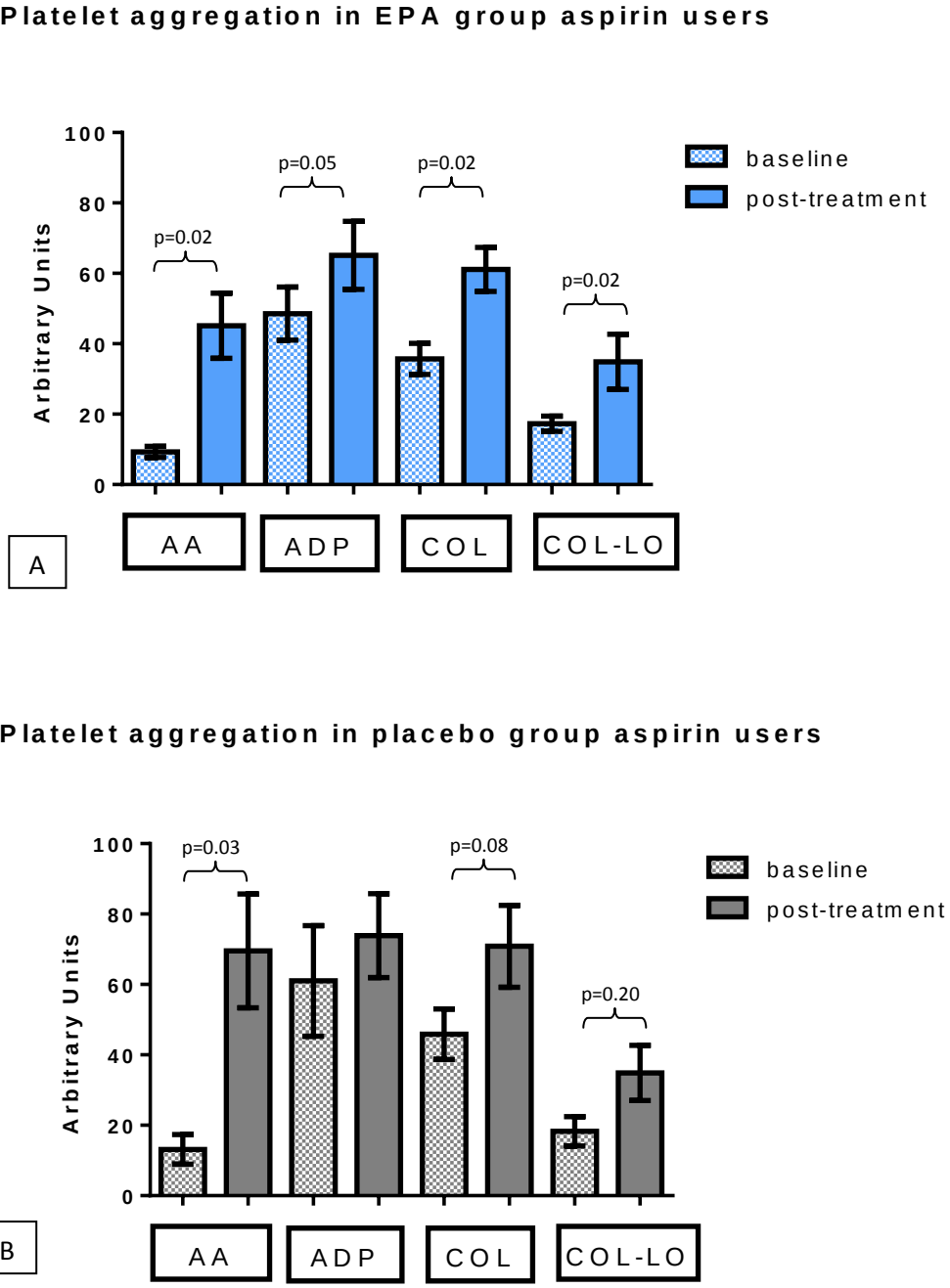


Figure 5.31 Subanalysis over time of platelet aggregation in aspirin users. Seven patients in each group were taking aspirin during the study and had paired baseline and post-treatment samples available for analysis (A= EPA group, B=placebo group). Error bars represent mean +- SEM. Comparison of aggregation over time within each group was performed using Wilcoxon matched-pairs signed rank test. Selected p-values are presented, with all other p-values being non-significant. AA=arachidonic acid, ADP=adenosine diphosphate, COL=collagen 3.2µg/ml, COL-LO = low-dose collagen 0.64µg/ml.

5.15 Discussion of results from The EMT Trial

Safety and tolerability

The EMT Trial has demonstrated that pre-operative supplementation of EPA is safe and well-tolerated in patients with CRCLM awaiting liver resection. The type and frequency of side effects seen, namely gastrointestinal upset, are comparable to previous studies using the same preparation of EPA-FFA 2g daily. In a phase III study of six months EPA supplementation in patients who had undergone subtotal colectomy for FAP,(262) diarrhoea was reported by 31% of patients taking EPA and 35% of patients taking placebo. Withdrawal due to EPA intolerance was 3.4%. In an earlier phase II study in patients with previous colorectal adenoma, 3 months of EPA-FFA caused diarrhoea in 14% of patients who took 2g/day, but only in 2% of patients who took 1g/day.(257) The long-term tolerability of ω-3 PUFAs is further supported in large cardiovascular studies. The GISSI prevention study gave low dose (0.85g) ω-3 PUFA to 2836 patients for 3.5 years.(314) In this study gastrointestinal disturbance was reported by 4.9% of patients, nausea by 1.4% of patients, and only 3.8% of patients discontinued ω-3 PUFA supplementation.(314) Similar findings were seen in a study of higher dose (6.9g) ω-3 PUFA daily for 6 months.(315) Gastrointestinal disturbance was reported by 7% of 275 patients who received ω-3 PUFA supplementation, but also by 8% of patients who received the corn-oil placebo.(315)

EPA supplementation was not associated with any increase in post-operative bleeding or infective complications, and *ex vivo* platelet function tests showed no evidence of anti-platelet activity of EPA. The evidence concerning a risk of bleeding with ω-3 PUFA supplementation is mixed, with some studies raising concern that ω-3 PUFAs inhibit platelet aggregation, and others showing no effect. A cross-over study of dietary salmon and salmon oil supplementation versus an un-supplemented control diet in 11 healthy volunteers found that the salmon

supplemented diet was associated with an increase in bleeding time (10mins vs. 6.75mins, $p<0.05$) and a 24% reduction ($p<0.05$) in platelet aggregation in response to ADP (2µM) but not in response to AA or collagen.(266) In a study of Type II diabetic patients (n=40), 6 weeks of supplementation of 1.2g/day ω-3 PUFA vs. placebo was associated with 15% reductions in platelet aggregation in response to ADP and collagen (both $p<0.01$).(267) In contrast, a study of TPN following major abdominal surgery found that the addition of 0.2g/kg/day ω-3 PUFA to TPN had no effect on platelet aggregation as measured by resonance thrombography, and no effect on other measures of haemostasis including levels of the clotting factors VIIa and XII, the need for transfusion of packed red blood cells, or routine laboratory coagulation tests such as thromboplastin time and activated partial thromboplastin time.(316) Similarly, recent reviews of over 4000 patients in 19 trials of ω-3 PUFAs who were undergoing either arterial surgery or trans-arterial angiography and angioplasty found no evidence that ω-3 PUFAs were associated with an increased risk of bleeding.(268, 269)

Several recent studies have also investigated whether aspirin and ω-3 PUFAs in combination increases the risk of bleeding. In one study, subjects were given a single dose of aspirin (625mg) on day 1, then received ω-3 PUFA (4g/day) for 28 days, then took one day of combined ω-3 PUFA and aspirin on day 30.(317) Omega-3 PUFA supplementation alone did not inhibit aggregation in response to ADP, low- or high-dose collagen. Aspirin alone inhibited aggregation in response to low-dose collagen only. However, in combination, ω-3 PUFA and aspirin inhibited aggregation to all three agonists. Similar results were seen in a double-blind, randomised, placebo-controlled cross-over study which gave 25 healthy volunteers either 3.4g EPA/DHA, 81mg aspirin, or both, and took blood for analysis of platelet aggregation at baseline and 4hrs post-treatment.(318) They found that aspirin and ω-3 PUFA in combination, but neither alone, inhibited platelet aggregation

measured by the PFA-100 (Siemens, Deerfield, IL) system. In a further study, addition of ω-3 PUFA potentiated the anti-platelet effect of combination aspirin and clopidogrel therapy in patients who had undergone coronary angioplasty.(319)

There were only a small number of patients in The EMT Trial taking aspirin and ω-3 PUFAs, but a possible potentiation of platelet inhibition with combination therapy was still seen. At baseline, platelet aggregation was significantly lower in the small number of patients who were taking fish oil supplements and aspirin compared to patients who were taking aspirin alone. However, there was no significant difference in platelet aggregation in response to any of the four agonists between the EPA and placebo groups at the end of the period of supplementation. It is also important to remember that there was no difference between the two groups in the rate of blood transfusion (10% vs. 8%), which for both groups was less than the 17% transfusion rate reported in the last review of transfusion requirement (2004-2008) in the Leeds Hepatobiliary Unit (320). Whilst blood transfusion rates have likely fallen in the 5 years since this study, the 10% transfusion rate in The EMT Trial is considered to be in keeping with current transfusion requirements within the Unit.

Therefore, despite the complexity of the platelet aggregation pathway and the lack of consensus regarding the techniques and agonists to best measure platelet aggregation, the results from The EMT Trial support the hypothesis that EPA does not significantly inhibit platelet aggregation and does not cause an increased risk of bleeding during liver resection, although the possibility that EPA might potentiate the platelet inhibitory effect of aspirin when given in combination should still be kept under consideration.

Tumour PUFA content

Treatment with EPA was associated with significantly higher tumour EPA content compared to placebo. This is the first study to demonstrate that oral EPA supplementation leads to incorporation into CRC or CRCLM. An inherent limitation of The EMT Trial is that only post-treatment tumour tissue was available for analysis. It is unethical to biopsy liver metastases before surgery because of the risk of tumour seeding, and MRI scan of the liver with liver-specific contrast such as Primovist® is the accepted modality for diagnosis of CRCLM. Pre-operative biopsy is therefore only performed in the rare situations when there is doubt about the diagnosis of a liver lesion. Comparison of individual patients' change in tumour PUFA content pre- and post-treatment was therefore not possible. Instead, only comparison of post-treatment tumour PUFA content between the EPA and placebo groups could be performed.

A further limitation of the study is that the median duration of supplementation was relatively short. Few clinical studies have examined the effect of short-duration ω-3 PUFA supplementation (Table 5.11), and only one of these examined ω-3 PUFA uptake by solid gastrointestinal tumours.(321) In this study of 40 patients, five days of pre-operative oral ω-3 PUFA supplementation (3.7g ω-3 PUFA/day) versus un-supplemented controls was associated with significant increases in the EPA content of liver tissue (1.25% vs. 0.4%), gastrointestinal mucosa (1% vs. 0.25%) and tumour tissue (0.75% vs. 0.25%). This 0.5% absolute increase in tumour EPA content is comparable to the 0.65% absolute increase seen in The EMT Trial. The remaining clinical data which is available for comparison comes from colonic mucosa biopsy studies, with periods of supplementation of up to 6 months (Table 5.11). These studies showed greater colonic mucosal uptake of EPA than the CRCLM uptake of EPA seen in The EMT Trial. This includes studies of short duration (2-6 weeks)(254, 255, 273, 322) and two studies which used the same

preparation of EPA as in The EMT Trial (257, 262), albeit in these two studies EPA was given for 3-6 months rather than the median 30 days in The EMT Trial. Notably, the level of AA in tumours in the placebo group in The EMT Trial was much higher than the baseline levels of AA in colorectal mucosa in other studies. There are no studies reporting the AA content of solid GI tumours available to know whether there is a difference in AA content between human colonic mucosa and solid tumours. It is also noticeable that in all the studies which performed serial colonic biopsies, mucosal EPA levels increased early, within 2-4 weeks, whereas mucosal AA levels did not fall until 12 weeks or more.(254, 255, 273) The same trend of an early rise in EPA/DHA, with a delayed fall in AA was also seen in a separate study of the PUFA content of plasma and blood cells at 0, 2 and 12 weeks from eight healthy volunteers taking 1.4-4.2g EPA/day.(323)

The study by Gee *et al* investigated the effect of fish oil supplementation (1.4g EPA + 1.0g DHA/day) vs. placebo on 49 patients prior to colorectal surgery.(322) Mean duration of supplementation was 12.3 days. They found a significant increase in EPA content in the colorectal mucosa of patients in the fish oil group compared to placebo, but no difference in PUFA levels in mesenteric adipose tissue. However, patients who self-reported use of FO supplements prior to the trial did have higher levels of EPA and higher ratios of ω-3:ω-6 PUFA in adipose tissue compared to patients who did not report previous FO supplementation. This mirrors the findings of the similar subanalysis of patients already exposed to ω-3 PUFA intake in The EMT Trial.

It is clear that the uptake of ω-3 PUFAs and reduction in AA is time dependent, and may vary between different tissues. Whilst the results from The EMT Trial demonstrate an increase in tumour EPA content following EPA supplementation, it is likely that the duration of supplementation was too short to reach either the maximal EPA incorporation, or to observe a reduction in AA content.

Study	Duration	Tissue	Daily ω-3 PUFA (EPA/DHA)	Baseline			Post-treatment Placebo group			Post treatment ω-3 PUFA group		
				EPA	DHA	AA	EPA	DHA	AA	EPA	DHA	AA
Senkal 2005(321)	5 days	GI mucosa	3.7g	-	-	-	0.3			1.0		
		Liver		-	-	-	0.3			1.3		
		GI tumour		-	-	-	0.3			0.8		
Gee 1999*(322)	12 days	Colorectal mucosa	2.4g (1.4g/1.0g)	-	-	-	0.5	0.5	4.0	1.0	0.8	5.5
		Adipose					0.1			0.1		
Anti 1992(255)	2 weeks	Colorectal mucosa	7.7g (4.1g/3.6g)	1.3-1.5	1.5-1.8	5.7-6.1	1.3	1.9	6.3	2.6	1.9	5.0
	12 weeks						1.8	1.6	6.2	2.3	1.8	4.4
Hillier 1991(273)	3 weeks	Colorectal mucosa	5.4g (3.2g/2.2g)	0.3-0.4	1.7-2.1	8.5-10.0	0.4	1.7	8.6	3.2	3.3	7.9
	6 weeks						0.4	1.7	7.5	3.1	3.1	8.3
	12 weeks						0.2	1.0	5.1	3.1	2.9	5.6
Anti 1994(254)	4 weeks	Colorectal mucosa	2.5g (1.4g/1.1g)	0.8-1.2	1.3-2.0	5.8-6.5	1.1	1.5	6.1	1.6	2.2	5.0
			5.1g (2.7g/2.4g)							1.9	2.6	3.6
			7.7g (4.1g/3.6g)							2.5	4.0	4.2
	6 months		2.5g (1.4g/1.1g)							1.5	2.1	3.6
Courtney 2007(257)	3 months	Colorectal mucosa	2g EPA-FFA	0.8-1.2	1.7-2.4	8.4-9.4	0.8	1.9	9.1	2.7	2.5	8.0
West 2010(262)	6 months	Colorectal mucosa	2g EPA-FFA	0.7-1.0	1.4-1.9	8.5-9.6	1.3	1.4	9.9	2.5	1.7	8.8
Sorensen 2013 (324)	7 days	Colorectal mucosa	2.g EPA + 1g DHA				0.2	0.9	2.0	0.6	1.3	2.6
EMT Trial	**4 weeks**	**CRCLM**	**2g EPA-FFA**	**-**	**-**	**-**	**1.3**	**2.9**	**12.8**	**1.8**	**2.6**	**12.0**

Table 5.11. Clinical studies reporting the change in PUFA content of colonic mucosal or gastrointestinal tumours following ω-3 PUFA supplementation. Levels given are the ω-3 PUFA content as a % of total fatty acids. *approximate figures read off graph

Tumour PGE_2 and urinary PGE-M

In this trial there was no difference in tumoural PGE_2 levels between the EPA and placebo groups, despite previous *in vitro* and mouse models showing that EPA incorporation is associated with a reduction in PGE_2 production.(149, 209) Notably, PGE_2 levels varied widely in both the EPA and placebo groups. There are no previous studies reporting PGE_2 production in human CRCLM available for comparison. Two studies have investigated this in human gastrointestinal mucosa.(259, 325) In a study by Bartram *et al,*(259) 12 healthy volunteers received either fish oil (4.4g ω-3PUFA/day) or corn oil for two 4-week periods, with a 4 week washout period, in a double-blind crossover study. They found no significant difference in ω-3 PUFA content of rectal biopsies after fish oil supplementation compared to after corn oil supplementation (4.00% vs. 4.03%), and a non-significant increase in ω-6 PUFA content after corn-oil supplementation (19.23% vs. 14.96%, p=0.11). PGE_2 levels in rectal biopsies were approximately 671 ng/g tissue after corn oil and 435.5 ng/g tissue after fish oil supplementation ($p<0.05$). This suggests that corn oil supplementation led to increased ω-6 PUFA and PGE_2 content, rather than fish oil supplementation leading to lower PGE_2 content. In a study by Mehta *et al*,(325) 52 patients with Barrett's oesophagus were randomised to receive 1.5g EPA-FFA/day or placebo for 6 months, with biopsies of the Barrett's oesophagus taken at baseline and post-treatment. In the EPA group there was a significant increase in mucosal EPA post-treatment (2.4% vs. 0.8%, $p<0.01$), but no change in mucosal AA (6.4% vs. 6.0%, p=0.14) or mucosal PGE_2 (36.1pg/µg protein vs. 33.1pg/µg protein).

Median PGE_2 levels in CRCLM in The EMT Trial were 6.62ng/g in the EPA group and 5.87ng/g in the placebo group. This is lower than the PGE_2 levels seen in the Bartram and Mehta studies, although methodological differences make direct comparison between the studies difficult. In the Bartram study PGE_2 was

measured in culture medium by radioimmunoassay after 2 hrs incubation with the rectal biopsies (259) and in the Mehta study PGE_2 was standardised to protein content of the sample.(325)

Notably, of the *in vivo* and clinical studies that have demonstrated a reduction in tumour/mucosa PGE_2 with fish oil supplementation and also measured tissue PUFA content, all demonstrated a significant reduction in AA in addition to significant increases in EPA, resulting in AA:EPA ratios ranging between 0.59:1 and 3.74:1(151, 209, 227, 236, 241, 259, 326) which are lower than the 7.58:1 AA:EPA ratio in tumours in the EPA group of The EMT Trial. This may explain why there was no difference in tumour PGE_2 levels in The EMT Trial, and again, may reflect the duration of supplementation having been too short to achieve maximal tumour EPA incorporation and AA reduction.

In the limited number of *in vivo studies* that have measured tumour PGE_3, levels were between 2-20 times lower than corresponding PGE_2 levels.(151, 209, 326, 327) In The EMT Trial, PGE_3 was detected in only one tumour, that with the highest level of PGE_2 which was one of the placebo group tumours. PGE_3 in this tumour (21.10ng/g) was present at 20 times lower concentration than the level PGE_2 (447ng/g). The largest concentration of PGE_2 in the EPA group was 33.40ng/g. Therefore any PGE_3 in tumours in the EPA group, at a similar 2-20 times lower concentration than PGE_2 might have been present below the limit of detection (10ng/g) of LC-MS/MS, making it impossible to draw any conclusions about PGE_3 production from this study.

In addition to measuring tumoural PGE_2 I also assayed urinary PGE-M, which being a measure of overall PGE_2 synthesis, allowed a longitudinal analysis of the changes in PGE_2 synthesis associated with EPA supplementation. Baseline levels of PGE-M in this study ranged from 7.93-28.93ng/mg of creatinine, with a median of

16.49ng/mg in the EPA group and 18.24ng/mg in the placebo group. Studies of healthy volunteers have shown mean PGE-M levels of approximately 5-10ng/mg, (81, 82, 328) with levels being higher in men than in women (81-83) and higher in smokers than in non-smokers.(328) Mean levels in patients with non-small cell lung cancer were approximately 20-30ng/mg,(81, 329, 330) and in the one study found of PGE-M in patients with CRC, 17 patients with inoperable or metastatic CRC had a mean PGE-M of 25ng/mg.(331) The PGE-M levels in patients with operable CRCLM in The EMT Trial therefore seem to be appropriately between those of healthy controls and patients with advanced inoperable disease.

No other studies of the effect of ω-3 PUFAs on urinary PGE-M were found for comparison. Although the 27% reduction in PGE-M between baseline and post-treatment in the EPA group did not reach statistical significance, the magnitude of reduction is plausible, being lower than the 40-60% reductions seen with the more potent COX-2 inhibitor celecoxib (81, 83, 329) but higher than the 14% reduction in PGE-M seen in a study of 36 healthy volunteers who were supplemented with 7.5g of ω-3 PUFA for 10 weeks.(332) PGE-M levels rose again in the EPA group at follow-up, having stopped EPA supplementation, but stayed the same in the placebo group, providing further evidence for an effect of EPA on systemic PGE_2 production. Interestingly PGE-M levels did not fall in either group at follow-up despite this being after removal of their cancer. Together with the finding that tumoural PGE_2 levels were unaffected by EPA, this might suggest that systemic PGE_2 production is a greater contributor to urinary PGE-M than tumoural PGE_2 production. This hypothesis is supported by a study of patients with unresectable or metastatic CRC which found no difference in urinary PGE-M between those patients who did and those who did not respond to dual therapy with celecoxib and cetuximab.(331) Alternatively, it might be that the six week interval between surgery and follow-up was too short for the systemic inflammation caused by

surgery to have subsided, and that this masked any reduction in PGE_2 synthesis attributable to the removal of the tumour. Such an inflammatory response to surgery might be expected to have subsided within 1 week (333, 334) although there are no studies examining this beyond 1 week. An additional confounder that could have masked a reduction in PGE-M after removal of the tumour is that hepatic COX-2 expression and PGE_2 synthesis increase following liver resection as part of the liver regeneration response.(335-337) Whilst the duration of PGE_2 rise following hepatectomy is unstudied in humans, liver regeneration would still be ongoing at six weeks, potentially contributing to elevated systemic PGE_2, and hence raised urinary PGE-M, secondary to liver regeneration.

Nevertheless, EPA therapy was associated with a small but statistically significant reduction in PGE-M indicative of an effect on PGE_2 production, whether this be of tumoural or systemic origin. This anti-inflammatory effect is consistent with the known COX inhibitory activity of EPA.(338)

PBMC NFκB activation

EPA therapy inhibited basal and LPS-stimulated NFκB activation in PBMCs, which similar to the PGE-M data, was reversible after cessation of EPA therapy. This provides further evidence of a systemic anti-inflammatory effect of EPA therapy.

Whilst there are no previous studies of PBMC NFκB activation in patients with either CRC or CRCLM, these results are consistent with other clinical studies which have demonstrated uptake of ω-3 PUFAs by PBMCs (339-341) with resultant anti-inflammatory effects. (342-349) The rate of uptake and washout of different PUFAs in these studies mirrors that seen in other cells and tissues in clinical studies discussed earlier in this section. In a study of parenteral fish oil supplementation (three days, total 42g ω-3 PUFA), there was a significant increase in PBMC content of EPA and DHA, but not AA.(340) EPA levels returned to normal by eight days,

with DHA and DPA levels remaining high up to day 11. This is consistent with the ω-3 PUFA content in plasma and erythrocytes in the Leicester trial of parenteral supplementation in patients with CRCLM.(211) In the study by Yaqoob *et al* of 12 weeks oral ω-3 PUFA supplementation (2.1g EPA + 1.1g DH/day) peak EPA content of PBMCs occurred by 4 weeks, with a reduction in AA only detected at 12 weeks.(341) This is consistent with the majority of human mucosal biomarker studies (Table 5.11) and the CRCLM PUFA data in The EMT Trial. Omega-3 PUFA incorporation into PBMCs has been demonstrated to reduce pro-inflammatory gene expression, including several NFκB target genes, pro-inflammatory cytokines and genes involved in eicosanoid synthesis.(342, 343) It has also been associated with a reduction in PBMC production of $PGF_{2\alpha}$, PGE_2, IFN-gamma, IL-1, IL-6 and TNFα,(344-349) and a reduction in paw oedema *in vivo*.(348) A minority of studies have failed to show any effect of ω-3 PUFAs on PBMC cytokine production.(340, 341)

The finding in The EMT Trial that EPA inhibits NFκB activation in isolated human PBMCs is also consistent with pre-clinical studies of the effect of ω-3 PUFAs on NFκB activation in other cell lines *in vitro* and *in vivo*. In human CRC cell lines, ω-3 PUFA supplementation has been shown to inhibit IκB phosphorylation, reduce NFκB activation and reduce NFκB p65 expression.(178, 179) Omega-3 PUFA supplementation has also been shown to inhibit NFκB activation in HUVECs.(180) Similar findings have been shown in pancreatic and breast cancer cell lines,(182, 350, 351) in transgenic mouse prostate cancers,(181) and in a nude mouse model of breast cancer.(182)

Therefore, whilst no previous studies have directly investigated the effect of ω-3 PUFAs on NFκB activation in PBMCs, the results from The EMT Trial would be consistent with separate studies of the effects of ω-3 PUFAs on PBMCs and of ω-3 PUFAs on NFκB activation in other cell lines. Bringing these results together

supports the hypothesis of EPA having an anti-inflammatory effect, and potential anti-CRC activity, through inhibition of NFκB signalling.

Immunohistochemistry

In contrast with findings from the recent MC-26 mouse CRCLM model,(209) EPA was not associated with a change in either tumour proliferation or apoptosis. Subanalysis did, however, reveal reduced tumour vascularity in the EPA group compared to the placebo group after exclusion of patients who were either taking fish oil supplements or who had a high weekly intake of oily fish prior to the study. Whilst there are no other studies of the effect of EPA on CRCLM available, limited comparisons can be made with immunohistochemical findings in other studies of CRCLM. The range of Ki-67 PI seen in The EMT Trial (mean 70.7% in the EPA group, 73.0% in the placebo group) is in keeping with the range of PIs seen in other studies. Veremeulen *et al.* found a mean PI of 62.9% (35.8-93.5%) in 26 historic FFPE CRLM specimens,(352) and Tatebe *et al.* found a mean PI of 60.7% (55.3-70.4%) in 15 historic FFPE CRCLM specimens.(313) In a similarly designed study of the effect of rofecoxib on CRCLM by Fenwick *et al*, mean (range) PI in CRCLM tumours was 54.7% (0-96%) in the rofecoxib group and 52.6% (17-94%) in the placebo group.(271) The lower mean PIs in the rofecoxib study may be due to the subjective choice of a high powered field of view for analysis (an area of high apoptosis was chosen for counting AI, then PI was counted from the equivalent field of view in an adjacent section of tumour), compared to the method of analysis in The EMT Trial which counted proliferation at a standardised point at the tumour edge. It is plausible that the advancing edge of a tumour has a higher rate of proliferation than more central areas of the tumour which might display higher apoptosis counts. Another study of 90 patients with resected CRCLM found that Ki-67 PI was >50% in only 29% of tumours.(353) However, these resected tumours were analysed in a tissue microarray, with non-necrotic sections of tumour placed in

the array in triplicate. The random sampling from throughout the tumour might again explain why PI is lower in this study than in The EMT Trial which analysed PI from the edge of the tumour. For comparison with other cancers, only one clinical trial was found which observed a 32% reduction in prostate cancer Ki-67 PI in patients supplemented with 5g ω-3 PUFA/day preoperatively for 4-6 weeks compared to those given placebo.(354) The study of the effect of rofecoxib by Fenwick *et al* is the only other study found which measured either apoptosis or vascularity in CRCLM.(271) Similar to the effect of EPA in The EMT Trial, rofecoxib had no effect on tumour apoptosis but was associated with a 29% reduction in MVD, although as with EPA, this failed to reach statistical significance (p=0.15).

Chapter 6: Results – Studies of the effects of EPA on Human Umbilical Vein Endothelial Cells (HUVECs)

6.1 MTT proliferation assay

6.1.1 Assay optimisation

Initial dose-finding experiments investigating the effect of EPA on HUVEC proliferation were performed using a 96-well plate with 6 replicates for each cell condition (Experiments 1 and 2). There were no viable cells seen at 24hrs when cells were supplemented with EPA concentrations greater than 50µM, and MTT optical density readings at these concentration were equivalent to the no-cell controls (Figures 6.1 and 6.2). This suggested that EPA ≥ 50µM was cytotoxic to cells. There appeared to be a dose-dependent effect of EPA on HUVEC proliferation between EPA 5-50µM. In Experiment 2, duplicate 96-well plates were seeded. In one, culture media was left unchanged for 72hrs, and in the other, culture media in each well was changed after 48hrs. Changing the culture media at 48hrs was associated with increased proliferation across all cell conditions compared with not changing the culture medium (Figure 6.2). However, because this affected all cell lines equally it was decided for future experiments not to change the culture media during the experiment in order to a) simplify the experiment, b) minimise wastage of expensive culture medium, and c) minimise error in repeatedly making up small volumes of EPA containing media.

The assay was then scaled up to a 48 well format to increase the number of cells per well and allow better visualisation of the cells under microscopy (Experiment 3). The dose range of EPA was narrowed to 0-30µM. Similar to the 96-well format, a dose-dependent reduction in HUVEC cell proliferation was observed (Figure 6.3).

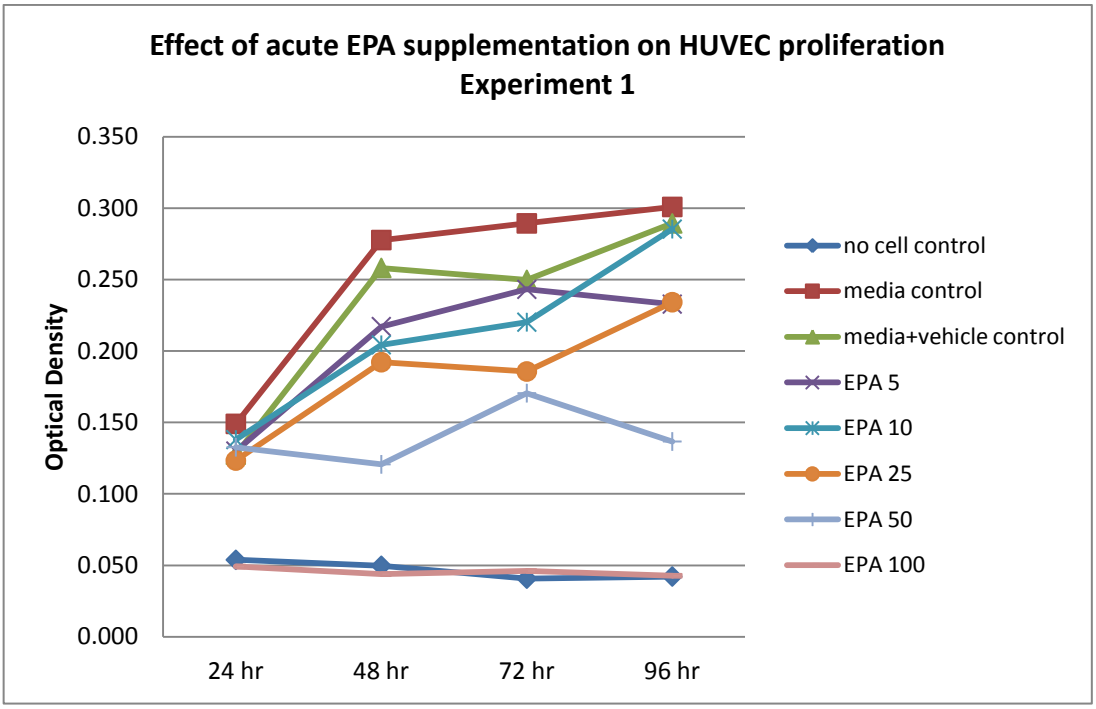


Figure 6.1. The effect of EPA on HUVEC proliferation: Experiment 1. Each data point is the mean of 6 replicate wells.

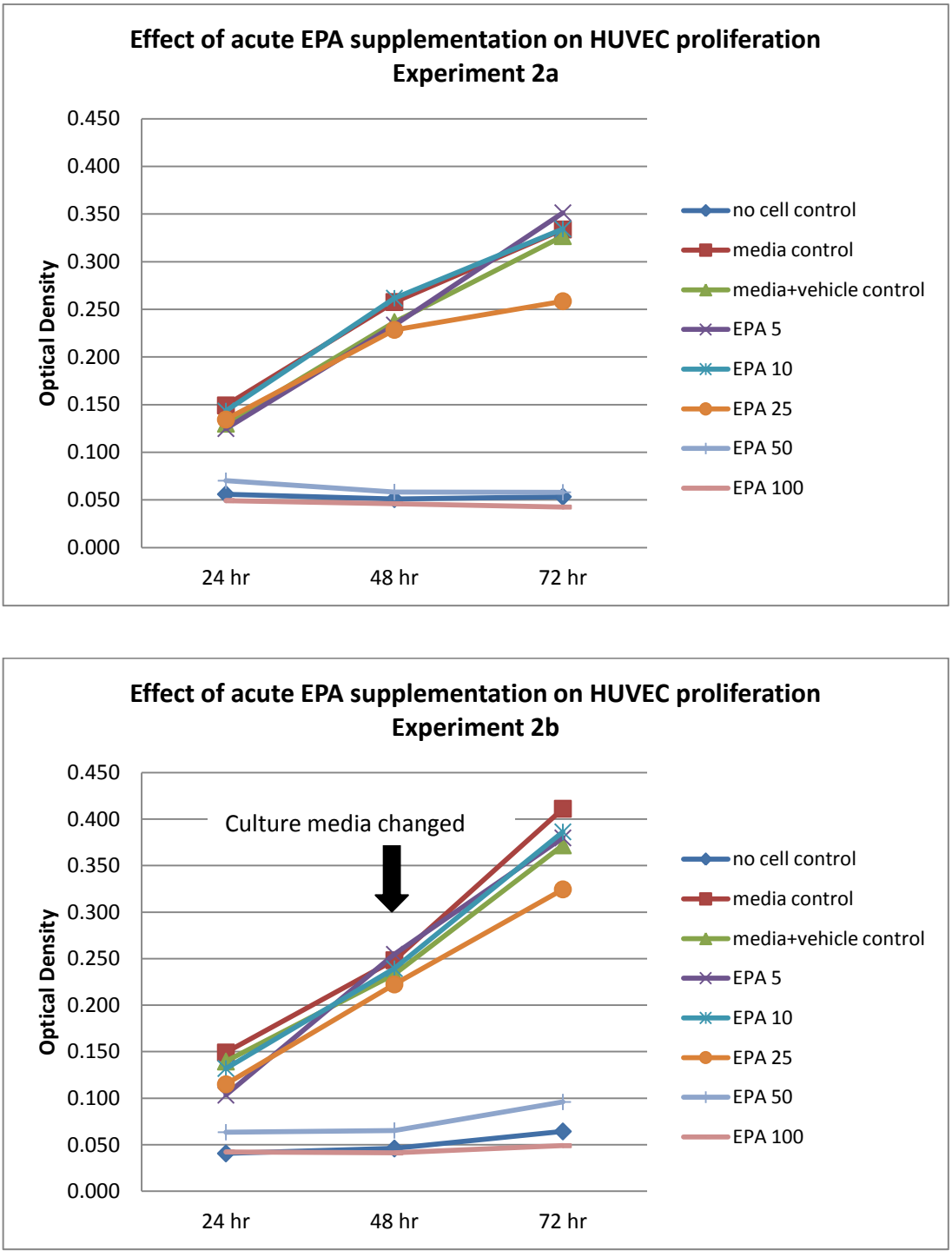


Figure 6.2. The effect of EPA on HUVEC proliferation: Experiment 2.

In experiment 2a culture media was left for the duration of the experiment, whereas in 2b the culture media in each well was aspirated and replaced with fresh medium at 48hrs. Each data point is the mean of 6 replicate wells.

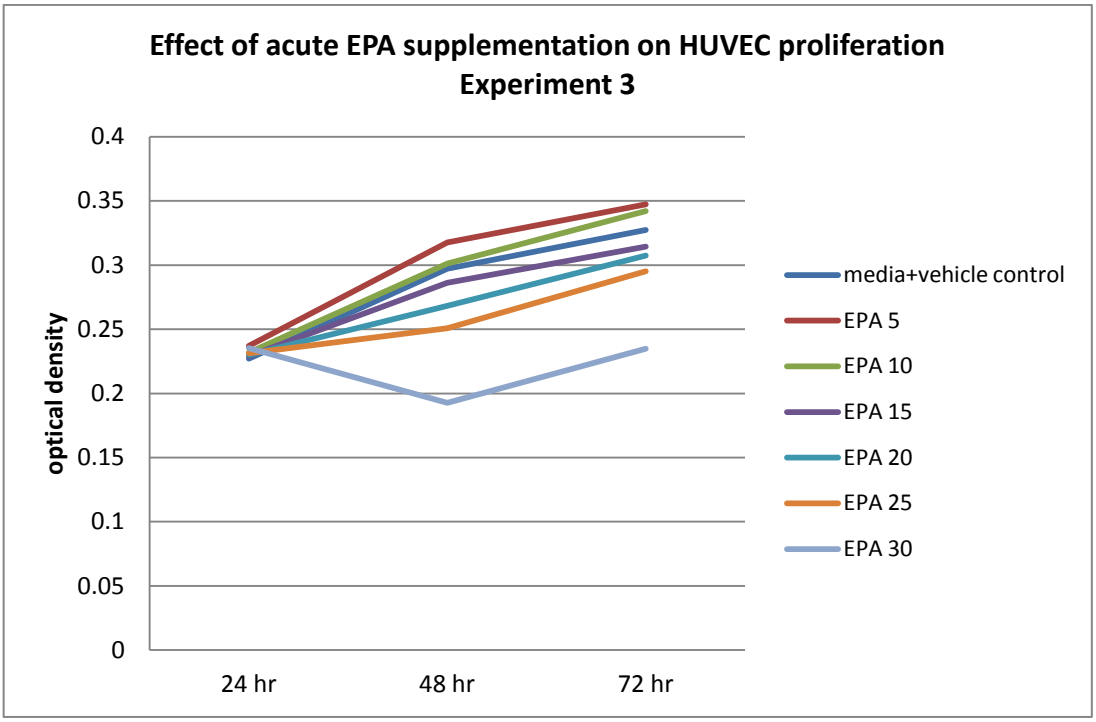


Figure 6.3. The effect of EPA on HUVEC proliferation: Experiment 3. In this experiment the effect of EPA 5-30μM was investigated in a 48-well format. Each data point is the mean of 12 replicate wells.

After scaling up the experiment to the 48-well format and observing similar results to the 96-well format, it was decided to validate the MTT assay before performing further experiments in the 48-well format. The purpose of this was two-fold. Firstly, to confirm that the MTT assay OD readings were proportional to cell number, and secondly to exclude the possibility that EPA had a direct effect on the assay itself by interfering with mitochondrial metabolism of MTT and the solubilisation of formazan crystals by propan-1-ol.

To investigate whether MTT assay OD readings were proportional to cell number. serial 2-fold dilution of cells were plated at densities of 0 - 32000 cells/well and incubated for 24hrs before performing an MTT assay. Figure 6.4 demonstrates that OD was proportional to seeded cell number. This relationship was linear up to a cell number of 16000 (OD 0.43). At the next cell density of 32000 cells, this relationship plateaued.

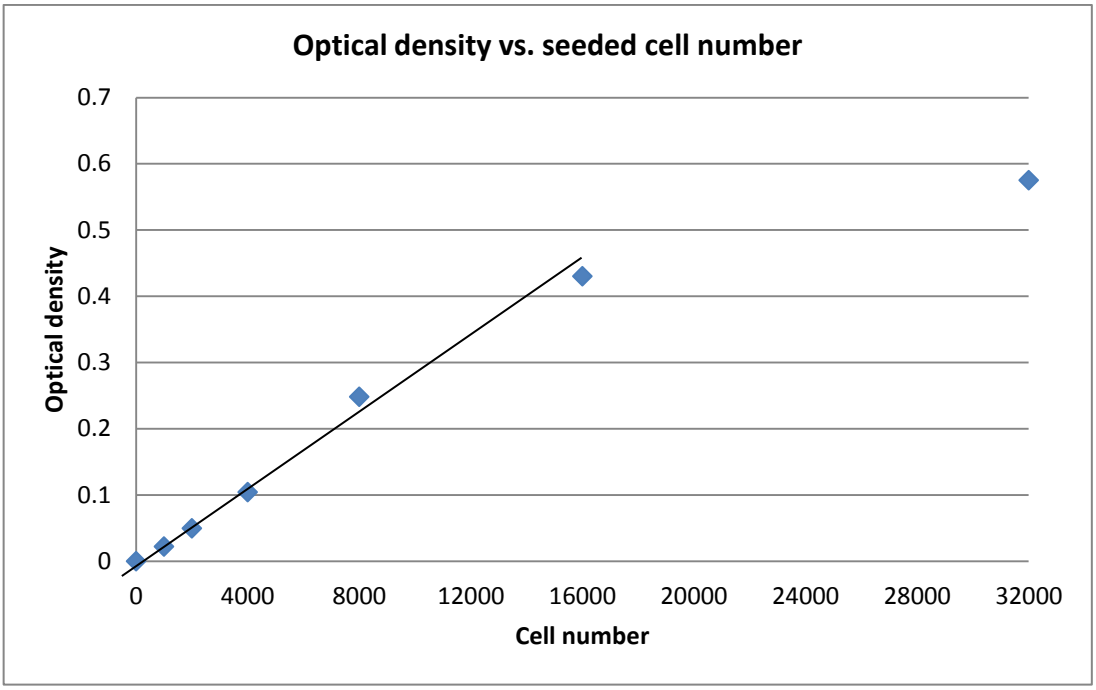


Figure 6.4. Optical density vs. seeded cell number. Serially diluted HUVECs were plated and allowed to attach for 24hrs. MTT assay was then performed and optical density plotted against seeded cell number. A line of best fit has been added between cell densities of 0-16000 cells. Each data point is the mean of 6 replicate wells.

To investigate whether EPA directly affects the MTT assay rather than affecting cell proliferation, $1x10^4$ cells were incubated in plain culture medium for 24hrs. EPA 100µM or an equivalent volume of ethanol carrier were added to one row each at the same time as adding the MTT to the cultured cells, and again to separate rows at the time of solubilising the formazan crystals with propan-1-ol. These experimental conditions are summarized in Table 6.1.

Figure 6.5 demonstrates that the addition of ethanol carrier had no effect at any stage of the MTT assay. Addition of EPA at the MTT step caused a reduction in OD, whereas addition of EPA at the propan-1-ol step had no effect on OD. Under microscopy (Figure 6.6) it was clear that the cells which received MTT+EPA were less viable than those which received MTT alone, and resembled the cells seen previously when grown in media supplemented with EPA50-100µM. It is therefore likely that 100µM EPA had a cytotoxic effect on cells within the 3 hours incubation

period when added with the MTT, rather than EPA directly affecting the performance of the assay by preventing the metabolism of MTT by viable cells.

	Culture conditions	MTT step	Propan-1-ol step
No-cell control	No cells	MTT	Propan-1-ol
Standard assay	Cells + plain media	MTT	Propan-1-ol
MTT + EtOH	Cells + plain media	MTT + EtOH	Propan-1-ol
MTT + EPA 100µM	Cells + plain media	MTT + EPA 100µM	Propan-1-ol
Propan-1-ol + EtOH	Cells + plain media	MTT	Propan-1-ol + EtOH
Propan-1-ol + EPA 100µM	Cells + plain media	MTT	Propan-1-ol + EPA 100µM

Table 6.1. Experimental conditions for each row of the 48-well plate. These conditions were replicated in 6 wells per row.

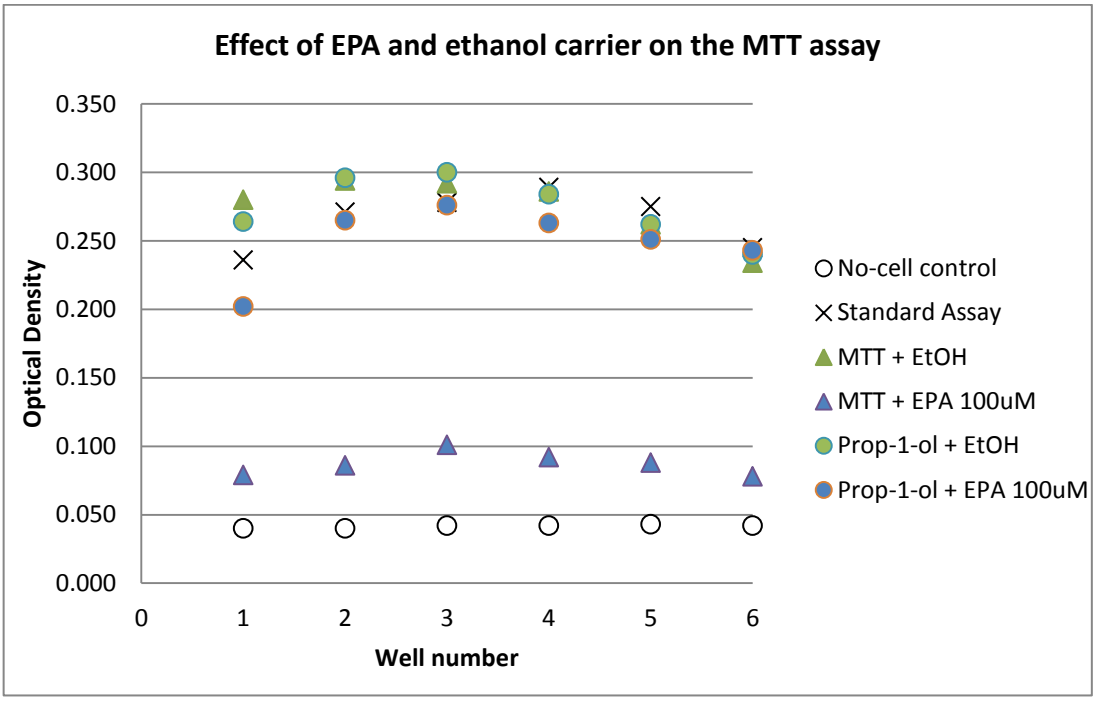


Figure 6.5. Effect of EPA and ethanol (EtOH) carrier on the performance of the MTT assay. Each data point represents a single value.

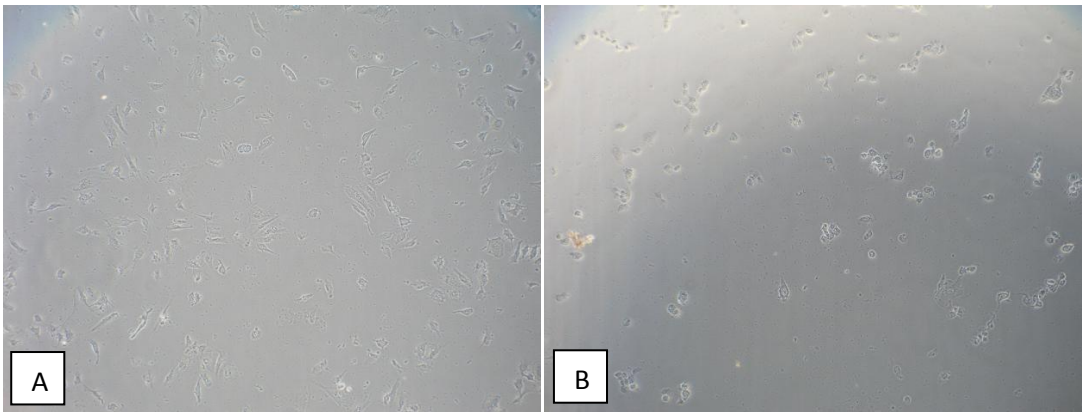


Figure 6.6. Representative photos of cells seeded in a 48-well plate. Photograph of cells (x10 magnification) remaining in wells after MTT incubation, from wells treated with (a) MTT alone as per the standard assay and (b) MTT + 100µM EPA.

6.1.2 Acute EPA supplementation

The effect of acute EPA supplementation on HUVEC proliferation was repeated using the 48-well setup and the methodology was altered slightly. Cells were plated in replicate 48-well plates containing plain media and left for 24hrs. MTT assay was performed on one plate after 24hrs of EPA-free culture (Time 0). The media in the remaining plates was then changed to experimental EPA-containing media and cultured for 24, 48, or 72hrs before performing the MTT assays. The change in OD relative to the baseline OD at Time 0 could then be calculated, whereas in the previous methodology no baseline was calculable because the cells had already been exposed to experimental EPA-containing media for 24hrs at the time of the first MTT assay.

This methodology was performed on two separate occasions (Experiments 4 and 5). In both experiments there was a dose-dependent EPA inhibition of HUVEC proliferation. These results were pooled to give a mean of 12 replicate wells (Figure 6.7). HUVECs supplemented with 10µM EPA showed approximately 26% less growth than un-supplemented cells at 24hrs ($p=0.02$), 33% less growth at

48hrs (p<0.01) and 33% less growth at 72hrs (p<0.01). Cells supplemented with 20µM EPA showed 34% less growth than un-supplemented cells at 24hrs (p<0.01), 38% less growth at 48hrs (p<0.01), and 41% less growth at 72hrs. At 48 and 72hrs, cells supplemented with 25-30µM EPA showed 64-69% less growth than un-supplemented cells (p<0.01). All statistical analyses were performed using unpaired t-tests.

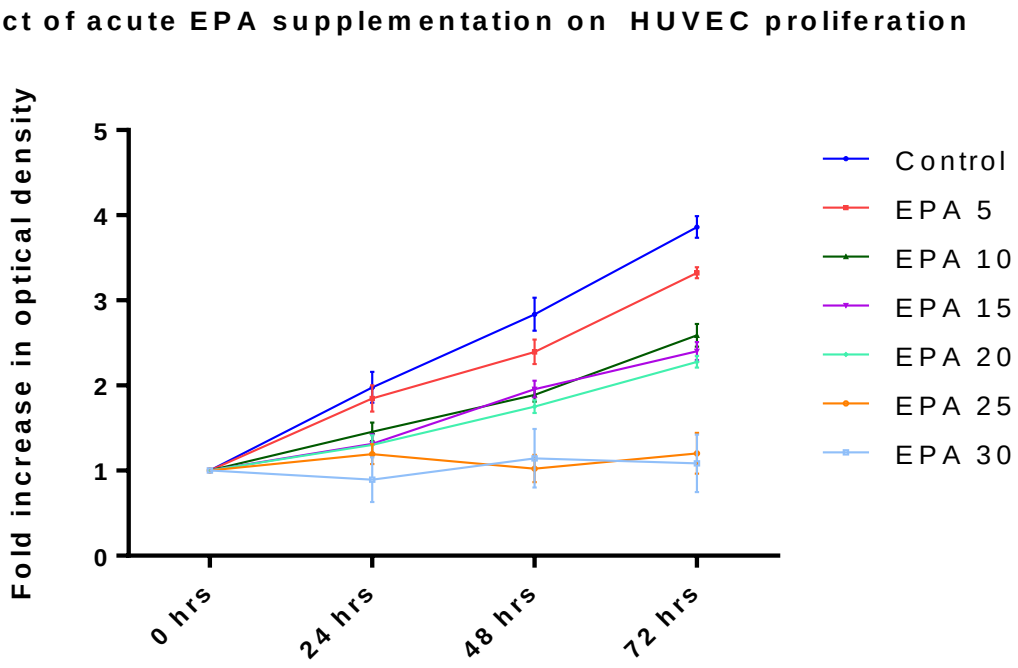


Figure 6.7. Effect of EPA on HUVEC proliferation. Each data point is the mean of 12 replicate wells. Note that for these experiments cells were plated for 24hrs in plain media, and then culture media change to EPA-containing media and cells incubated for a further 24-72hrs. One plate was read immediately prior to exposing cells to EPA, allowing optical density at each time point to be compared to mean optical density of the corresponding row of the baseline 48-well plate and expressed as a fold-increase. Error bars represent mean+- SEM.

6.1.3 Chronic EPA supplementation

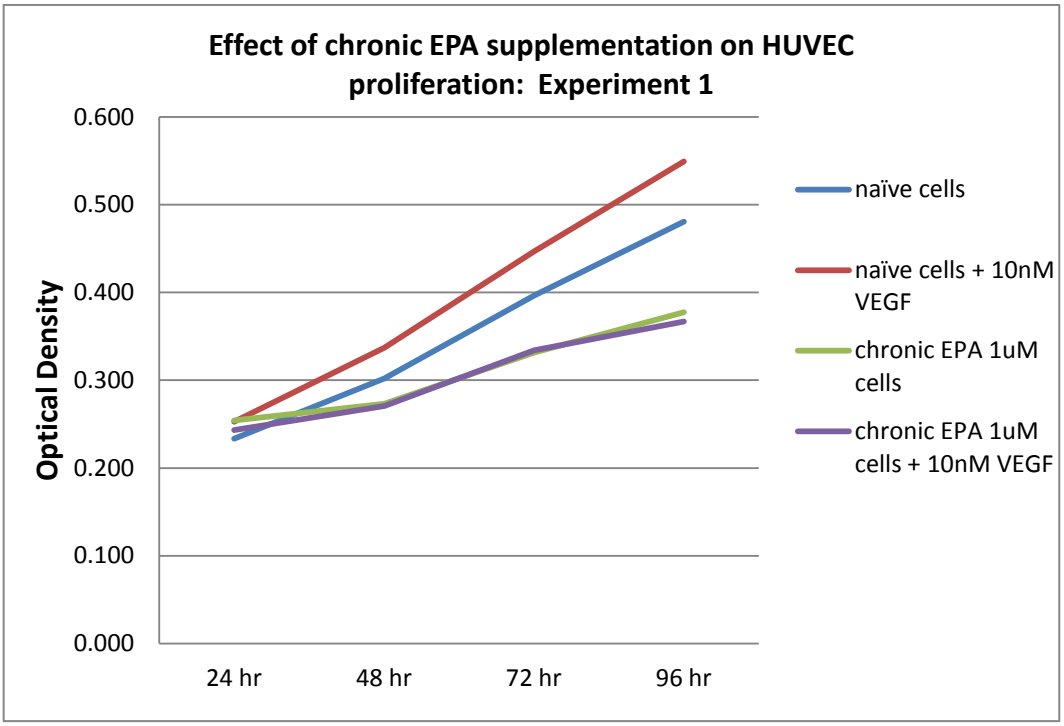
Whilst the acute EPA supplementation experiments provided a simple, reproducible assay, the short period of HUVEC exposure to EPA was not representative of the duration of EPA supplementation in *in vivo* and human studies. I therefore decided to also investigate the effect of a longer-duration, lower-dose EPA supplementation as a closer representation of conditions in *in vivo* and human studies. HUVECs were incubated for two weeks in either plain medium or medium supplemented with 1µM EPA. Media was changed every 48 hrs. After two weeks, cells were trypsinised, counted and seeded in 48-well plates and an MTT assay performed as in the acute EPA supplementation experiments. This was performed on two separate occasions (Experiment 1 and 2). In contrast to the acute EPA supplementation experiments, a baseline MTT assay could not be performed prior to EPA exposure because the cells had already been exposed to experimental medium for two weeks. Results of the chronic EPA supplementation experiments are therefore quoted as the mean OD, rather than as the fold-change in OD compared to baseline.

Cells chronically supplemented with EPA showed reduced proliferation compared to EPA naive cells at all time points in both experiments. The proliferation curve was steeper in Experiment 2 than in Experiment 1, but the same trends were observed in both experiments (Figure 6.8). At 96hrs, OD in EPA-supplemented cells was 16.4% lower than in EPA-naive cells in Experiment 1 ($p<0.01$), and 33.9% lower in Experiment 2 ($p<0.01$).

VEGF is known to stimulate HUVEC proliferation, and inhibition of VEGF-stimulated angiogenesis has been proposed as one mechanism by which EPA might inhibit angiogenesis. To further investigate the mechanistic effect of chronic EPA supplementation on HUVEC proliferation, I decided to supplement cells with VEGF at the time of seeding onto the 48-well plates to test the hypothesis that EPA-

supplemented cells would have an attenuated response to VEGF stimulation. Supplementation of VEGF 10nM to culture media stimulated proliferation in the EPA-naive cells in both Experiment 1 and Experiment 2 compared to VEGF unstimulated cells at 96hrs (12.7% and 18.9% increases in proliferation, $p<0.01$ and $p=0.01$ respectively). However, VEGF had no significant effect on proliferation of cells chronically supplemented with EPA compared to VEGF unstimulated cells at 96hrs (0.8% increase and 3% increase in proliferation, $p=0.77$ and $p=0.56$ respectively). The response to VEGF was indeed attenuated in the EPA-supplemented cells.

VEGF is known to be upregulated by PGE_2,(73, 186) so EPA inhibition of the COX-PGE_2 pathway is a potential mechanism by which EPA might inhibit VEGF-stimulated angiogenesis. I therefore decided to supplement cells with PGE_2 at the time of seeding onto 48-well plates to test the hypothesis that the addition of PGE_2 would restore proliferation in the EPA-supplemented cells to the same levels as that seen in control cells (i.e. a PGE_2 rescue effect). In Experiment 2, cells were supplemented with 1µM PGE_2. In the EPA-naive cells, PGE_2 supplementation stimulated proliferation (20.6%, $p=0.01$), to a similar extent to that seen with VEGF supplementation. PGE_2 also stimulated proliferation in cells chronically supplemented with EPA, but this response was attenuated compared to the proliferation of EPA-naive cells and did not reach statistical significance (12.9%, $p=0.08$). PGE_2 supplementation therefore failed to show a rescue effect in cells chronically supplemented with EPA.



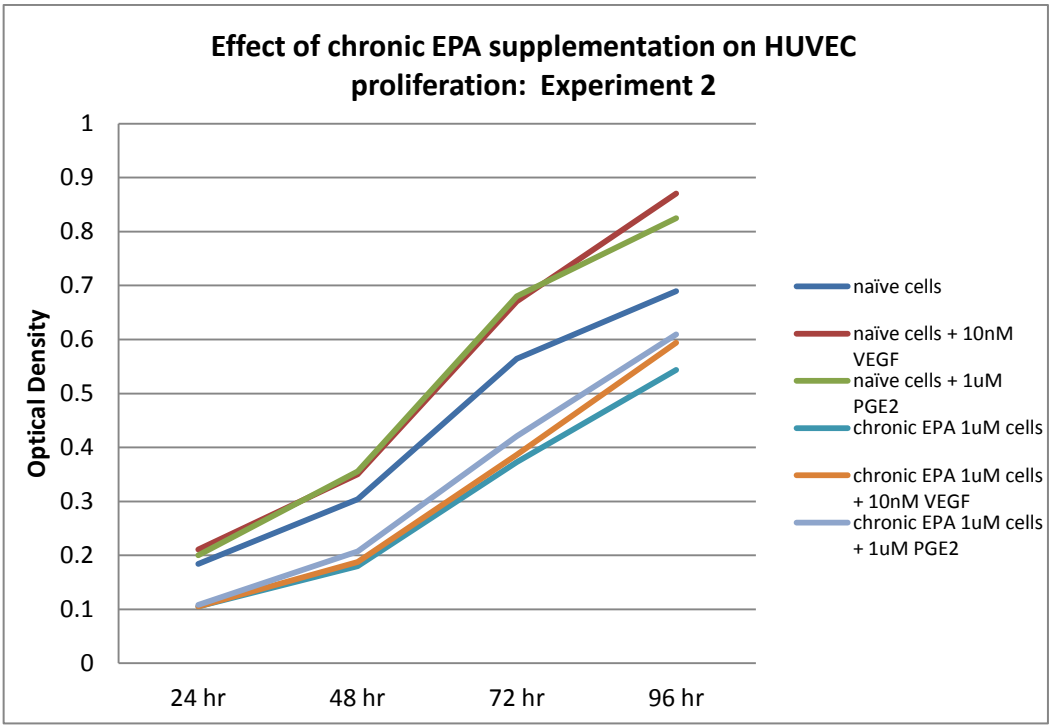


Figure 6.8. The effect of chronic EPA supplementation on HUVEC proliferation (Experiments 1 and 2). The effect of VEGF supplementation is shown in Experiment 1 and 2, and the effect of PGE_2 supplementation is shown in Experiment 2. Each data point is the mean of 6 replicates.

6.1.4 Concentration of PGE_2 and 6-keto-$PGF_{1\alpha}$ in HUVEC culture medium

HUVEC cell-conditioned medium was collected from acute EPA supplementation Experiment 4 and Experiment 5 at 72 hours, and from chronic EPA supplementation Experiment 1 at 96 hours. For each EPA supplementation condition in each experiment, medium was collected from the six replicate wells and pooled and analysed as a single sample. This was because it was impractical and prohibitively expensive to analyse PGE_2 in the cell conditioned medium of each replicate well separately. PGE_2 was not detectable in any of the samples by LC-MS/MS, most likely because PGE_2 was present in pg/ml concentrations, i.e. below the 10ng/ml limit of detection of PGE_2 by LC-MS/MS (Loadman *et al*, unpublished data).

However, PGI_2 rather than PGE_2 is considered the main prostaglandin product of AA metabolism in vascular endothelium and previous studies in HUVEC cultures have shown greater levels of PGI_2 than PGE_2 production.(299-301) I therefore decided to measure the levels of 6-keto-$PGF_{1\alpha,}$ a stable product of PGI_2 , in cell-conditioned media. The same batches of cell-conditioned media were used and levels of 6-keto-$PGF_{1\alpha}$ measured using an enzyme immunoassay (EIA) as described in section 4.8.1.

The standard curve of 6-keto-$PGF_{1\alpha}$ concentration versus percentage binding for the assay is shown in Figure 6.9.

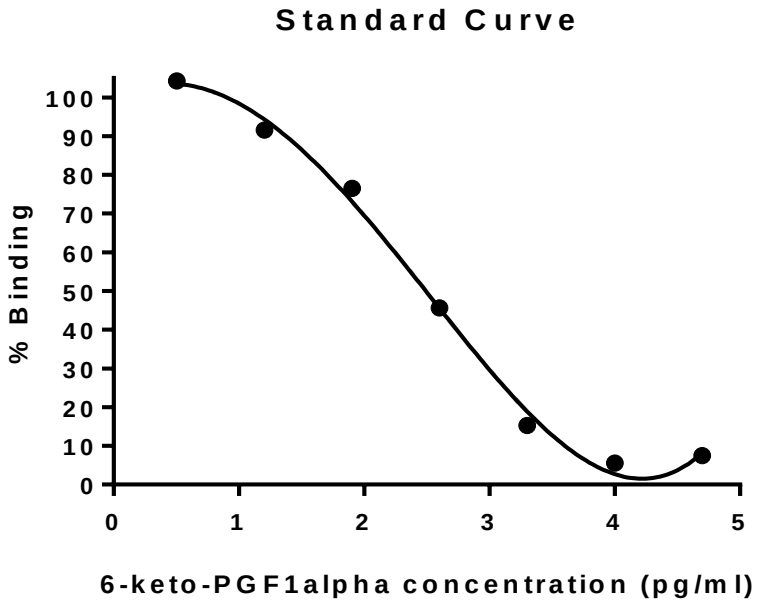


Figure 6.9. Standard curve of 6-keto-$PGF_{1\alpha}$ concentration versus percentage binding

The results of the 6-keto-$PGF_{1\alpha}$ analyses are presented alongside the corresponding MTT experiment data in Figures 6.10 - 6.12. In the acute EPA supplementation experiments, the concentration of 6-keto-$PGF_{1\alpha}$ in culture medium at 72 hours in Experiment 5 (Figure 6.10) was highest in those cells supplemented with the highest concentrations of EPA (20 and 30µM) and lowest in the control cells which were not supplemented with EPA (0um). By contrast, in Experiment 6 (Figure 6.11) EPA supplementation had no effect on 6-keto-$PGF_{1\alpha}$ concentration in the culture medium. The range of 6-keto-$PGF_{1\alpha}$ concentrations (17.9-26.7pg/ml) was small, and similar to the 6-keto-$PGF_{1\alpha}$ concentration seen in the culture medium of EPA un-supplemented cells in Experiment 5 (22.1pg/ml). In the chronic EPA supplementation experiment (Figure 6.12), 6-keto-$PGF_{1\alpha}$ concentration in the culture medium of EPA supplemented cells was 39.8% lower than that in the culture media of control cells. The concentration range in this experiment (42.7 - 71.3pg/ml) more closely matches that of Experiment 5 of the acute EPA supplementation experiments.

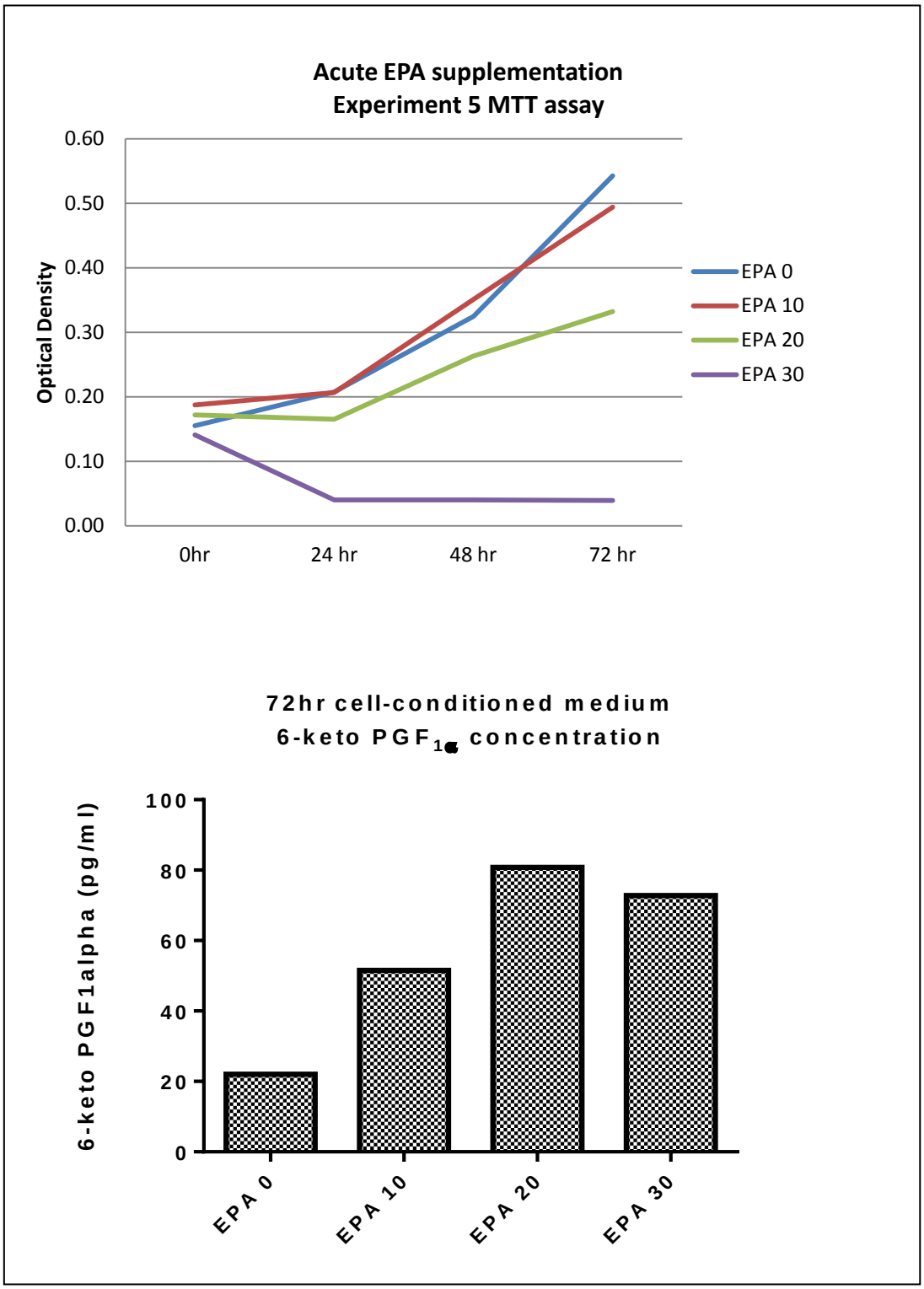


Figure 6.10. 6-keto-$PGF_{1\alpha}$ concentration in cell-conditioned media: Acute EPA supplementation Experiment 5. Results from the MTT assay of the effect of acute EPA supplementation on HUVEC proliferation (Experiment 5, each data point is the mean of 6 replicates) are shown together with the concentration of 6-keto-$PGF_{1\alpha}$ in the cell conditioned medium when aspirated from the 48-well plate of the MTT assay at 72hours (each data point is a single replicate of pooled media).

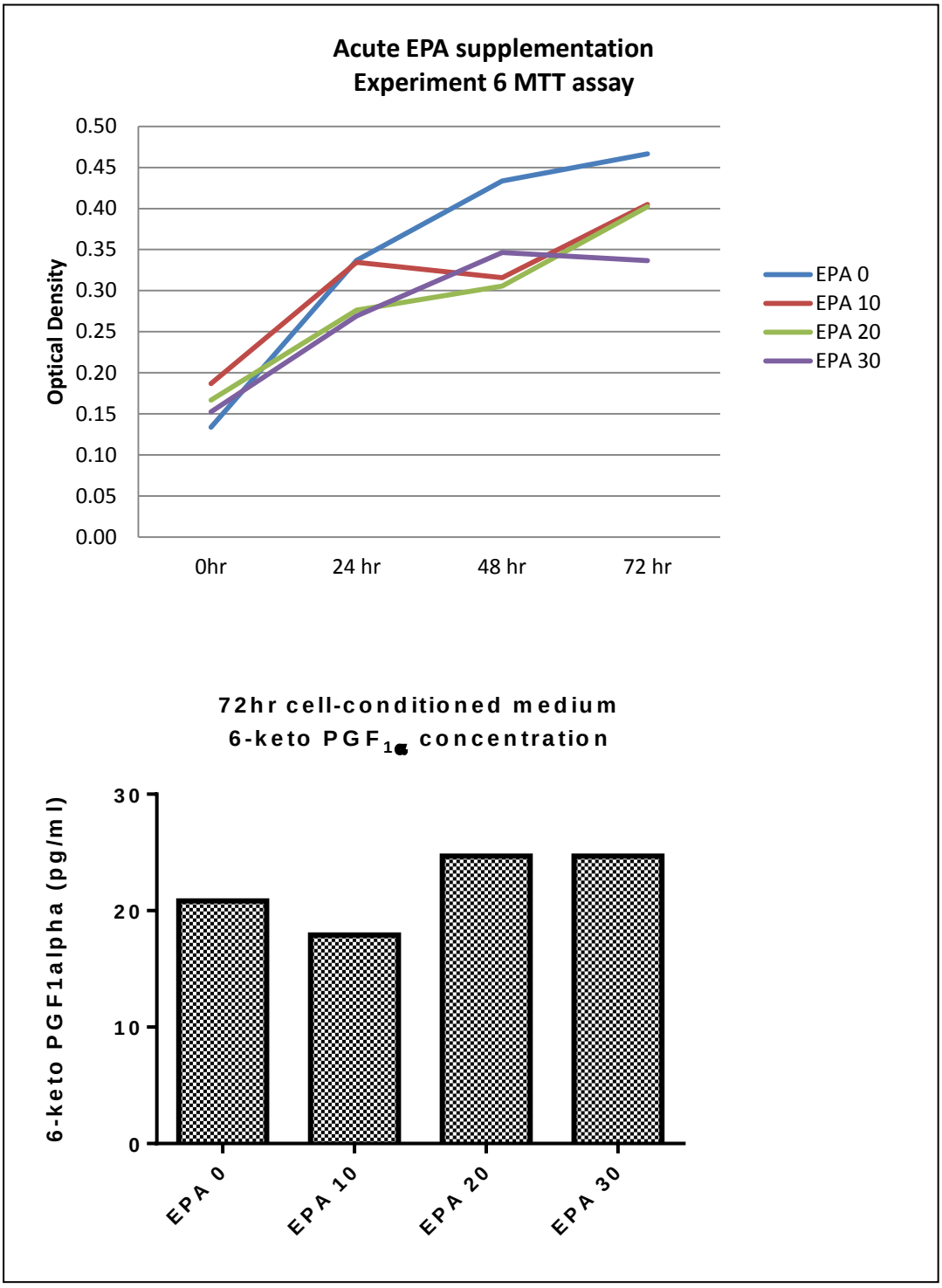


Figure 6.11. 6-keto-$PGF_{1\alpha}$ concentration in cell-conditioned media: Acute EPA supplementation Experiment 6. Results from the MTT assay of the effect of acute EPA supplementation on HUVEC proliferation (Experiment 6, each data point is the mean of 6 replicates) are shown together with the concentration of 6-keto-$PGF_{1\alpha}$ in the cell conditioned medium when aspirated from the 48-well plate of the MTT assay at 72hours (each data point is a single replicate of pooled media).

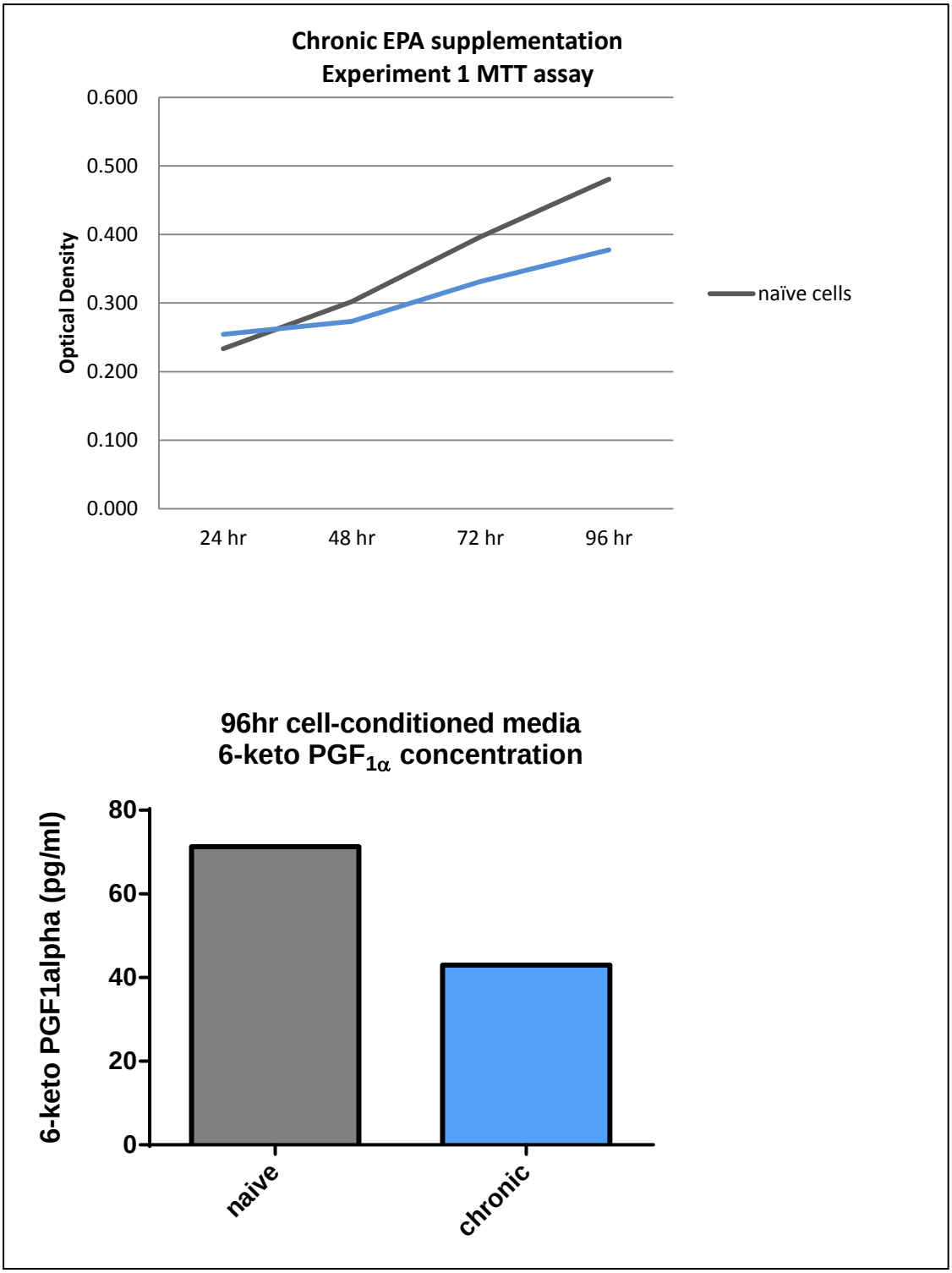


Figure 6.12. 6-keto-$PGF_{1\alpha}$ concentration in cell-conditioned media: Chronic EPA supplementation Experiment 1. Results from the MTT assay of the effect of chronic EPA supplementation on HUVEC proliferation (Experiment 1, each data point is the mean of 6 replicates) are shown together with the concentration of 6-keto-$PGF_{1\alpha}$ in the cell conditioned medium when aspirated from the 48-well plate of the MTT assay at 72hours (each data point is a single replicate of pooled media)

It is not possible to draw any conclusions about the effect of EPA on PGI_2 production based on the inconsistent findings of this small number of experiments each with a small number of replicate readings.

6.2 Wound migration assay

6.2.1 Assay optimisation

Several experiments were performed to optimise the technique of wound scoring in this assay. The most consistent wound score of an appropriate width was achieved with a yellow pipette tip. Other sized pipette tips produced scores that were too thin, too thick, or caused excessive detachment of cells from the sides of the wound. A gentle technique of slowly aspirating and replacing culture media was required to prevent cells detaching from the plate in large sheets. Lifting of cells from the plate was also minimised by aspirating culture medium from the cells, scoring the dry cell monolayer, then adding fresh culture medium, rather than scoring cells in the presence of culture medium.

6.2.2 Acute EPA supplementation

In the first dose finding experiment of the effect of acute EPA supplementation on HUVEC wound migration (Figure 6.13), 60µM EPA caused cell death, with cells seen floating in the culture medium and causing an artificial increase in the size of the measured wound. At EPA concentrations up to 40µM, there appeared to be little difference in the closure of wounds, although the variability in wound closure in the 3 wells of the upper row of the 6 well plate was higher than that in the 3 wells of the lower row, which seemed to have more consistent wound closure. On closer examination of the wound photographs there was a noticeable "shouldering" effect in some of the photos (Figure 6.14), with the wound closing at the lower end (nearest the centre of the well), but not at the upper end (nearest the edge of the well). Those cells at the peripheral edge of the well appeared less healthy than

those more centrally placed. The assay was therefore refined so that the parallel reference lines for taking photographs of the wound crossed the well more centrally, avoiding taking photos towards the periphery and therefore avoiding this peripheral edge effect.

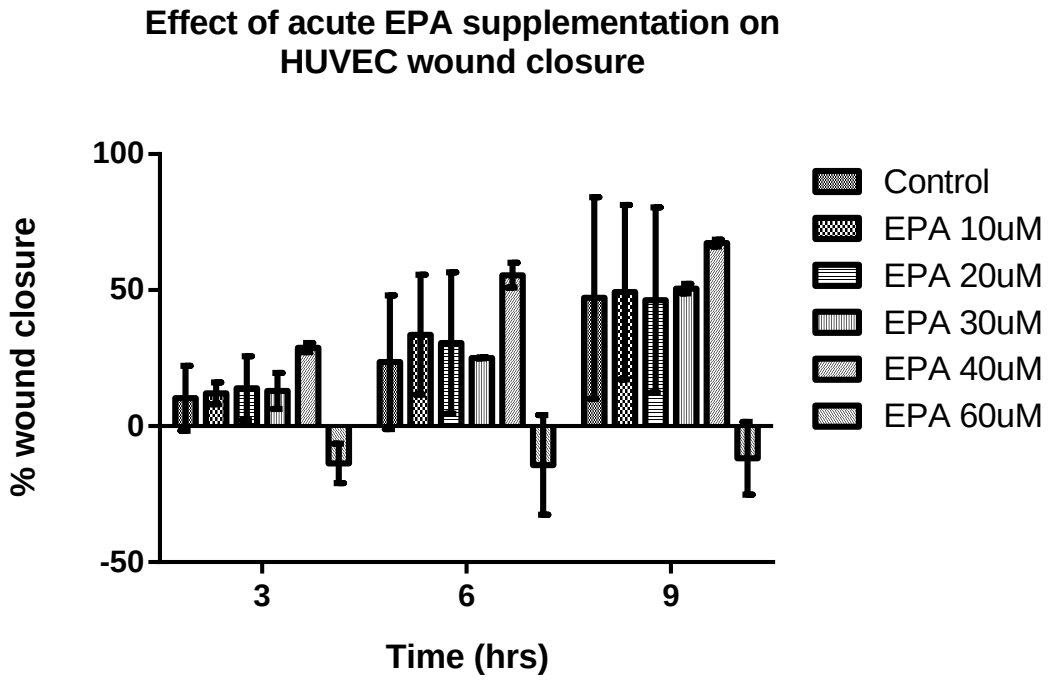


Figure 6.13. Dose-finding experiment of the effect of acute EPA supplementation on HUVEC wound closure. This was performed on a single 6 well plate with 2 photos taken per well. Error bars represent mean +- SEM of the two photos taken per well.

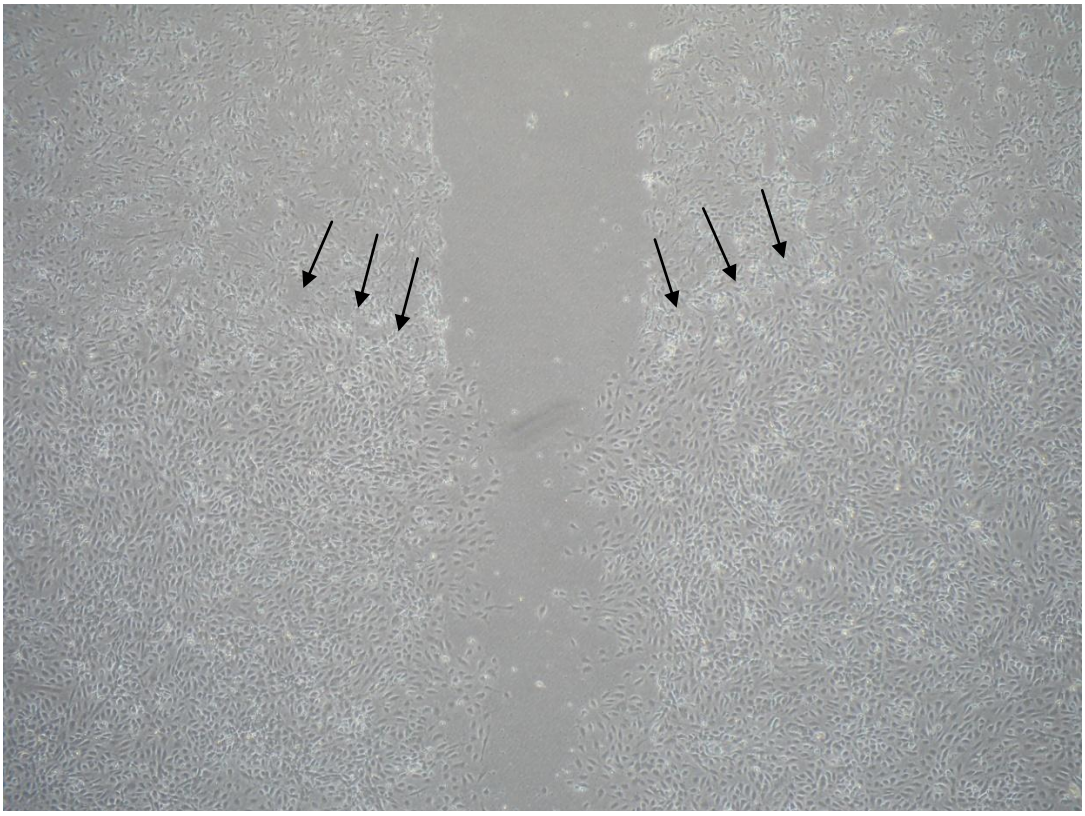


Figure 6.14. Photograph of the shouldering effect seen in some of the wound scores. Note the less healthy cells and absence of wound closure at the well periphery (top edge of photo) compared to the healthier cells and wound closure more centrally in the well (middle and bottom edge of photo). The transition is marked with arrows.

Two further experiments were performed in triplicate 6-well plates with EPA doses 10-50μM. In the first experiment (Figure 6.15 & 6.16) there was no appreciable wound closure at 3 hours. At 6 hours, control cells showed 45.8% wound closure. In the cells treated with 20, 30 and 40μM EPA, wound closure was approximately 24% lower than that in controls, with wound closures of 35.0% ($p=0.01$), 37.5% ($p=0.04$) and 34.9% ($p<0.01$) respectively. Wound closure in cells treated with 50μM EPA was half of that in controls, with a wound closure of 23.6% ($p<0.01$). At 9 hours, wound closure in the control cells was 75%. Wound closure was approximately 13% lower in cells treated with 10, 20 and 30μM EPA than controls ($p=0.17$ to $p=0.29$). Cells treated with EPA 40μM EPA showed almost one third less wound closure than controls with a wound closure of 54% ($p<0.01$), and cells treated with 50μM EPA showed two-thirds less wound closure than controls with a wound closure of 24.4% ($p<0.01$). All statistical comparisons were performed using unpaired t-tests.

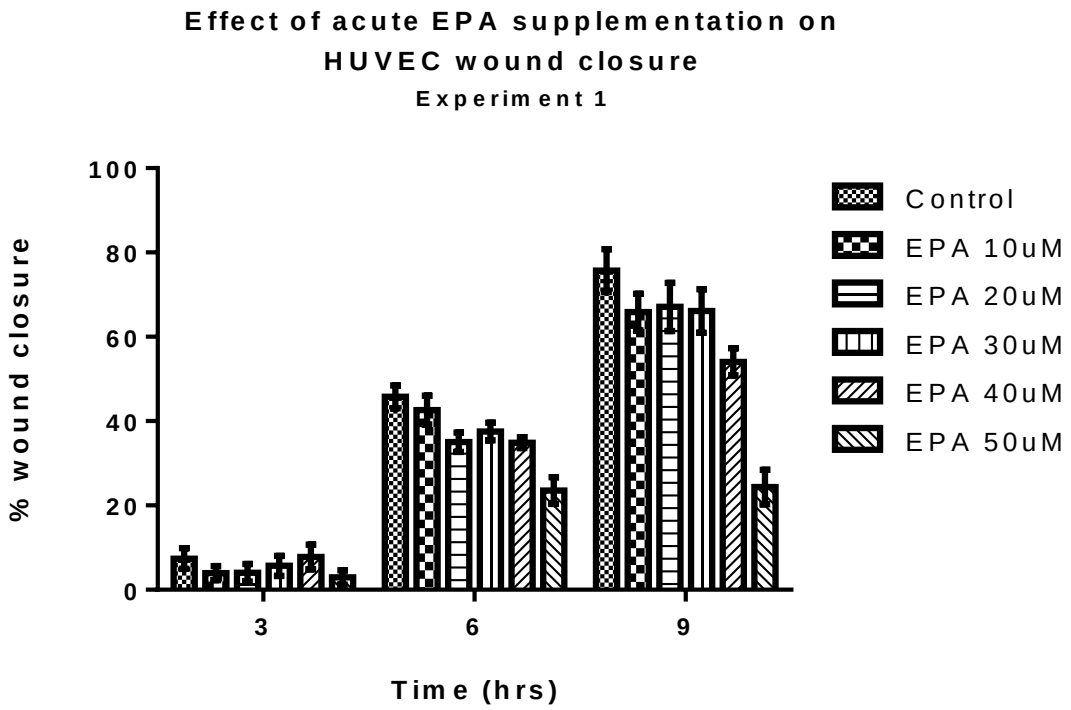


Figure 6.15. Effect of acute EPA supplementation on HUVEC wound closure: Experiment 1. Wound closure over time is shown as the percentage reduction in the width of the wound compared to width at baseline. Each bar is the mean of 6 photos from 3 separate wells, except for the EPA 50µM group where two of the wells showed a shouldering effect at one of the reference lines. These photos were discarded and bars in the EPA 50µM group are therefore the mean of 4 photos from three wells. Error bars represent mean+- SEM.

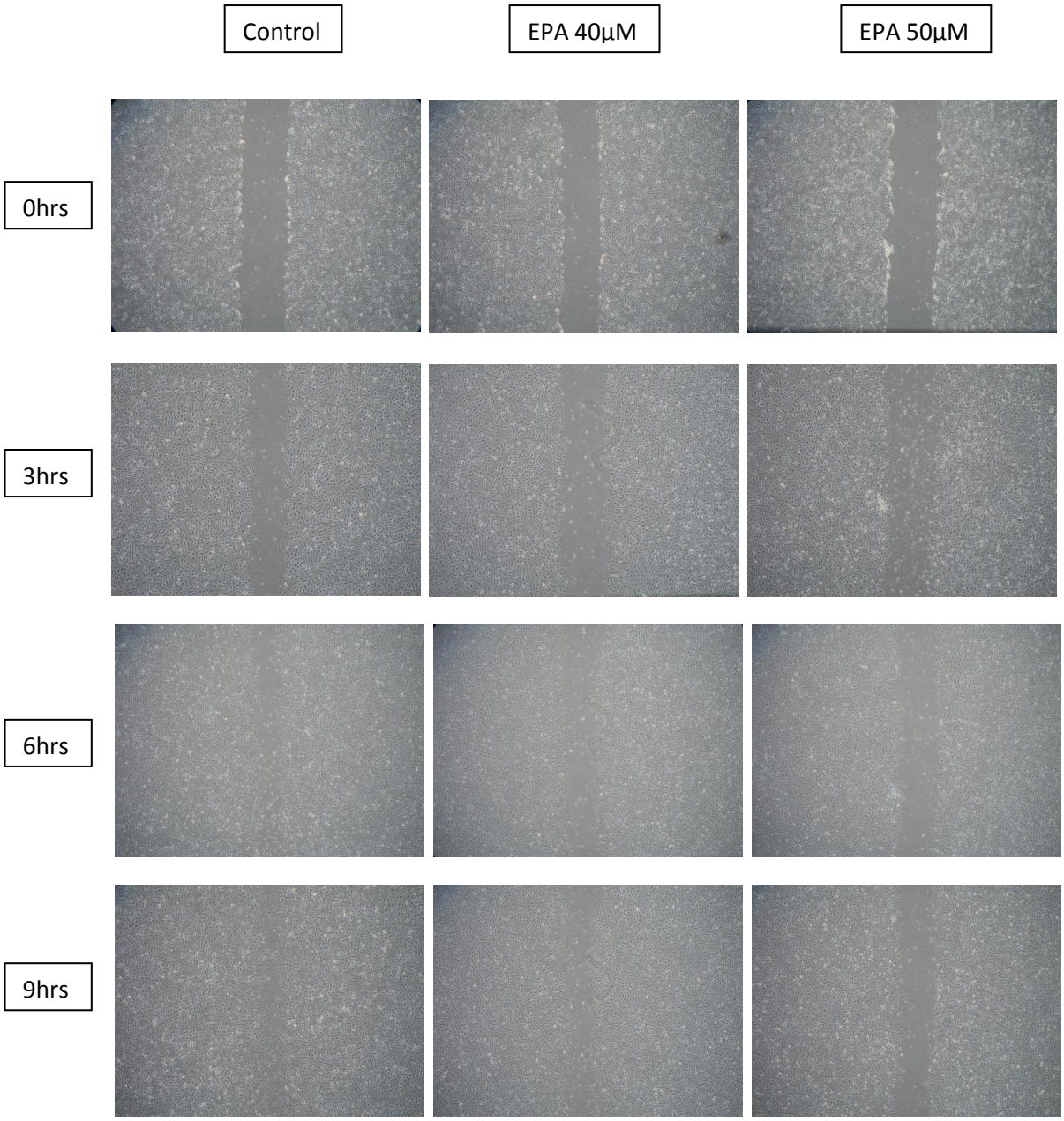


Figure 6.16. Photographs of HUVEC wound closure over time: Experiment 1. Wound closure at 0, 3, 6 and 9hrs in control cells and cells acutely supplemented with 40µM and 50µM EPA. Note the progressive closure of the wound over time in the control group (left column), with less pronounced closure in the EPA supplemented groups (centre and right column).

In the second experiment, supplementation of cells with EPA concentration >20µM resulted in cells lifting off the plate and floating in the culture medium within three hours. The reason for this unexpected variability in the dose of EPA which resulted in cells lifting off the plate between the two experiments is not clear. Perhaps the technique of wound scoring was too vigorous in the second experiment, resulting in more disruption of the HUVEC monolayer. Alternatively, it could have been due to an incorrect concentration of EPA being used in the second experiment, either due to an error in the mixing of EPA-supplemented media, or because a new batch of EPA capsules was used for this experiment. Wound closure was only calculable for doses of EPA of 10 and 20µM (Figure 6.17 and Figure 6.18). Supplementation with EPA 10µM resulted in a 13.8% reduction in wound closure compared to controls at 6 hours ($p=0.32$), and 13.1% reduction at 9hrs ($p=0.20$). EPA 20µM resulted in 37.8% reduction in wound closure compared to controls at 3hrs ($p=0.04$), 82.5% reduction at 6hrs ($p<0.01$) and 67.8% reduction at 9hrs ($p<0.01$).

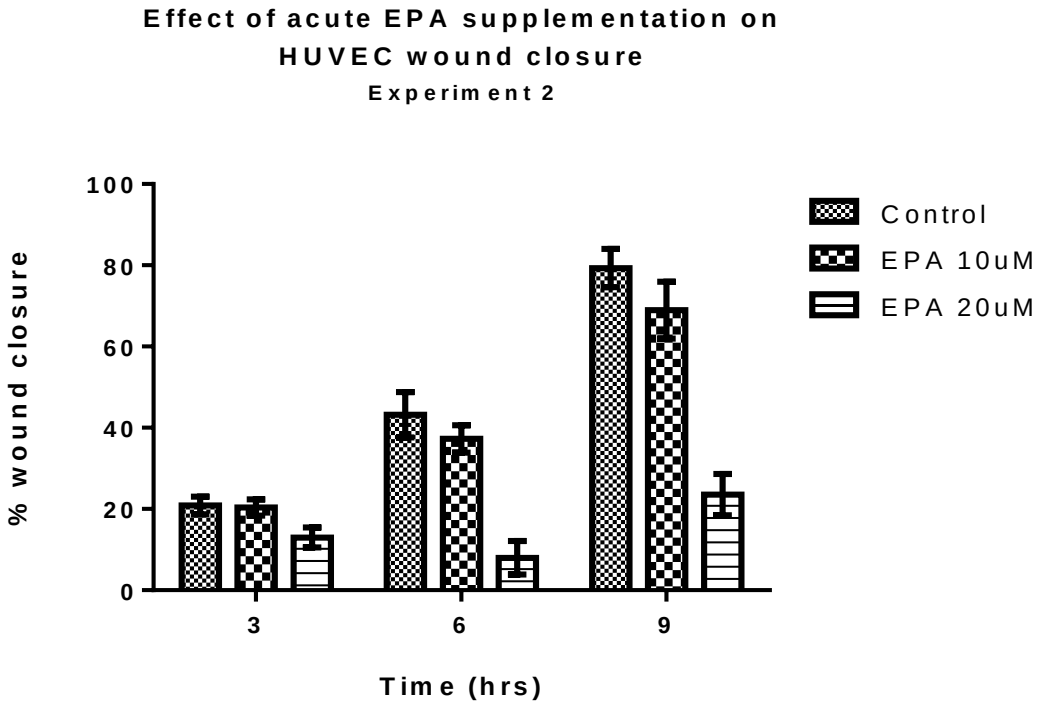


Figure 6.17. Effect of acute EPA supplementation on HUVEC wound closure: Experiment 2. Wound closure over time is shown as the percentage reduction in the width of the wound compared to width at baseline. Each bar is the mean of 6 photos from 3 separate wells. Error bars represent mean+- SEM.

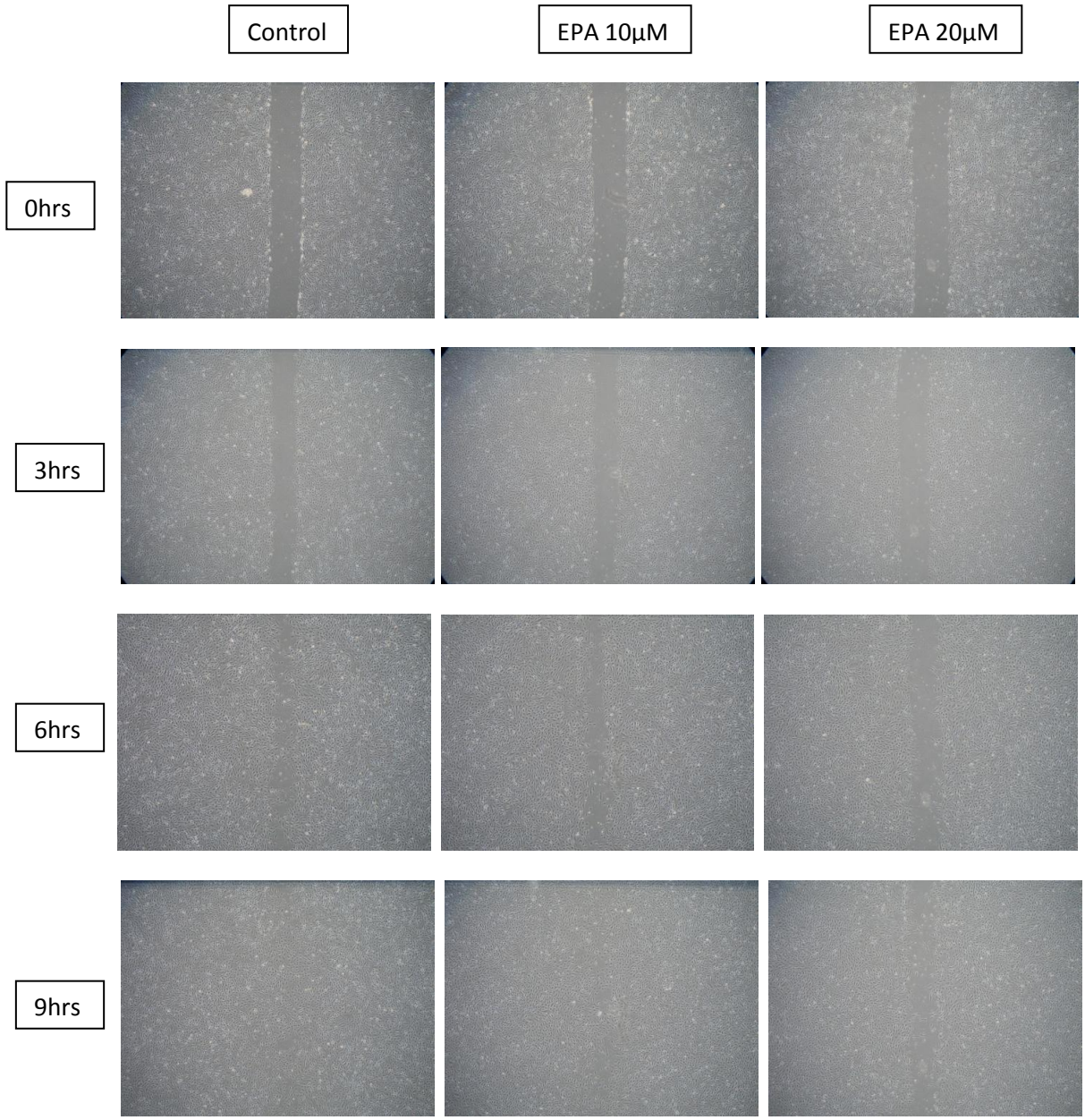


Figure 6.18. Photographs of HUVEC wound closure over time: Experiment 2. Wound closure at 0, 3, 6 and 9hrs in control cells and cells acutely supplemented with 10µM and 20µM EPA

6.2.3 Chronic EPA supplementation

In the first experiment of the effect of chronic EPA supplementation (1μM EPA for 2 weeks) on HUVEC wound closure, the effect of adding 10nM VEGF to the culture medium immediately after performing the wound score was also investigated. The results are shown in Figure 6.19. There was no statistically significant difference in wound closure between EPA-supplemented cells and EPA-naive cells, although wound closure in the EPA-supplemented cells was 15.2% greater at 6hrs (p=0.15) and 20.2% greater at 12hrs (p=0.07) compared to the EPA-naive cells. Addition of VEGF did not increase wound closure in either the EPA-naive or EPA-supplemented cells.

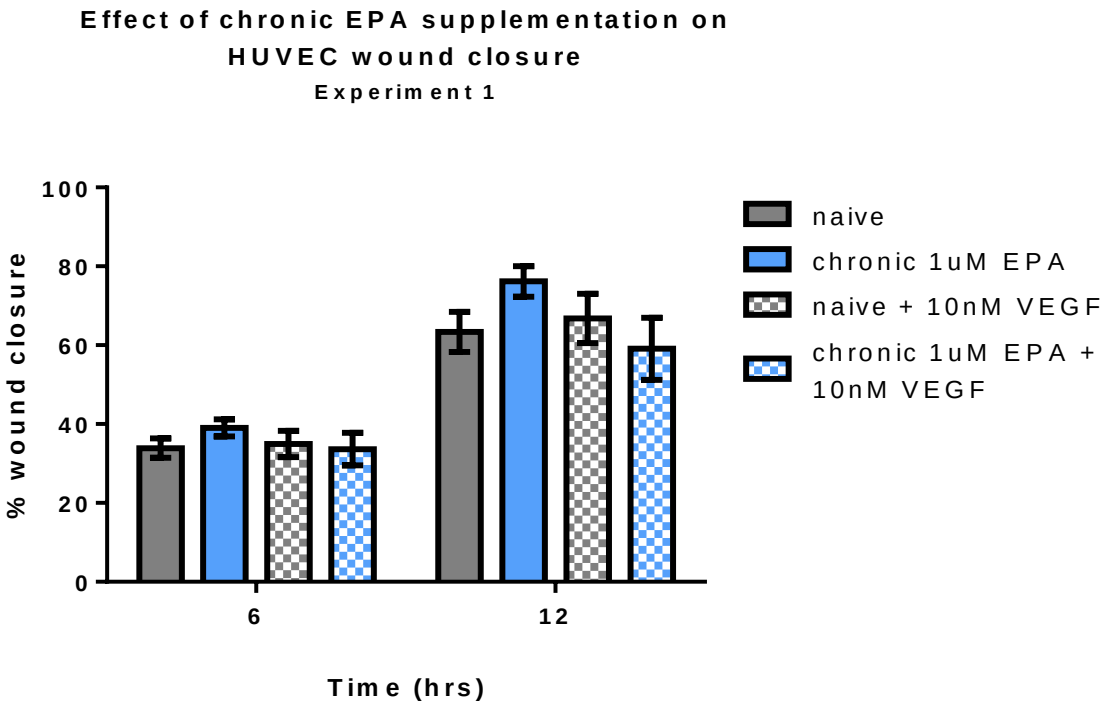


Figure 6.19. The effect of chronic EPA supplementation on HUVEC wound closure: Experiment 1 Wound closure over time is shown as the percentage reduction in the width of the wound compared to width at baseline. In the chequered bars, 10nM VEGF was added immediately after creating the wound scores. Each bar is the mean of 6 photos from three separate wells. Error bars represent mean +- SEM.

Two further replicate experiments investigating the effect of chronic EPA supplementation on HUVEC wound migration were performed (Experiments 2 and 3). In each experiment, triplicate 6 well plates were plated up, with three wells per plate containing EPA naive cells and 3 wells per plate containing EPA-supplemented cells. In Experiment 2 (Figure 6.20) EPA-supplemented cells showed slower wound closure compared to controls. Wound closure compared to controls was 16.2% less at 6hrs (unpaired t-test, p=0.03) and 14.7% less at 9hrs (unpaired t-test, p=0.06). In Experiment 3 (Figure 6.21) there was no significant difference in wound closure between the EPA-naive and EPA supplemented cells. The variability in these three experiments is difficult to explain, with one showing increased wound closure in the EPA group, another showing reduced closure, and the third showing no difference in closure compared to the EPA-naiive cells. However, on the basis of this limited number of experiments, it appears that chronic EPA-supplementation does not affect wound closure.

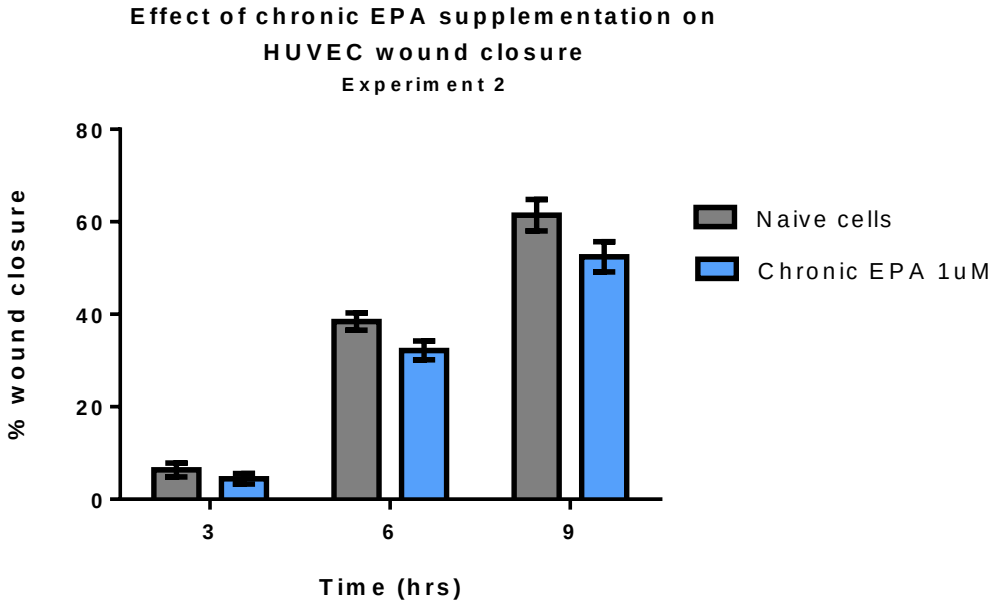


Figure 6.20. The effect of chronic EPA supplementation on HUVEC wound closure: Experiment 2. Wound closure over time is shown as the percentage reduction in the width of the wound compared to width at baseline. Each bar is the mean of 18 photos from 8 replicate wells. Error bars represent mean +- SEM.

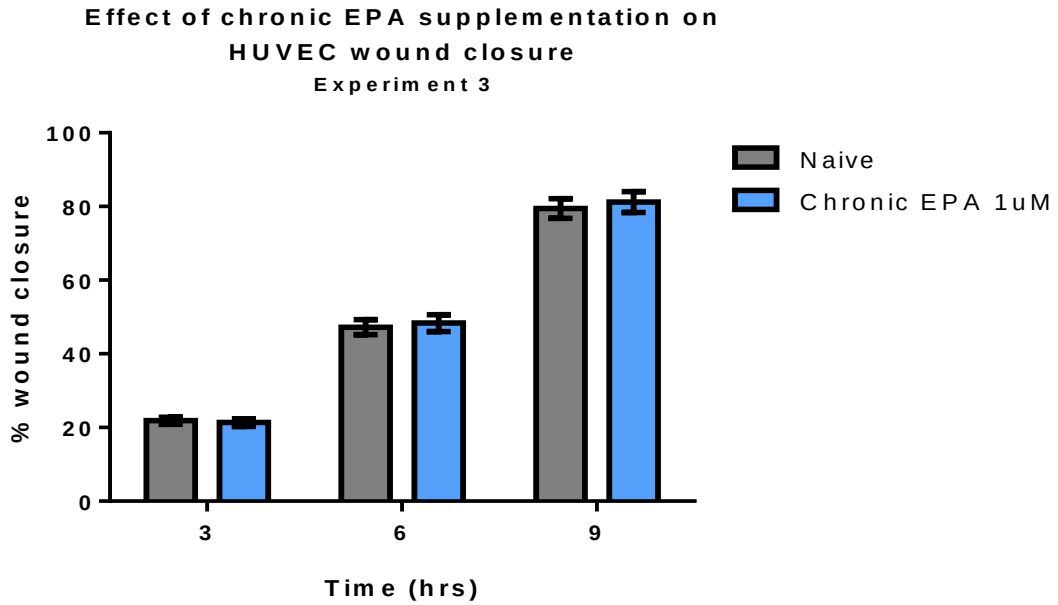


Figure 6.21. The effect of chronic EPA supplementation on HUVEC wound closure: Experiment 3. Wound closure over time is shown as the percentage reduction in the width of the wound compared to width at baseline. Each bar is the mean of 18 photos from 8 replicate wells. Error bars rep[resent mean +- SEM.

6.3 Matrigel tubule formation assay

6.3.1 Assay optimisation

Matrigel was thawed and diluted with PBS to give concentrations of Matrigel ranging from 1 in 1 to 1 in 20. Diluted Matrigel was spread evenly in a 6-well plate and left for 30 minutes at 37^{o}C until solidified into a consistent gel layer. HUVECs were seeded at 2x10^{5} cells/well in 2ml of culture medium, and tube formation assessed at 4hrs and 24hrs. Matrigel at concentrations of 1 in 15 and 1 in 20 resulted in an uneven and patchy layer of Matrigel when viewed under the microscope at low power, with correspondingly poor HUVEC seeding and no tubule formation (photos not shown). Tubule formation at higher concentrations of Matrigel are shown in Figure 6.22. A Matrigel concentration of 1 in 3 resulted in good tubule formation. Matrigel at a concentration of 1 in 2 gave similar results to 1 in 3, whereas concentrations less than 1 in 3 resulted in poor to no tubule formation. A Matrigel concentration of 1 in 3 was therefore chosen for use in subsequent experiments.

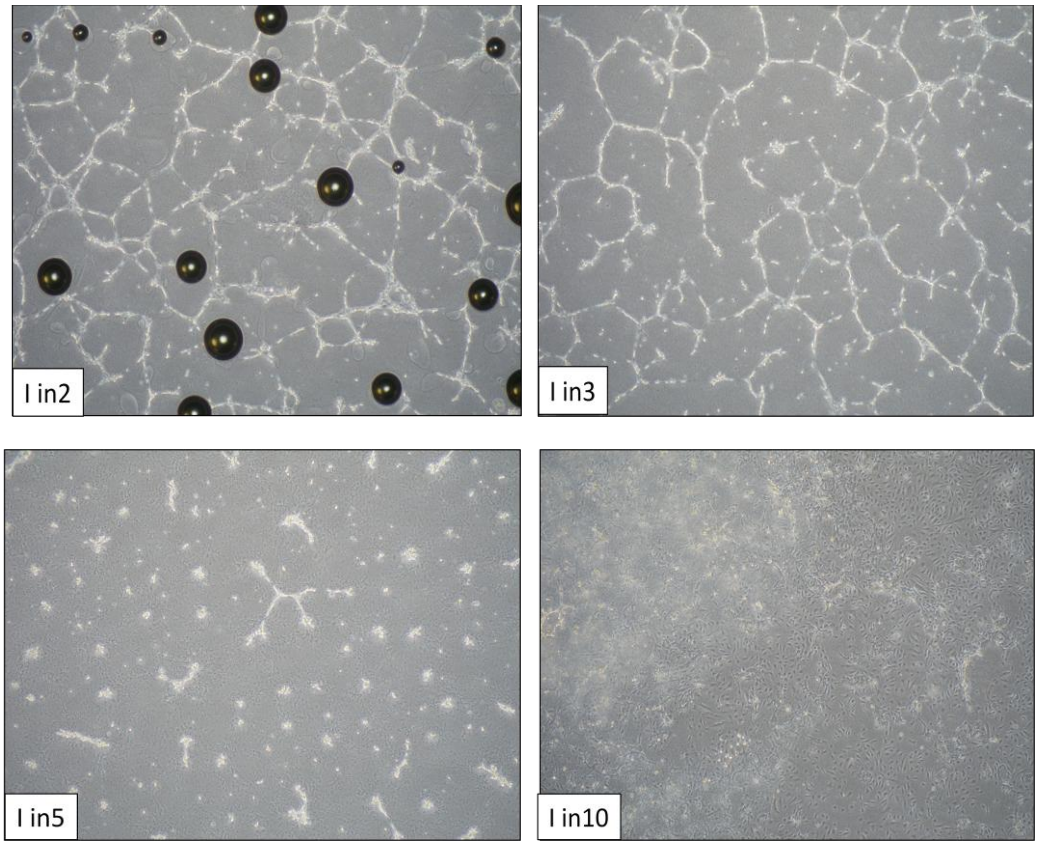


Figure 6.22. Formation of tubules when HUVECs were seeded on different dilutions of Matrigel.

The Matrigel assay was less consistent and reproducible than either the MTT assay of HUVEC proliferation or the wound score assay of HUVEC migration. Small adjustments to the technique of Matrigel dilution and plating into a 6-well plate improved the consistency and reproducibility of the Matrigel gel layer, and minimised the formation of air bubbles that were noticeable in the 1:2 dilution photo in Figure 6.23. This was primarily achieved by ensuring that all reagents and equipment was cooled to 4^{o}C and then kept on ice at all stages until the Matrigel had been plated into the 6 well plate. Even following this, the formation of tubules varied between experiments. In some experiments, at 24hrs only a small number of short tubules formed with healthy HUVECs remaining seeded on the Matrigel layer, whereas in other experiments almost all HUVECs had formed long branching tubules within 6 hours. In two experiments it was noted that tubules formed only in the centre of the well whereas in the periphery HUVECs remained as a typical cobbblestone-appearance cell monolayer.

On the advice of colleagues in another department who were performing Matrigel angiogenesis assays with HUVEC and fibroblast co-cultures, Growth Factor Reduced BD Matrigel Basement Membrane Matrix (BD Biosciences, Cat no. 356231) rather than High Concentration BD Matrigel Basement Membrane Matrix (BD Biosciences, Cat no. 354248) was ordered and used for subsequent experiments. In an effort to further improve the reproducibility of the assay by improving the consistency of the Matrigel layer, for subsequent experiments the concentration of Matrigel was increased from 1 in 3 to 1 in 2 dilution with PBS, and 0.5ml rather than 0.3ml of diluted Matrigel were plated in each well of the 6-well plate. This improved the number of tubules formed, with more even tubule formation across the whole well, and improved the reproducibility between experiments. The results of subsequent acute and chronic EPA supplementation experiments using these modifications to the protocol are detailed below.

6.3.2 Acute EPA supplementation

One acute EPA supplementation experiment was performed after the modifications to the protocol described above. Two 6-well plates were prepared with a Matrigel layer as described above. HUVECs in culture medium supplemented with EPA 0-30µM were placed into separate falcon tubes. A post-doctoral fellow then seeded the HUVECs into the two 6 well plates (2 wells for each EPA concentration) so that I remained blind to the treatment allocation. Well allocation was only revealed to me when the image-analysis results were returned from Wimasis. An example of the photograph of tubules taken and uploaded to Wimasis for analysis, together with an overlay of the image-recognition of tubules performed by Wimasis is shown in Figure 6.23.

The results of tubule formation at 6hrs are shown in Figure 6.24. There was no significant difference in total tubule length between controls and any concentration of EPA (control vs. EPA 30µM p=0.32). There was no significant difference in the number of tubule branching points between controls and any concentration of EPA (control vs. EPA 20µM p=0.14, control vs. EPA 30µM p=0.11). EPA was associated with a reduction in the number of tubule loops compared to controls, with this narrowly missing statistical significance at the highest dose of EPA (control vs. EPA 20µM, p=0.11; control vs. EPA 30µM, p=0.06). All statistical comparisons were performed using unpaired t-tests.

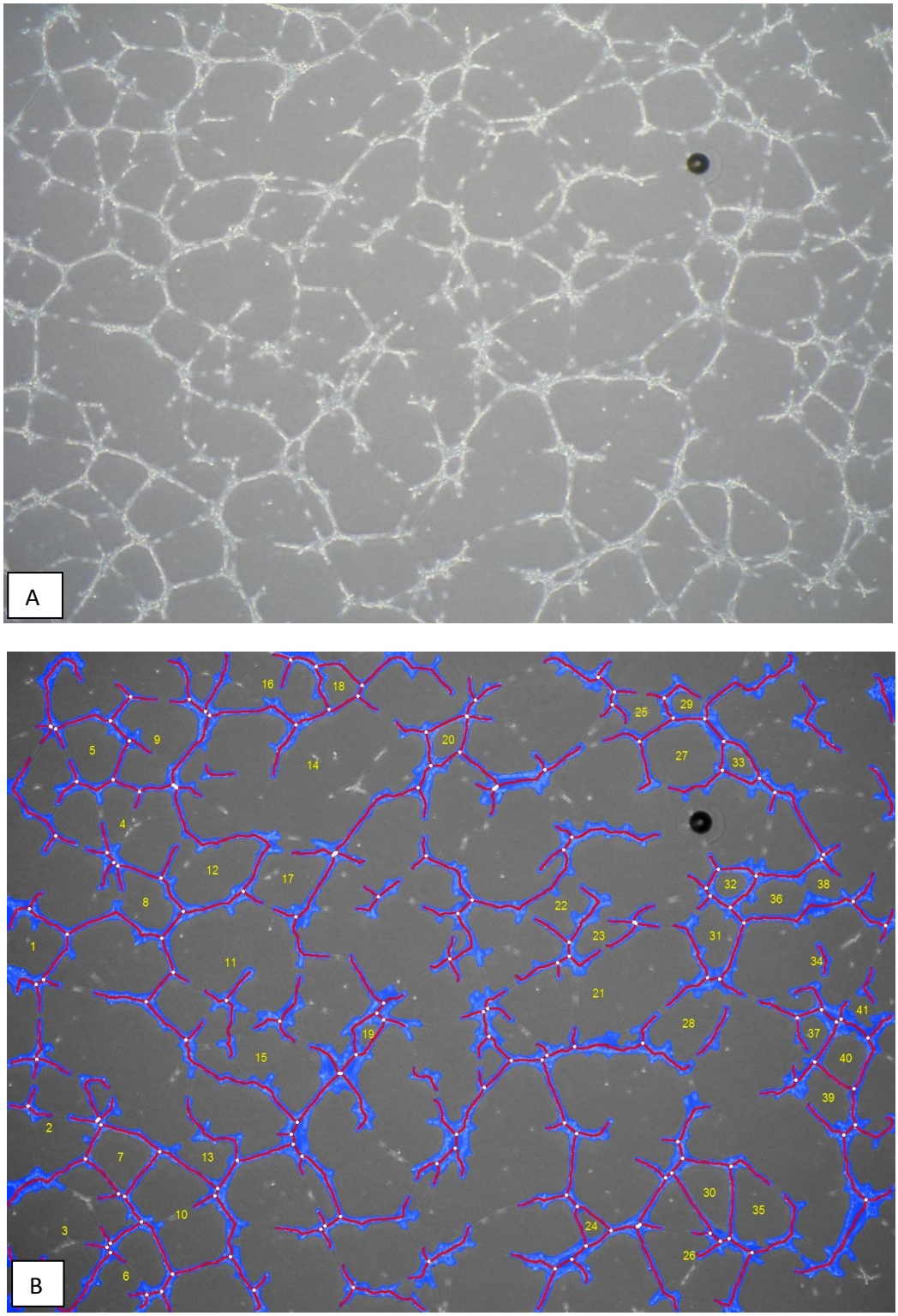
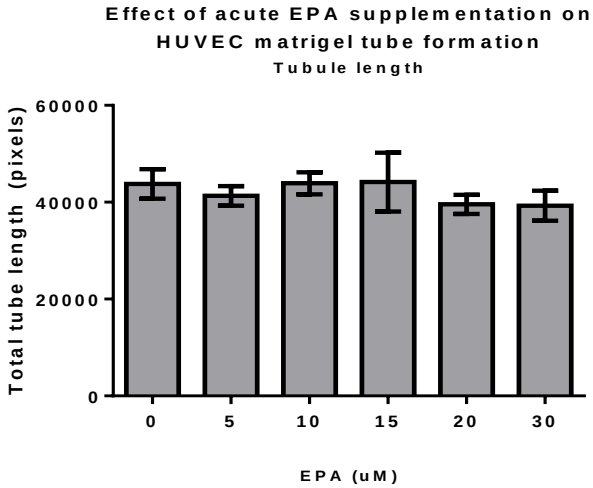
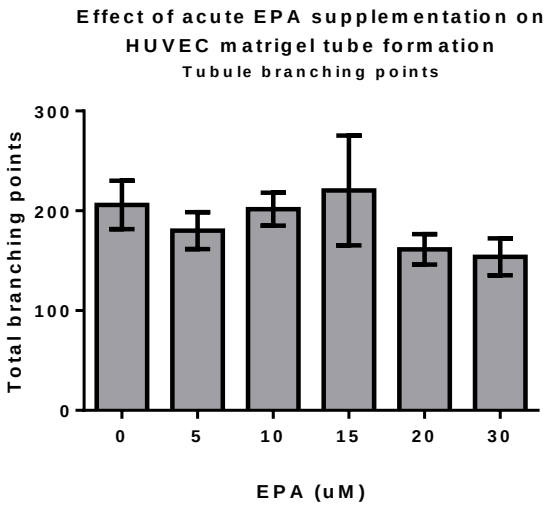


Figure 6.23. Example of Wimasis scoring of tubules. a) Photograph of HUVEC tubules. The image was uploaded to Wimasis for automated image recognition and analysis. b) HUVECs were identified by binary thresholding (blue). Individual tubules were identified by a skeletonization algorithm (pink). Tubule branching points were identified (white dots). Total tubule number, branching points and loops were calculated.





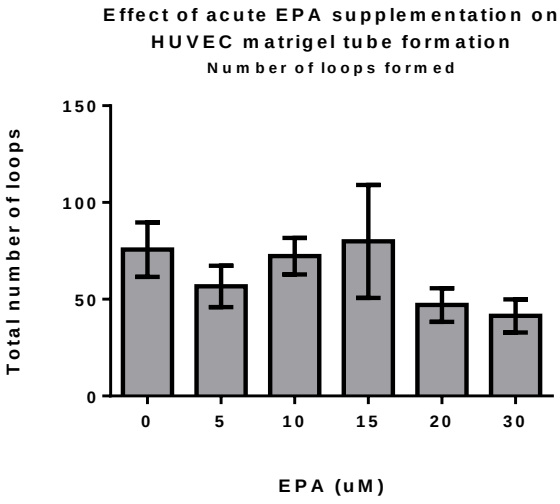


Figure 6.24. The effect of EPA on tubule length, branching and loop formation. Each bar is the mean of 8 images (4 representative photos from each of 2 replicate wells). Error bars represent mean +- SEM.

6.3.3 Chronic EPA supplementation

The effect of chronic 1µM EPA supplementation on HUVEC tubule formation was performed in three experiments following the protocol modifications described in 6.3.1. In the first experiment the effect of 10nM VEGF supplementation at the time of plating HUVECs onto the Matrigel was also investigated. Two wells were plated for each experimental condition (naive, naive+VEGF, chronic EPA, chronic EPA+VEGF). Peak tubule formation was observed at 6 hours and a total of 3 representative photos taken from the two wells of each experimental condition. Example photographs are shown in Figure 6.25. The results are shown in Figure 6.26. There was no significant difference between EPA-naive and EPA-supplemented cells in either tubule number (p=0.41), number of branching points (p=0.40) or number of loops formed (p=0.52). Addition of VEGF had no significant effect on any of these three measures of tubule formation in either the EPA-naive cells (p=0.77, p=0.75, p=0.73 respectively) or the EPA supplemented cells (p=0.15, p=0.13, p=0.19 respectively).

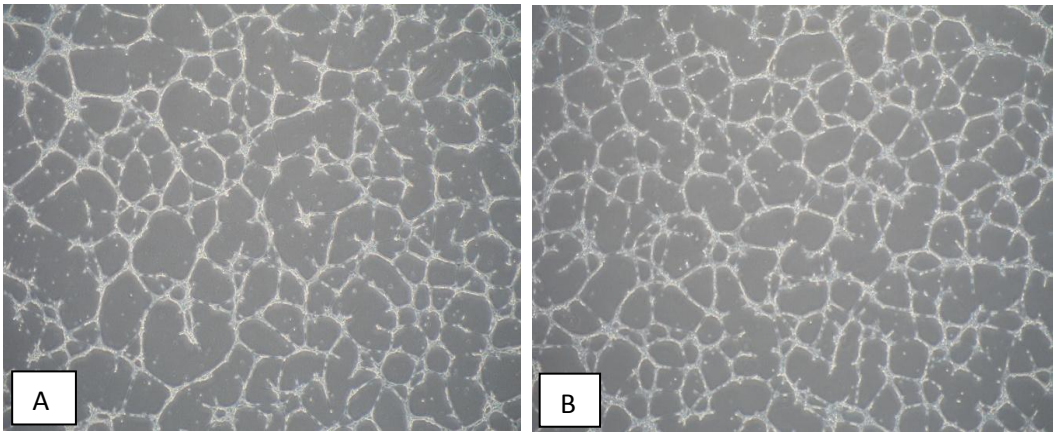
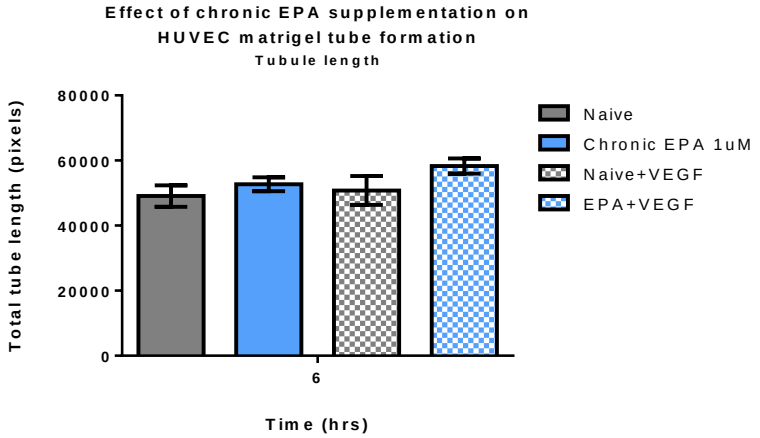
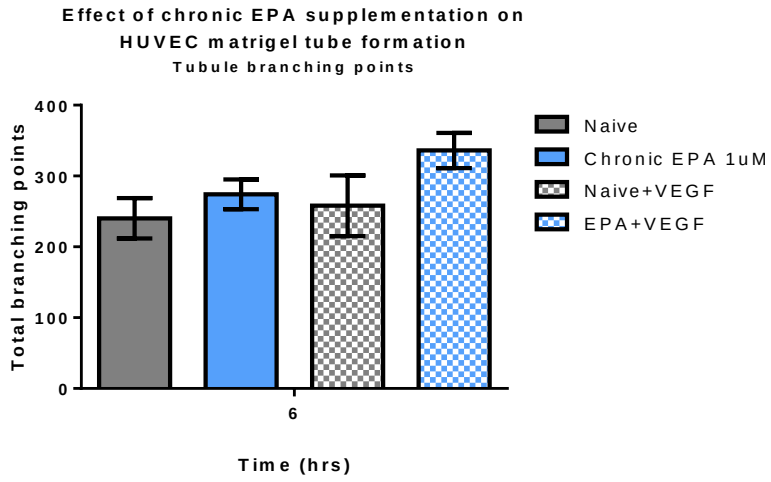


Figure 6.25. Example photographs of the effect of chronic EPA supplementation on HUVEC tubule formation: Experiment 1. Photographs taken 6 hours after seeding a) Naive cells and b) EPA-supplemented cells onto Matrigel®.





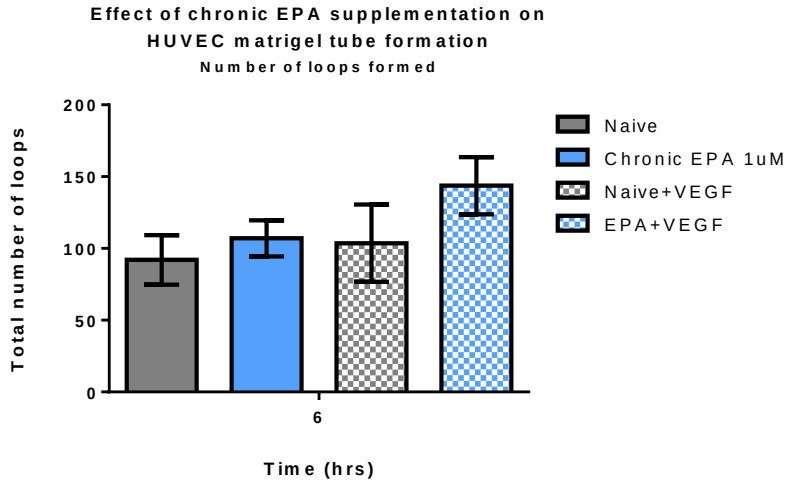


Figure 6.26. The effect of chronic EPA-supplementation in HUVEC tubule formation: Experiment 1. Tube formation, branching points and total number of loops formed were scored 6 hours after seeding cells onto Matrigel. In the chequered bars, VEGF was added at the time of seeding cells onto Matrigel®. Each bar is the mean of 3 representative photos from 2 wells (i.e. n=3). Error bars represent mean +- SEM.

In the second experiment a single 6-well plate was seeded with naive cells (3 wells) and EPA supplemented cells (3 wells). Tubule formation was much poorer than in the first experiment (Figure 6.27). One representative photo from each well was analysed and the results shown in Figure 6.28. Tubule formation was reduced in the EPA-supplemented cells compared to the EPA-naive cells across all three measures of tubule formation at both time points. At 6 hours the EPA-supplemented cells showed a 45.7% reduction in tubule length (p=0.01, unpaired t-test) and a 60.5% reduction in tubule branching points (p=0.05, unpaired t-test) compared to EPA-naive cells.

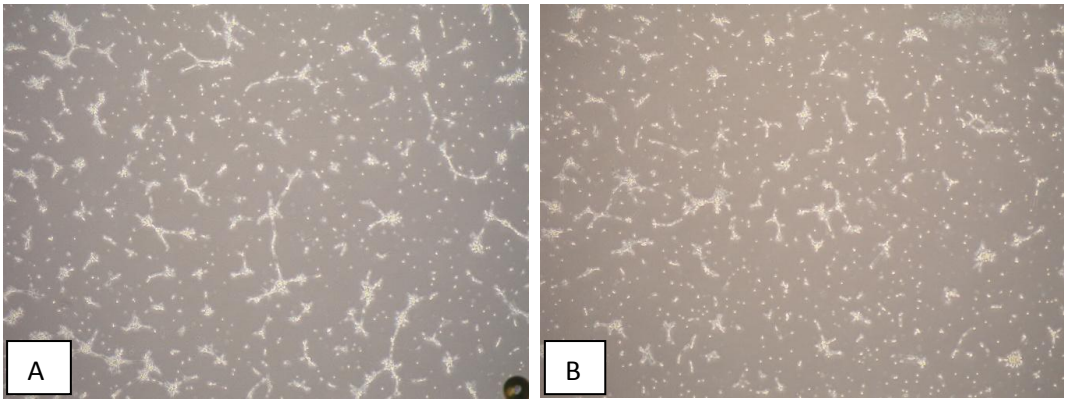
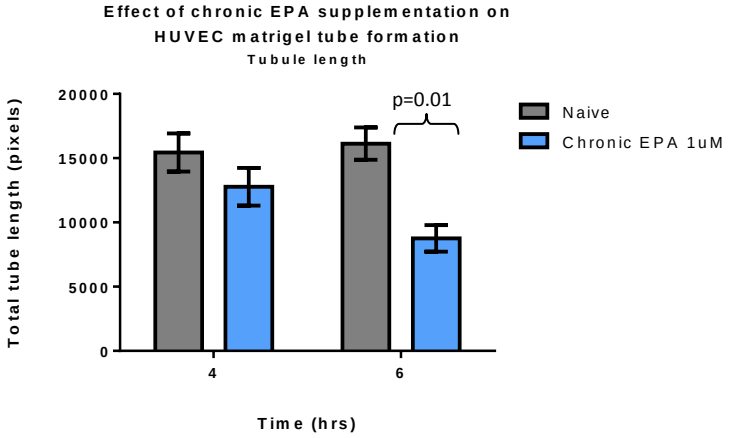
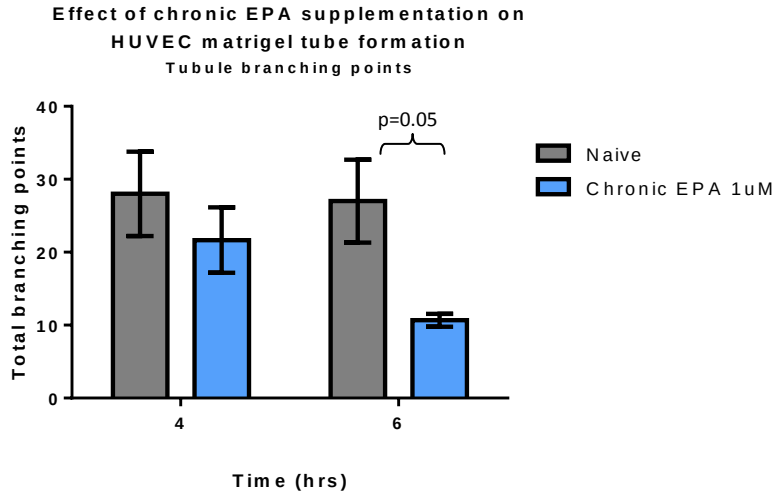


Figure 6.27. Example photographs of the effect of chronic EPA supplementation on HUVEC tubule formation: Experiment 2. Photographs taken 6 hours after seeding a) Naive cells and b) EPA-supplemented cells onto Matrigel®.





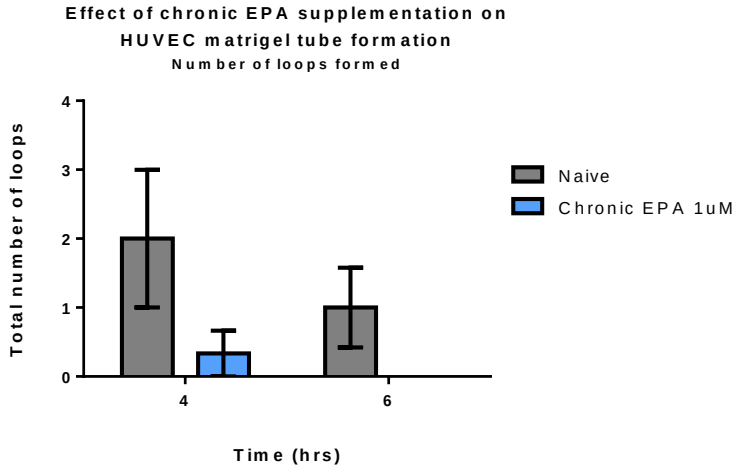


Figure 6.28. The effect of chronic EPA-supplementation in HUVEC tubule formation: Experiment 2. Tube formation, branching points and total number of loops formed were scored 6 hours after seeding cells onto Matrigel. In the chequered bars, VEGF was added at the time of seeding cells onto Matrigel®. Each bar is the mean of 1 representative photo from 3 wells (i.e. n=3). Error bars represent mean +- SEM.

In the third experiment, one six well plate was seeded with naive cells (3 wells) and EPA-supplemented cells (3 wells) by a post-doctoral research fellow so that I remained blind to the well allocation. Well allocation was only revealed to me when the image-analysis results were returned from Wimasis. Four representative photos were taken from each well and analysed. Example photos are shown in Figure 6.29. All four photos from each well were analysed and the results are shown in Figure 6.30. At 6 hours the EPA-supplemented cells showed an 18.3% reduction in tubule length (p=0.03), a 30.9% reduction in tubule branching points (p=0.04) and a 41.9% non-significant reduction in the number of loops formed (p=0.11) compared to EPA-naive cells. All statistical comparisons were performed using unpaired t-tests.

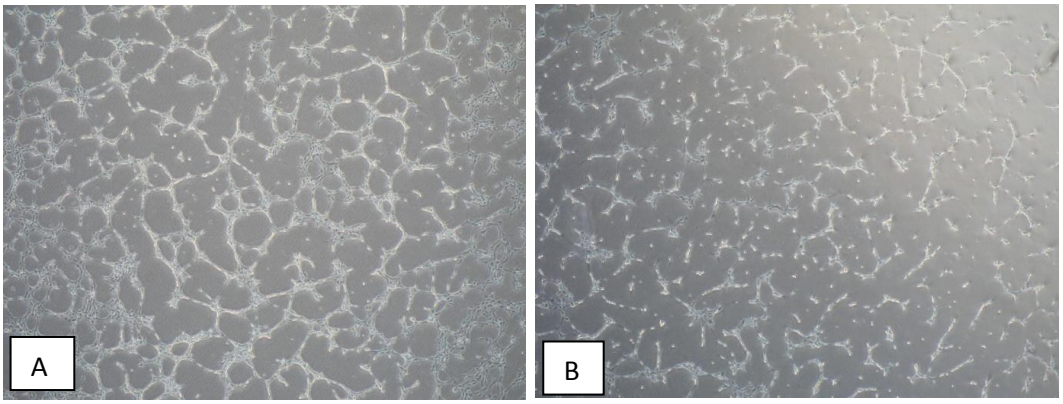
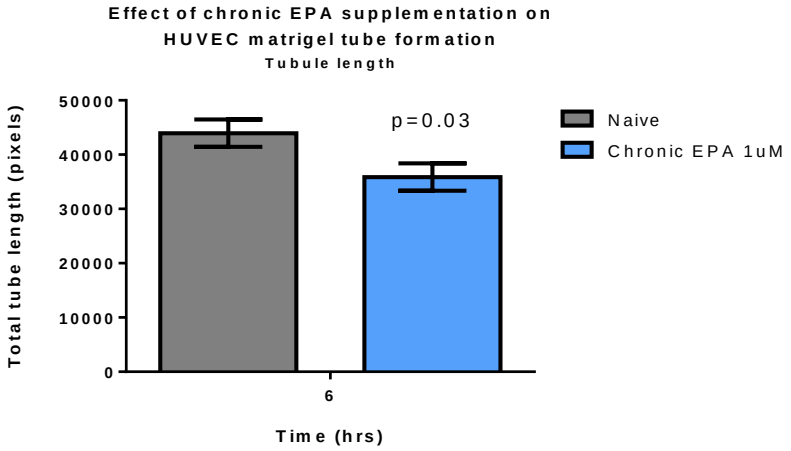
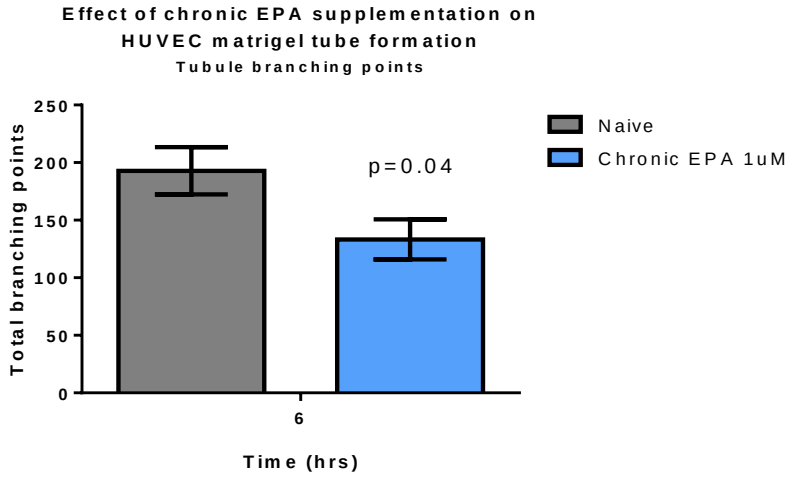


Figure 6.29. Example photographs of the effect of chronic EPA supplementation on HUVEC tubule formation: Experiment 3. Photographs taken 6 hours after seeding a) Naive cells and b) EPA-supplemented cells onto Matrigel®.





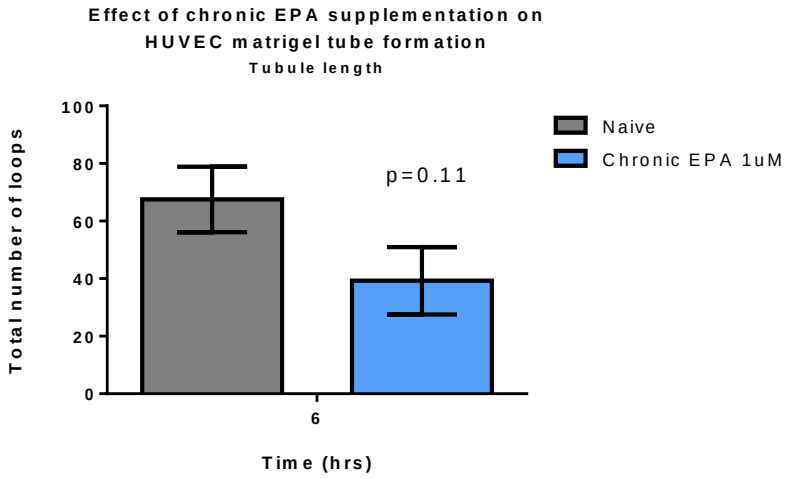


Figure 6.30. The effect of chronic EPA-supplementation in HUVEC tubule formation: Experiment 3. Tube formation, branching points and total number of loops formed were scored 6 hours after seeding cells onto Matrigel. In the chequered bars, VEGF was added at the time of seeding cells onto Matrigel®. Each bar is the mean of 4 representative photos from each of 3 wells (i.e. n=12). Error bars represent mean +- SEM.

6.4 Summary of HUVEC data

A summary of the effects of acute and chronic EPA supplementation on measures of angiogenesis *in vitro* is shown in Table 6.2. Acute EPA supplementation resulted in a dose-dependent inhibition of HUVEC proliferation over concentration ranges comparable to other endothelial cell studies,(195, 355, 356) and chronic low dose EPA supplementation (1µM for 2 weeks) caused a similar magnitude reduction in HUVEC proliferation to that seen with 10-20µM acute supplementation. HUVEC wound migration was inhibited by acute EPA supplementation in the range of 20-50µM EPA but the results of the chronic EPA supplementation were more equivocal, with a trend to reduced wound closure in two out of three experiments, but which did not reach statistical significance in either experiment. HUVEC tubule formation in Matrigel® was inconsistently inhibited by chronic EPA supplementation but not acute EPA supplementation.

	Acute EPA	**Chronic EPA**
Proliferation	Dose dependent inhibition with 40-70% ↓ proliferation Equivocal effect on 6-keto-$PGF_{1\alpha}$ in cell-conditioned media, with dose dependent ↑ in one experiment and no change in another.	16-33% ↓ proliferation Attenuated response to VEGF stimulation of proliferation No convincing PGE_2 rescue ↓6-keto-$PGF_{1\alpha}$ in cell-conditioned media
Wound closure	Dose dependent inhibition with 24-67% ↓ wound closure	Non-significant ↓ wound closure in 2 out of 3 experiments VEGF did not stimulate closure in either EPA-supplemented cells or controls
Tube formation	No effect	↓tubule length and branching points in 2 out of 3 experiments

Table 6.2 Summary of the effect of acute and chronic EPA supplementation on measures of angiogenesis in HUVECs.

Anti-angiogenic activity of ω-3 PUFAs has been demonstrated in previous *in vitro* endothelial cell studies. Omega-3 PUFA supplementation has been shown to reduce expression of VEGFR-2,(195, 196, 356) reduce cell migration,(194, 196) and reduce VEGF-stimulated microtubule formation.(193, 194, 196) Omega-3 PUFA supplementation has also been associated with reduced tumour vascularity *in vivo*. (186, 204, 205) However, similar to the findings in my assays, previous *in vitro* studies have also shown mixed results. HUVEC wound migration was arrested by acute supplementation with EPA but not DHA in a study by Tonutti *et al*, (357) and by conjugated EPA but not by EPA in a study by Tsuzuki *et al*.(194) In alternative types of migration assays which involved measuring the number of cells that migrate out of a Matrigel droplet or the number of cells that migrate across a Boyen chamber, neither EPA nor DHA reduced the number of migrating cells compared to controls whereas AA did significantly increase the number of migrating cells.(194, 355) Some studies have shown that HUVEC tubule formation is inhibited by EPA,(193, 194, 356) DHA,(358) and conjugated EPA,(194) whilst another study showed that tubule formation was inhibited by 24hr EPA, DHA or DPA only when tubule formation was stimulated with 20μM VEGF.(196) AA, by contrast, increased tubule formation compared to controls in both the VEGF-stimulated and unstimulated experiments. One further study, without VEGF supplementation, showed no effect of 10 μM EPA or DHA on HUVEC tubule formation (355). It may be that EPA exerts anti-angiogenic activity through both direct activity on endothelial cells, and indirect activity mediated, for example, by effects on VEGFR expression.

The reasons for the differing effects of EPA on the three angiogenesis assays used in my study are unclear. Compared to the simple MTT assay of cellular proliferation, the processes involved in cell migration (e.g. requiring lamellipoia extension and actin-myosin complex contraction) and tubule formation (e.g.

requiring motility, cytoskeletal rearrangement and interaction with adjacent cells) are more complex. Different pathways are likely to be upregulated in each process. The relative contributions of such pathways, for example the COX-PGE_2 and VEGF pathways, to each of these processes and the relative speed at which EPA supplementation affects these pathways might explain some of the conflicting results. These factors might also explain the different effects seen with acute and chronic EPA supplementation. It is conceivable that chronic EPA supplementation has different biological effects than acute EPA supplementation. Chronic supplementation might lead to greater EPA incorporation in cell membranes, or greater down-regulation of cell surface VEGFR-2 expression. Acute EPA supplementation meanwhile (0-6hrs in the wound migration and Matrigel assays) might not have had time to down-regulate the number of VEGFR-2 receptors, and instead exert effects in this timeframe predominantly by inhibition of the COX pathway and reduction in PGE_2 production (and production of 3-series prostaglandins).

PGE_2 is known to be a strong stimulant of cell proliferation. An alternative acute supplementation methodology for the wound migration and Matrigel assays would have been to pre-condition HUVECs with EPA acutely for 24-48hrs prior to wound scoring or prior to plating on Matrigel rather than adding EPA-containing media after the scoring or plating of cells. Whilst this would have more closely mirrored the conditions of the MTT proliferation assay, I already knew that EPA inhibited HUVEC proliferation, and decided that the addition of EPA to culture medium 24-48hrs prior to wound scoring might have affected the HUVEC confluence at time of scoring and therefore biased the results. I did perform a few preliminary experiments investigating the effect of PGE_2 rescue on the proliferation of HUVECs chronically supplemented with EPA. PGE_2 stimulated proliferation in EPA-supplemented cells but did not return proliferation to the baseline proliferation of EPA-naive cells. This

might suggest that PGE_2 rescue was under-dosed, although PGE_2 stimulated an even greater proliferation in the EPA-naive cells suggesting that EPA inhibition of proliferation was not solely PGE_2 dependent. Interestingly, the concentration of 6-keto-PGF1α in the cell-conditioned medium of cells chronically supplemented with EPA was lower than that of the EPA-naive cells, whilst acute EPA supplementation seemed to have no effect on 6-keto-PGF1α levels. This might suggest that chronic supplementation inhibits COX metabolism of 2-series prostaglandins to a greater extent than acute supplementation. However, these assays were only performed on culture medium from single MTT assays, and the results would need to be reproduced before drawing any conclusions.

A few studies were also performed supplementing cells with VEGF. Whilst VEGF stimulated proliferation in the EPA-naive cells, cells chronically supplemented with EPA showed no response to VEGF stimulation, suggesting either that VEGFR-2 receptors were down-regulated or that one mechanism of inhibition by EPA occurs downstream of the VEGF receptor. When the same experiment was performed in the wound migration model, supplementation with VEGF failed to stimulate migration in either the EPA-naive or chronically EPA-supplemented cells, raising the possibility that pathways other than the VEGF pathway are more important in endothelial cell migration.

Without more detailed mechanistic analyses, which may include measuring mucosal EPA content, cell surface VEGFR-2 expression or further investigation of 2-series and 3-series prostaglandin levels in cell-conditioned media, any explanation for the difference in the effect of acute and chronic supplementation is speculative. Nevertheless, taken together the results from these HUVEC experiments do point towards an inhibitory effect of EPA on angiogenesis, which is supported by the available literature, and supports the hypothesis that EPA might reduce tumour microvessel density by inhibiting angiogenesis.

Chapter 7: Discussion

7.1 Safety profile of EPA

The high rate of uptake of patients into The EMT Trial (96% of eligible patients) demonstrates that ω-3 PUFAs are considered by patients to be safe and that potential gastrointestinal side effects such as diarrhoea, dyspepsia and nausea do not deter patients from accepting ω-3 PUFAs as a potential adjuvant treatment for CRCLM. In The EMT Trial, EPA-FFA 2g/day was demonstrated to be safe and well-tolerated. The rate of compliance with trial medication was over 90%, and the rate of gastrointestinal upset was similar to that seen in other ω-3 PUFA trials.(262, 314, 315) Importantly, very few patients were withdrawn due to side effects of medication (4.7%), further demonstrating the tolerability of study medication.

Whilst it is easy to identify pre-operative side effects that can be attributable to study medication, the effect of pre-operative interventions on post-operative morbidity is harder to measure, and harder still to demonstrate causality. Few institutions formally classify the severity of complications according to a recognised grading system, such as the Dindo-Clavien classification system.(359) This makes comparison between the often fastidious collection of morbidity data within a clinical trial and the morbidity data published in retrospective reviews of institutional databases difficult. Nevertheless, the rate of post-operative morbidity in the EMT Trial was comparable to that of other published series,(307, 308) providing further reassurance that EPA supplementation is safe in patients undergoing liver resection.

There have been concerns in the literature about the risk of bleeding with ω-3 PUFA supplementation.(269) This concern has not been realised clinically, with a recent review highlighting that excessive bleeding was virtually non-existent in over

4000 patients taking ω-3 PUFAs who underwent coronary artery bypass grafting, carotid artery endarterectomy or percutaneous coronary angioplasty.(268) Similarly, no excessive bleeding has been seen in studies of patients taking ω-3 PUFA supplements who are admitted with acute myocardial infarction (typically requiring heparinisation or thrombolysis),(360) or in patients having spinal surgery.(361) Nevertheless, liver resection involves parenchymal transection which predisposes the patient to bleeding from the cut surface of the liver. Any small increase in bleeding tendency as a result of EPA supplementation might therefore be expected to result in a clinically apparent bleed following liver resection. However, liver resections are routinely performed on patients who are still taking aspirin, which has a well established anti-platelet activity, without any concern about bleeding risk and without additional pre-operative or intra-operative measures to minimise bleeding. The clinical risk of bleeding with EPA supplementation in patients undergoing liver resection in The EMT Trial was therefore considered to be negligible. This opinion was supported by the finding that EPA supplementation had no effect on either laboratory analysis of whole blood platelet aggregation, or on clinical bleeding parameters such as the need for packed red cell transfusion and the need for drainage of postoperative collections.

This excellent safety and tolerability profile makes EPA a strong candidate agent for the treatment and/or prevention of CRC and CRCLM. Whilst both aspirin and COX-2 inhibitors have been shown to have anti-CRC activity,(127-130, 135, 136, 138, 139) the risk of bleeding and GI ulceration with prolonged aspirin therapy (140) and the risk of myocardial infarction or thrombosis with prolonged COX-2 inhibitor therapy (132, 134) has prevented their long-term use for the chemoprevention of CRC and CRCLM. EPA, in contrast, appears to have no such side effects with long term use.(314)

7.2 Patient characteristics and tumour biology

Two factors which play an important role in the outcome of CRCLM, and could potentially impact on the results of this trial are the "biology" of the disease and the use of chemotherapy. Of all prognostic markers, lymph node status of the primary cancer and size of the CRCLM are consistently the most important independent predictors of survival following resection of CRCLM.(362-365) Together with factors such as Dukes' stage and synchronicity of disease, these are considered surrogate markers for the tumour biology. The EPA and placebo groups were well matched across all of these factors. Just under half of patients in both groups had received chemotherapy at some stage prior to presentation, with no difference between the groups in the interval between chemotherapy and enrolment into The EMT Trial. A higher proportion of patients in the placebo group presented with recurrent liver metastases (24% vs. 14%). This could be interpreted as an indication of more "aggressive" disease in the placebo group. However, this was not a statistically significant difference, and secondly, it is known that disease-free and overall survival after resection of recurrent liver metastases is similar to that after the first resection of CRCLM. The difference in the proportion of patients with recurrent disease in this trial is perhaps of limited clinical significance. Therefore, as far as can be determined the tumour characteristics of the two groups at baseline were comparable.

Although the two groups were well matched across all other baseline characteristics, two potentially confounding variables were identified. Firstly, approximately 22% of patients in each group were taking aspirin, which as an inhibitor of the COX enzyme acts on the same pathway as one of the proposed mechanisms of action of EPA. Aspirin use was deliberately not cited as an exclusion criterion for the study because of the interest in the novel anti-inflammatory compound resolvinE1, which is metabolised from EPA by acetylated

COX-2 (see Figure 1.5 and Section 1.7.4) (164). Inclusion of aspirin-users therefore provided the opportunity to look for resolvin E1 in the plasma and tissue of patients who were taking both aspirin and EPA. These analyses were not performed as part of this MD project due to cost and time implications, and samples have been stored for future analysis. Sub-analyses were performed excluding concurrent aspirin users. These showed that aspirin use did not affect the results of either the tumour immunohistochemistry or the mechanistic analyses.

Secondly, patient use of fish oil supplements was not cited as an exclusion criterion for participation in the trial. One in five patients in the placebo group and one in eight patients in the EPA group were taking an oral fish oil supplement at the time of enrolment. This previous fish oil exposure introduced a potential confounder into the study, but did present the opportunity to study those patients with a prolonged exposure to fish oil supplementation prior to entering the trial. In retrospect, the trial methodology would have been more robust if patients who were taking fish oil supplements at the time of screening for the trial had been excluded. One possible limitation of the trial is not being able to determine whether patients enrolled into the trial decided to either purchase their own over-the-counter ω-3 PUFA supplements or increase their oily fish consumption after being told about the potential benefits of ω-3 PUFAs. The FFQ attempted to control for this by monitoring patients' oily fish consumption and nutritional supplement use during the trial. Within the limitations of a FFQ, which relies on patients accurately completing the questionnaire, patients did not increase their oily fish consumption or start to take over-the-counter supplements during the trial. Measurement of the fatty acid content of red blood cells on each study visit by gas chromatography would have been one way of monitoring this more objectively, but to perform these analyses on three occasions for 88 patients would have been prohibitively expensive, and other mechanistic analyses were instead prioritised.

7.3 Choice of primary endpoint

Immunohistochemistry for tumour Ki-67 proliferation index was chosen as the primary endpoint in The EMT Trial, with tumour apoptosis and microvessel density also investigated as secondary endpoints. Ki-67 proliferation index (PI), has been widely studied as a prognostic marker of survival in many types of cancer.(366) Specifically, the Ki-67 PI has been evaluated as a surrogate for disease-free and overall survival in resectable CRC and CRCLM. In the largest single-centre study of Ki-67 PI in patients with resection of CRCLM (n=221), multivariate analysis identified Ki-67 PI as the most significant independent prognostic indicator of survival, with a 2.8 RR of cancer death in patients with Ki-67 PI >50% compared to Ki-67 Pi <50%.(367) A larger Chinese study of Ki-67 PI in CRC biopsies before and after adjuvant regional chemotherapy (n=509) demonstrated a reduction in Ki-67 PI following chemotherapy from 48.6% to 38.4% (21% reduction, $p<0.05$). At mean follow up of 42 months the chemotherapy group demonstrated a significant improvement in median survival (45 months vs. 40 months; p=0.02), disease free survival (74% vs. 62%; p=0.02) and overall survival (81% vs. 60%; p=0.01) compared to the surgery alone group.(368) The association between reduction in Ki-67 post-chemotherapy and survival has been most extensively studied in breast cancer, where paired pre- and post-chemotherapy tissue is commonly available. Many papers have shown that Ki-67 is prognostic for overall and disease-free survival in breast cancer, and that the post-treatment PI is more predictive of survival than either the baseline PI or the percentage change in PI.(369)

Although there were no human studies of the effect of EPA on CRC or CRCLM proliferation on which to base a power calculation for The EMT Trial, many *in vivo* studies had demonstrated a reduction in PI in colonic mucosa and colorectal tumours of rodents supplemented with ω-3 PUFAs.(370) Only one of these studies evaluated tumour vascularity, with a 50% reduction in MVD of HT-29 subcutaneous

tumours in nude mice fed 1g/kg EPA versus controls.(186) Similarly, in nine human studies of ω-3 PUFA supplementation, colonic mucosal PI was reduced (13-70% reduction) in all but two studies,(370) and apoptosis index increased in all three studies that measured AI (50%, 57% and 700% increases, one study did not reach statistical significance).(370) Based on *in vitro* and *in vivo* findings, a 30% reduction in PI with EPA supplementation was predicted.(186, 199) This magnitude of reduction in PI has been shown to correlate with increased patient survival in studies of chemotherapy agents which are now well established treatments for CRC and CRCLM. In a trial of neoadjuvant regional 5-FU therapy for CRC, a 21% difference in Ki-67 PI of post-treatment CRC tissue between the control and chemotherapy groups was associated with an 18% improved disease-free survival and 35% improved overall survival.(368) Likewise, in a trial of a single intravenous 5-FU bolus prior to resection of CRCLM, there was a reduction in Ki-67 PI of 48% and 30% in patients receiving 5-FU at 2 hours and 46 hours pre-surgery respectively compared to no-chemotherapy controls (312). A 30% reduction in PI therefore seemed an appropriate size of treatment effect to use in the power calculation for The EMT Trial.

Because pre-operative biopsy of liver cancers is contraindicated due to the risks of biopsy, in particularly the risk of tumour seeding, histological analysis of pre-and post-treatment CRCLM tissue was not possible in this study. Immunohistochemistry analysis was therefore restricted to the comparison between placebo and EPA groups of post-treatment tumour samples only. Immunohistochemistry of the original CRC specimen as a baseline for each patient was considered, but it would not have been helpful because of the well-documented heterogeneity in characteristics between CRCs and their paired CRCLMs. There is no evidence that Ki-67 PI in the original CRC is comparable to the PI in the subsequent CRCLM, with some studies showing a reduction in PI in the CRCLM

compared to PI in the CRC specimen,(371) some showing an increase,(372) and some showing no difference.(313)

7.4 Discrepancy between *in vivo* data and CRCLM immunohistochemistry

EPA supplementation had no effect on either tumour PI or AI levels in The EMT Trial, although there was a trend towards reduced tumour vascularity in the EPA group. Why then, when there is strong evidence for EPA reducing proliferation and increasing apoptosis in rodent models (151, 161, 162, 186, 199, 225, 226, 238, 244) and in human mucosal biomarker studies,(232, 254-259, 261) did this not translate into similar changes in CRCLM tissue in this study? The limitations of animal models as representations of human disease and the difficulties of translating *in vivo* findings into clinical effects are well known.(373) The use of higher doses of study medications in animal models than would be achievable in humans is often a factor, with high doses often being given to accentuate mechanistic effects. For example, the study of MC-26 mouse CRC cells in a model of CRCLM used the same preparation of EPA as in The EMT Trial, at a dose of 2.5-5% EPA as a percentage of total dietary intake.(209) This represents approximately 6-12 g/kg mouse body weight and was comparable to the dose used in other rodent models of CRC.(370) This is 200-400 times higher than the 2g/day dose of EPA used in The EMT Trial which equates to approximately 30mg/kg human body weight. Even adjusted for the difference in mouse and human body surface areas, as recommended when translating animal into human dose equivalents,(374) the mouse dose is still 15-30 times higher than that given in The EMT Trial. Tumour proliferation in this mouse study was measured using 5-bromo-2-deoxyuridine (BrdU) incorporation rather than Ki-67 expression as the marker of proliferation. However, this is unlikely to have had any bearing on the translatability of *in vivo* findings to clinical studies. Several studies have shown good correlation between proliferation indices measured by BrdU and Ki-67 staining in rodent and

human tissues.(375-377) Furthermore, an anti-proliferative effect of EPA has been demonstrated using the Ki-67 assay in both rodent models (186) and in human mucosal biomarker studies.(257, 261)

Aside from the difference in dose of study medications, other possible explanations for the discrepancy between *in vivo* findings and the findings in The EMT Trial might include the heterogeneity of tumours in The EMT Trial, the duration of EPA supplementation, and the timing of EPA supplementation. Each is discussed in more detail below.

Tumour heterogeneity

There was significant heterogeneity in morphological appearance between the tumours resected in The EMT Trial. As illustrated in Figure 5.12, some tumours were well differentiated with densely packed proliferating cells, whereas other tumours were predominantly mucinous with large mucin lakes and very few cells. Mucinous CRCs are believed to have distinct genetic profiles and clinico-pathological characteristics compared to non-mucinous CRCs.(378-380) They have also been associated with poorer outcomes, including higher rates of lymph node involvement, more advanced stage of disease, higher rates of metastasis, and poorer overall survival.(381, 382) The morphological differences between mucinous and non-mucinous CRCLMs will have impacted on the scoring of PI, AI and MVD due to the density of cells and vessels. Similarly, some tumours had a large stromal component or large areas of inflammatory cell infiltrate, whereas other tumours contained very little stroma. It is recognised that the tumour microenvironment interacts with metastatic cells and influences the growth of tumours, and whilst these interactions are not fully understood they are of undoubted importance to the growth and survival of cancers such as through the secretion of pro-angiogenic growth factors or the secretion of MMPs.(383) It is difficult to quantify how this tumour heterogeneity might have affected tumour PI, AI

and MVD, but it is worth noting that in most *in vivo* models of CRC and CRCLM, well-established and characterised cell lines are used which will produce a more homogeneous cohort of tumours, with a more consistent morphology and biology for which to investigate the effects of any intervention. In The EMT Trial tumour heterogeneity could not be quantified, suffice to say that the distribution of different tumour types seemed to be equally spread between the placebo and EPA groups. Sub-analysis of PI after exclusion of mucinous tumours was performed, but this did not reveal any difference in the PI between the two groups.

Duration of supplementation

Another explanation for the negative PI and AI findings in The EMT Trial is that the period of supplementation may have been too short to observe either the maximal incorporation of EPA into the tumour, or to begin to see a reduction in tumoural AA content. On the limited evidence available from colonic mucosa studies, a reduction in tissue AA content does not seem to occur until around 12 weeks, whereas EPA content seems to peak much earlier at around 4 weeks.(254, 255, 273, 323)

It remains unclear why mucosal EPA peaks before the AA content starts to fall, but this would suggest that EPA incorporation is not simply a direct substitution of EPA for AA. EPA can be converted to DHA, via DPA, in a two-stage process involving elongase and desaturase activity. Limited evidence from clinical trials suggests there is no significant EPA to DHA conversion in human colorectal mucosa,(257, 262) Interestingly, there was a significant increase in tumoural DPA, but not DHA, content in the EPA group in the EMT Trial. Similar findings of comparable EPA and DPA increases, without a rise in DHA, were seen in the equivalent *in vivo* study of MC-26 mouse CRCLMs using the same preparation of EPA as used in the EMT Trial.(209) This suggests that the conversion between EPA and DPA in CRCLM is greater than first appreciated, and greater than that seen in human colonic mucosa.

If mucosal EPA content reaches a threshold beyond which EPA is converted to DPA and DPA levels continue to rise and diplace AA, this may explain why the EPA content seems to plateau before a reduction in AA content is seen. Interestingly, DPA has recently been shown to have anti-CRC activity(384) and has previously been shown to inhibit endothelial cell migration.(196) Future work might consider testing the following hypotheses:

- Mucosal incorporation of EPA occurs at the expense of fatty acids other than AA
- Changes in mucosal fatty acid composition stimulate liberation of AA from the mucosa, rather than the direct substitution of one fatty acid (EPA) for another (AA)
- There is a threshold for mucosal EPA content beyond which additional EPA is converted into other ω-3 PUFAs such as DPA.
- DPA has anti-CRC activity

It was also observed that the tumoural AA content in The EMT Trial was higher than the baseline levels of AA in previous human mucosal biomarker studies (Table 5.8). Perhaps the ratio of AA:EPA is more important than the absolute level of either fatty acid. The ratio of AA:EPA in The EMT Trial was certainly higher than the AA:EPA ratios in the human mucosal biomarker studies which demonstrated reduced proliferation and increased apoptosis. Alternatively, perhaps human mucosa has a faster rate of proliferation than CRCLMs, and therefore changes in PUFA metabolism due to EPA supplementation will manifest in changes in PI and AI more rapidly.

In the only other study of ω-3 PUFA supplementation in patients with CRCLM, 72hrs of pre-operative parenteral ω-3 PUFA supplementation was associated with an increase in plasma EPA and DHA levels immediately following supplementation compared to the control group, but no change in plasma AA levels and no

significant change in the ω-3 PUFA content of red blood cell membranes. Surgery for CRCLM was performed 5-12 days following ω-3 PUFA supplementation, by which time plasma EPA and DHA levels had returned to baseline levels. There was no significant difference in the EPA, DHA or AA content of tumours from the ω-3 PUFA supplemented group compared to those from the control group. The authors extrapolate their data backwards to suggest that the total ω-3 PUFA content of CRCLMs at the end of supplementation would have been approximately 8%, compared to a mean of 5.6% at the time of resection. This data really only provides evidence of a transient rise in plasma ω-3 PUFA content following parenteral supplementation. Extrapolating the tumour PUFA data back to estimate tumoural ω-3 PUFA at the end of supplementation is of limited clinical relevance. If the increase in tumoural ω-3 PUFA content was so transient as to have returned to baseline values within 5-12 days, what is the likelihood of this having had any clinically relevant effect on the tumour biology? By comparison, oral EPA supplementation in The EMT Trial was associated with a statistically significant increase in tumoural EPA, DPA, and total ω-3 PUFA (6.2%) content.

The two studies cannot be compared to assess the relative merits of oral or parenteral ω-3 PUFA supplementation on the speed of uptake of ω-3 PUFAs by CRCLM, or indeed its washout after cessation of therapy, because neither study assessed tumoural ω-3 PUFA content over time. What can be said by comparing the two studies, however, is that 4 weeks of oral supplementation was feasible, could be continued up to the day of surgery, was associated with significant changes in tumour ω-3 PUFA content, and that such supplementation could readily be applied to future clinical trials. By comparison, 72hrs of parenteral supplementation was not associated with significant changes in tumour ω-3 PUFA content, and to bring surgery closer to the end of parenteral supplementation on the assumption that the tumour ω-3 PUFA content would be higher would not only be

based on extrapolated and unproven data, but would also be logistically more challenging than oral supplementation in a clinical trial setting. Furthermore, neither trial saw any change in AA content of tumours, or of plasma or RBCs in the Leicester study, suggesting that a longer period of supplementation would be required for any future trial. This further commends the oral route as the more pragmatic for any future trial.

Timing of EPA supplementation

A third explanation for the discrepancy between the findings of pre-clinical studies and The EMT Trial is the timing of EPA supplementation in relation to the inducement of CRC/CRCLM. In The EMT Trial, EPA was given to patients with well established CRCLMs, which would have seeded in the liver and started growing before the original CRC was resected. EPA supplementation could therefore be considered as given late in the "life-cycle" of the CRCLM. By contrast, ω-3 PUFA supplementation in animal CRC treatment models is started either before, at the time of, or shortly after the subcutaneous injection of tumour cells. This does not accurately reflect the situation in The EMT Trial where supplementation is started in the presence of well-established CRCLMs. Those *in vivo* models which pre-load animals with ω-3 PUFA prior to chemical inititation of carcinogenesis, or prior to the injection of cancer cell lines, may increase EPA in the microenvironment before a tumour has even started growing, altering the cancer cell - microenvironment interaction and affect the tumour's growth characteristics. In these situations there may not be a clear distinction between "chemoprevention" and "chemotherapy". Similarly, those *in vivo* models that start ω-3 PUFA supplementation at, or shortly after, cancer cell injection are giving ω-3 PUFA much earlier in the "life-cycle" of the tumour than in The EMT Trial, which may therefore have a greater effect on the tumour's growth characteristics.

7.5 Tumoural and urinary prostaglandins

It is widely believed that one of the mechanisms of action of EPA is the inhibition of COX metabolism of AA, causing a reduction in the production of 2-series prostaglandins, whilst at the same time providing EPA as an alternative substrate for COX causing an increase in the production of 3-series prostaglandins. There is convincing evidence that EPA supplementation reduces pro-tumourgenic PGE_2 production *in vivo*. (151, 186, 209, 227, 236, 241, 244, 327) A "PGE_2 to PGE_3 switch" has been demonstrated *in vitro*,(145, 327) and in rodent models of CRC,(151) pancreatic cancer, (327) and more recently in mouse CRCLMs using the same preparation of EPA as used in the EMT Trial. However, as yet no studies have investigated the effect of ω-3 PUFA supplementation on the levels of PGE_2 in human CRC or CRCLM, and a PGE_2 - PGE_3 switch has not been demonstrated in any human tissue.

That there was no difference in tumoural PGE_2 levels between the two groups is perhaps unsurprising given that there was also no difference in tumoural AA content between the two groups. It is difficult to tease out from the literature whether it is the reduction in tissue AA, the inhibition of COX metabolism of AA, or a combination of both that accounts for the reduction in PGE_2 seen in pre-clinical studies of EPA therapy. Again, for the same reasons that the short duration of EPA supplementation might explain why there was no change in tumoural AA content with EPA supplementation, so too might it explain why EPA was not associated with a reduction in PGE_2 in this trial, in contrast to the findings of many pre-clinical studies.(151, 186, 209, 227, 236, 241, 244, 327) It is also unsurprising, given the low levels of PGE_2 in these tumours and the observation that PGE_3 tends to be present at 10-20 times lower concentration than PGE_2, that PGE_3 was detectable in only one tumour. PGE_2 levels were an order of magnitude lower in this study than in previous *in vivo* studies. This might be due to the biological characteristics of the

cell lines used in these *in vivo* studies. These can be high expressers of COX and produce vast quantities of PGE_2, unrepresentative of that seen in human tumours. These factors could make it difficult for any study to demonstrate a PGE_2 - PGE_3 switch in human tissue.

Interestingly, although EPA supplementation was not associated with a reduction in tumoural PGE_2, it did seem to cause a reduction in urinary PGE-M. Over the last 10 years there has been interest in the measurement of urinary PGE-M as a non-invasive measure of systemic PGE_2 synthesis, and therefore as a potential biomarker of CRC activity. Some studies have shown a correlation between PGE-M and the presence of colonic adenomas,(83, 385) and between PGE-M and CRC risk.(82) Other studies in non-small cell lung cancer have shown that celecoxib treatment is associated with a reduction in PGE-M,(330) and that patients who had the greatest reduction in PGE-M demonstrated improved overall survival.(329) Looking at the trends in the data in Figure 5.25, urinary PGE-M fell whilst patients were taking EPA, and then rose again to baseline levels after they stopped taking EPA. Although the change in the EPA group between baseline and post-treatment did not reach statistical significance, urinary PGE-M post-treatment was significantly lower in the EPA group than the placebo group. The small but statisttically significant reduction in PGE-M with EPA therapy indicates that EPA supplementation has an effect on PGE_2 synthesis. Whether this is tumour-derived PGE_2 or systemic PGE_2 cannot be determined. It would be expected that CRCLMs are the predominant source of PGE_2 production and therefore of PGE-M in the urine. However, the absence of any observed effect of EPA on tumour PGE_2 levels, and the observation that PGE-M did not fall following resection of the tumour would suggest that the urinary PGE-M was coming from systemic sources, for example endothelial cells.(386) One possible explanation could be that the systemic inflammatory response to surgery and liver regeneration contributed to elevated

systemic PGE_2 synthesis, and therefore elevated PGE-M levels, at the six week follow up. Future studies would need to monitor serial PGE-M levels in the immediate post-operative period and over a longer period of follow-up to investigate this. Given the strength of the preclinical data that EPA reduces tumoural PGE_2 levels, the relatively small changes in PGE-M seen in this study, and the limitations to the study which might explain why there was no change in tumoural PGE_2, these PGE-M results should not be taken as evidence that EPA does not inhibit tumoural PGE_2, only that there was absence of an effect of EPA on tumoural PGE_2 in this trial. The finding that EPA therapy was associated with small but statistically significant reversible reductions in both PGE-M and NFκB activation in PBMCs is evidence for a systemic anti-inflammatory effect of EPA, even if in this study it cannot be attributable to a direct anti-tumoural effect. Nevertheless, PGE-M may still have a role as a biomarker for CRC activity and justifies further investigation, either for the detection of CRC, for assessing and monitoring the response to treatment, or for the prognostication of outcome.

7.6 Angiogenesis

Previously published studies support the hypothesis that ω-3 PUFAs have anti-angiogenic activity,(387, 388) although the evidence is much more limited than that for its effects on the COX-PGE_2 pathway and on tumour proliferation. The mechanisms by which ω-3 PUFAs might inhibit angiogenesis are multi-factorial and remain unclear. Pro-angiogenic VEGF induces endothelial cell proliferation, migration and invasion, and increases vascular permeability.(19, 389) Production of VEGF is stimulated by inflammatory mediators including PGE_2.(390) Its effects are mediated by stimulating a variety of pathways including the MAPK, ERK, JNK and PIK3/Akt pathways.(389) VEGF and VEGFR-2 expression have been shown to correlate with CRC tumour vascularity, proliferation and metastasis.(391)

Omega-3 PUFAs have been shown to reduce endothelial cell expression of VEGFR-2 (195, 196, 356) and inhibit VEGF-induced activation of MAPK.(195) In another study, conjugated EPA, but not EPA, reduced VEGF-stimulated MMP-9 and MMP-2 mRNA expression and protein secretion at the same concentrations that inhibited HUVEC wound migration and tubule formation.(194) In a further study, ω-3 PUFAs suppressed VEGF- and bFGF-mediated expression of angiopoietin-2 and secretion of MMP-9, whereas ω-6 PUFAs increased angiopoietin-2 expression and MMP-9 secretion. These effects were COX-mediated, demonstrated by the abbrogation of these effects in the presence of the COX-inhibitor indomethacin and the finding that PGE_2, but not PGE_3, increased angiopoietin-2 expression. EPA and DHA have both been shown *in vitro* in HT-29 CRC cells to reduce VEGF and COX-2 expression, reduce PGE_2 levels and inhibit the ERK and HIF pathways which are associated with the induction of VEGF expression by PGE_2.(186) Similarly, when HT-29 cells were transplanted in nude mice EPA and DHA supplementation was associated with a reduction in VEGF and COX-2 expression in tumours, a reduction in tumoural PGE_2 levels and a reduction in tumour vascularity.(186) These studies support a role of the COX pathway as a mechanism for the inhibition of VEGF expression and angiogenesis by EPA.

The EMT Trial demonstrated a trend towards reduced tumour vascularity with EPA supplementation. Perhaps one would not expect any anti-angiogenic effect of EPA to manifest as a reduction in tumour vascularity after only 4 weeks of supplementation. The effect of EPA would most likely be on new vessel formation rather than causing disruption of existing vessels. A longer period of supplementation with more prolonged inhibition of new vessel formation might reveal a more pronounced effect on tumour vascularity.

Immunohistochemistry for MVD was performed using the well-established endothelial cell marker CD31. This protein is present on both large and small blood

vessels in both tumour tissue and normal tissue and is considered to be a pan-endothelial cell marker.(392, 393) CD105, by contrast, is thought to be a proliferation-associated protein expressed specifically by newly formed (neoangiogenic) blood vessels.(394, 395) CD105 has therefore been proposed as a better marker than CD31 for the evaluation of angiogenesis.(394, 395) Both CD31 and CD105 have been shown to be prognostic for metastatic risk and poor outcome in colorectal (396-398) and other types of cancers.(399-402) Despite the recent interest in CD105 as a better marker of neoangiogenesis, the specificity of CD105 for tumour blood vessels is not universally accepted and may be dependent on the type of tissue being studied.(395, 403) For example, high levels of CD105 staining of mature blood vessels has been seen in normal lung and brain tissue but not in normal breast tissue or gastric mucosa,(403) and high levels of CD105 staining of non-endothelial cells has been seen in normal liver and kidney tissue (35-70% non-specific expression) but not in normal lung, breast or colonic tissue.(395) Similar findings were observed when a batch of 20 tumours sections from The EMT Trial were stained for CD105 as a pilot to assess the quality of CD105 staining, using colon cancer tissue microarrays (TMA) which had shown good CD105 staining in an unrelated study as a positive control. The specificity of CD105 staining for endothelial cells was poor in the CRCLMs, with a high level of non-specific background staining compared to either the comparative CD31 staining of CRCLMs or the CD105 staining of the colon TMAs (data not shown). CD105 was therefore not explored further as a marker of MVD in The EMT Trial. These seemingly tissue-specific findings, together with variability in the particular antibody and staining methodologies used in different studies might explain the inconsistency in findings between studies and explain why no single endothelial cell marker has established itself as the marker of choice.

The trend to reduced tumour vascularity in The EMT Trial after only a short period of EPA supplementation is encouraging for an anti-angiogenic effect of EPA and is supported by the *in vitro* data from the HUVEC studies which demonstrated that both acute and chronic EPA supplementation inhibited endothelial cell proliferation, and also inhibited, less consistently, endothelial cell migration and tubule formation. The anti-angiogenic effects of EPA should be investigated further. Pre-clinical studies might focus on further elucidating the differences between acute and chronic EPA supplementation and the mechanistic basis underpinning these differences. Clinical studies of tumour vascularity should investigate the effects on MVD of longer periods of EPA supplementation, and should consider carefully the choice of endothelial cell marker, perhaps using a combination of markers such as CD31 and CD105 to differentiate between mature and neo-angiogenic vessels.

7.7 Tumour microenvironment

The communication between tumour microenvironment and the growth and metastasis of cancer cells is well recognised even if not fully understood. Inflammation at the site of a tumour is recognised as an important component of the tumour microenvironment, and causes a wide variety of cells to accumulate locally and infiltrate the tumour.(404) This has been particularly studied in relation to the importance of the COX pathway and pro-inflammatory prostaglandins to the initiation and promotion of CRC.(405) An in-depth discussion of the tumour microenvironment in CRC carcinogenesis can be found in the review by Peddareddigari *et al.*(383) Cells that accumulate and infiltrate CRCs include tumour associated macrophages (TAMs), mesenchymal stem cells, myeloid-derived suppressor cells, mast cells, neutrophils and platelets, amongst others.(383) Of these, TAMs are one of the most important components of the tumour, secreting a range of chemokines and growth factors which are involved in

inflammation, angiogenesis, epithelial-mesenchymal transition and immunosuppression.(383, 406, 407)

There is limited evidence for the effects of ω-3 PUFAs on the tumour microenvironment. In The EMT Trial, a PBMC model of TAMs showed that EPA supplementation was associated with inhibition of the transcription factor NFκB. NFκB activation is associated with pro-proliferative, anti-apoptotic and pro-metastatic pathways and its inhibition has been suggested as a potential therapeutic target.(176, 408) Although changes in NFκB activity in PBMCs cannot be taken as direct evidence for an effect of EPA on NFκB activity within CRCLM tissue, it is feasible that changes in PBMCs reflect changes in TAMs making the PBMC model a biologically plausible surrogate marker of changes within the tumour microenvironment. An alternative approach would have been to attempt to measure NFκB activation in CRCLM tissue. This would have required additional CRCLM tissue (which for many patients with small tumours would have been scarce), would have been subject to the heterogeneity of the tumour, and would have only permitted measurement of NFκB activation post-treatment. The PBMC model, by contrast, permitted a more controlled *ex vivo* analysis of NFκB activation and allowed comparison of NFκB activation pre- and post-treatment. Further studies are required to determine whether inhibition of NFκB signalling by EPA contributes to a direct anti-CRC cell activity, whether it exerts an indirect anti-CRC effect mediated by the tumour microenvironment, PBMCs or other circulating cells e.g. platelets, or whether its effects are more broadly due to a reduction in systemic inflammation. The effect of EPA on tumour microenvironment and circulating cells is very much understudied. Future work might investigate the effect of EPA on the number and localisation of macrophages within tumours, the effects of EPA on other cells within the tumour microenvironment, and the effect of EPA on signalling between circulating platelets and tumour cells which was recently described and shown to promote metastasis.(27)

7.8 Future clinical work

Now that two years have passed since the last patients recruited into The EMT Trial underwent liver resection, it would be an appropriate time to collect data for a comparison of disease-free survival and overall survival between the two groups. Whilst this was not explicitly stated as an endpoint in the trial, because the trial would have been underpowered for this as an endpoint, it was always planned to perform this analysis because the data would be readily available from the Leeds Hepatobiliary Unit's internal prospectively maintained database. The intention to perform such an analysis after 2 years of follow-up was included in the original application to Cancer Research UK for approval of The EMT Trial by the Clinical Trials Awards and Advisory Committee (CTAAC).

In the Rothwell meta-analyses of patients who participated in studies of the effect of aspirin on cardiovascular disease, there was a significant reduction in 20-year CRC incidence (138) and a reduction in all-cancer death after 10 years follow-up.(139) The protective effect of aspirin was not evident for 10 years because of the lag time between carcinogenesis and the development of clinically evident CRC. If EPA supplementation was associated with a similar separation or time-lag of the Kaplan-Meier curves for DFS or OS in The EMT Trial, this may indicate a biological effect of EPA which was not detectable by the short timeframe IHC and mechanistic endpoints measured in the trial.

Rothwell *et al*. were able to make use of large multi-centre trials of the effects of prolonged aspirin therapy on cardiovascular disease to look at CRC incidence in these patients many years after they completed participation in their respective trials.(138, 139) In the same way, it might be possible to perform a similar analysis of the chemopreventative effects of ω-3 PUFA supplementation using patients who participated in large trials of ω-3 PUFAs in cardiovascular disease such as the Italian GISSI trial. This would be dependent on there being accurate national cancer registries in the countries that took part in the original trials. The

coordination of ethical approval for this work may also prove an obstacle. However, given the convincing chemoprevention and cancer-mortality benefits that were shown in the Rothwell studies, (138, 139) a similar study for ω-3 PUFAs is worthy of investigation, especially given the more favourable long-term side effect profile of EPA than aspirin.

Having demonstrated in The EMT Trial that EPA is safe and well tolerated in patients with CRCLM, and confirmed for the first time in CRC/CRCLM at least some of the anti-neoplastic effects that have been seen in pre-clinical and mucosal biomarker studies, the question that remains is what context would EPA find most utility in the treatment of CRC and CRCLM. Potential uses of EPA include primary prevention of CRC or secondary prevention of CRCLM, neoadjuvant/adjuvant treatment of patients with established CRC and CRCLM who are undergoing surgery with curative intent, and management of incurable disease. The encouraging findings from The EMT Trial support progression to a large, multi-centre Phase III evaluation of EPA in patients with CRC or CRCLM. Since the duration of supplementation in The EMT Trial was perhaps too short to demonstrate some of the potential anti-CRC activity of EPA, Phase III evaluation should involve supplementation over a longer period of time, of at least 6 months. This could be a study of EPA supplementation following CRC resection, with outcomes of DFS and OS at 5 years. Alternatively, a similar study of EPA supplementation after CRCLM resection would perhaps require a shorter period of follow-up of between 2-5yr for DFS and OS endpoints since most CRCLM recurs within the first two years. There is also evidence to support a role for the use of ω-3 PUFAs in combination with established chemotherapeutic agents or with radiotherapy, and evaluation of this is already underway in other cancers including breast and pancreatic cancer. Omega-3 PUFAs also have a potential role as immunonutrition to improve cancer-related cachexia and to improve outcomes following cancer surgery. There is

therefore also scope to evaluate the use of EPA in combination with current neoadjuvant and adjuvant chemotherapy regimens for CRC and CRCLM. Such studies could include chemotherapy side effects and quality of life as outcomes in addition to DFS and OS endpoints.

7.9 Conclusions

The EMT Trial is the first RCT of an oral ω-3 PUFA in patients with CRCLM. EPA was safe and well tolerated in this population of patients, confirming the excellent safety and tolerability profile of ω-3 PUFAs which makes them an attractive candidate for the treatment and/or prevention of CRC and CRCLM. Previous concerns that ω-3 PUFAs may predispose patients to bleeding were dispelled, with no difference in platelet aggregation or in clinical endpoints of bleeding, transfusion rate or post-operative complications following liver resection.

The EMT Trial demonstrated that EPA supplementation was associated with an increase in tumour EPA content, and although there was not a reduction in tumoural AA or PGE_2 levels, it is conceivable that the period of supplementation was too short for this to have been observed. Similarly, although there was no reduction in tumour proliferation index or an increase in apoptosis index, the pre-clinical evidence for this is strong, and is further supported by the recent demonstration of chemopreventative efficacy of EPA in a Phase III trial in patients with FAP. Perhaps with a longer period of supplementation these effects may have been observed. There was a trend to a reduction in tumour vascularity, supported by supplementary *in vitro* angiogenesis experiments, which taken together provide encouragement that EPA might indeed reduce tumour angiogenesis. EPA supplementation was associated with a reduction in urinary PGE-M. Whether this simply represents a reduction in systemic inflammation or is representative of changes within the tumour cannot be determined based on the results from this

Phase II trial, but it does demonstrate a positive systemic effect of EPA supplementation and raises the possibility of urinary PGE-M as a non-invasive biomarker of disease progression or response to treatment. Finally, EPA was shown to inhibit PBMC NFκB activation, providing further evidence of a systemic anti-inflammatory effect of EPA, and raising the possibility of an effect of EPA on the tumour microenvironment.

Although no firm conclusions about the anti-CRC activity of EPA can be drawn from this study, the results from such a short period of supplementation are encouraging for there being a beneficial effect of EPA supplementation, and support further evaluation of EPA for the treatment and/or prevention of CRC and CRCLM. The mechanistic analyses from this trial also support the need for more pre-clinical studies to further elucidate the effects of EPA on tumour growth, angiogenesis, and on the tumour microenvironment. When available, it will be interesting to see the DFS and OS analysis from this trial, and this data may help to guide the design of any subsequent Phase III trial.

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List of Abbreviations

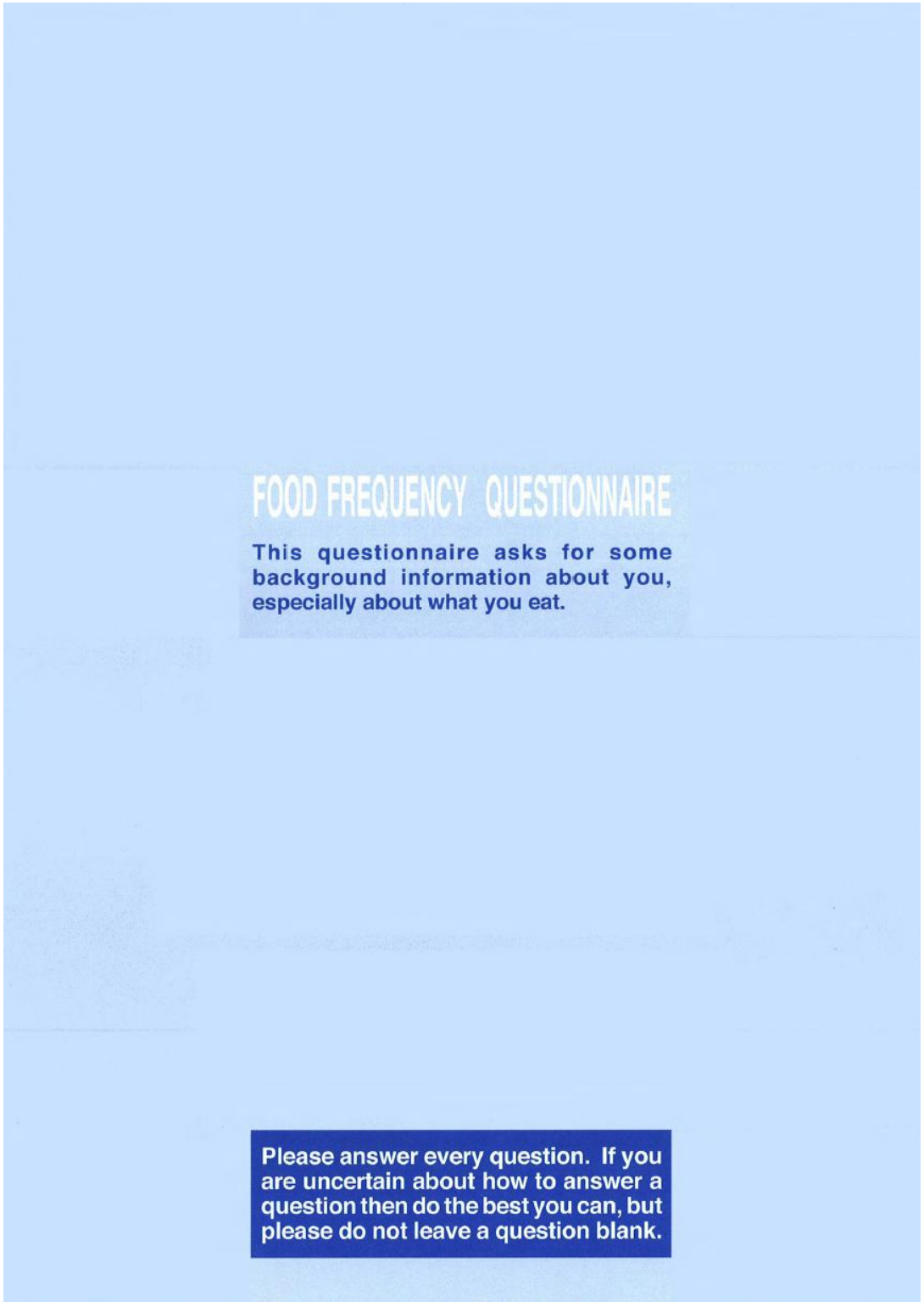
AA	arachidonic acid
ACF	abberant crypt foci
ADP	adenosine diphosphate
AE	adverse event
AI	apoptosis index
AICR	American Institute for Cancer Research
ALA	alpha-linolenic acid
APC	adenomatous polyposis coli
BAECs	bovine artery endothelial cell
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
CLB	complete lysis buffer
COX	cyclooxygenase
Cr	creatinine
CRC	colorectal cancer
CRCLM	colorectal cancer liver metastases
CYP450	cytochrome p459
DAB	diaminobenzedine
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DPBS	Dulbecco's phosphate buffered saline
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMT	EPA for Metastasis Treatment
EPA	eicosapentaenoic acid
ERK	extracellular signal-regulated kinase
FAP	familial adenomatous polyposis
FFA	free fatty acid
FFQ	food frequency questionnaire
FOLFIRI	folinic acid + fluorouracil + irinotecan
FOLFOX	folinic acid + fluorouracil + oxaliplatin
FOLFOXIRI	folinic acid + fluorouracil + oxaliplatin + irinotecan
FU	fluorouracil
GC	gas chromatography
GC-MS	gas chromatography - mass spectrometry
GCP	good clincial practice
GPCR	G protein-coupled receptor
HDU	high dependency unit
HNPCC	hereditary non-polyposis colorectal cancer
HPLC	high performance liquid chromatography

HR	hazard ratio
HRP	horseradish peroxidase
HTA	Human Tissue Authority
HUVEC	human umbilical vein endothelial cell
IHC	immunohistochemistry
IHPBA	International Hepato-Pancreatico-Biliary Association
IMP	investigative medicinal product
IQR	interquartile range
LA	linoleic acid
LC	liquid chromatography
LOX	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
LTA	light transmission aggregometry
MHRA	Medicines and Healthcare related products Regulatory Authority
MMP	matrix metalloproteinase
MRM	multiple reaction monitoring
MS/MS	tandem mass spectrometry
MTT	methylthiazolyldiphenyl-tetrazolium bromide
MVD	microvessel density
NAD	nicotinamide adenine dinucleotide
NFκB	nuclear factor kappa B
NICE	National Institute for health and Care Excellence
NSB	non-specific binding
OD	optical density
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PG	prostaglandin
PGDH	prostaglandin dehydrogenase
PI	proliferation index
pNpp	p-nitrophenyl phosphate
POF	perioperative outcome form
PPAR	peroxisome proliferator-activated receptor
PTFE	polytetrafluoroethylene
PUFA	polyunsaturated fatty acid
RCT	randomised controlled trial
REC	research ethics committee
RIA	radioimmunoassay
RNA	ribonucleic acid
ROS	reactive oxygen species
RR	relative risk
RT-PCR	real-time polymerase chain reaction
Rv	resolvin
RvE1	resolvin E1

SAE	serious adverse event
SEM	standard error of the mean
SPE	solid phase extraction
SUSAR	suspected unexpected serious adverse reaction
TAM	tumour associated macrophage
TBS	tris buffered saline
TBST	tris buffered saline Tween-20
TGF	transforming growth factor
TMA	tissue microarray
TPN	total parenteral nutrition
TSC	trial steering committee
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WCRF	World Cancer Research Fund

Appendix A

Pages from the EPIC Food Frequency Questionnaire that concern dietary fat intake were selected for use in The EMT Trial and are shown here.



Please estimate your average food use as best you can, and please answer every question – do not leave ANY lines blank. PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stew or slices									
Lamb: roast, chops or stew									
Chicken or other poultry eg. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, eg. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls									
Liver, liver paté, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, eg. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, eg. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, eg. crab, prawns, mussels									
Fish roe, taramasalata									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

This food frequency questionnaire (CAMB/PQ/6/1205) was originally designed for the EPIC-Norfolk Study.
www.epic-norfolk.org.uk

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DAIRY PRODUCTS AND FATS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, eg. Cheddar, Brie, Edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream(tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block or hard margarine, eg. Stork, Krona (teaspoon)									
Polyunsaturated margarine, eg. Flora, sunflower, soya spreads (teaspoon)									
Soft margarines,including olive oil based and dairy spreads, eg. Blue Band, Olivio/ Bertolli, Clover (teaspoon)									
Low fat spreads (less than 60% fat), eg. Outline, Gold (teaspoon)									
Very low fat spread (less than 30% fat) (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

YOUR DIET LAST YEAR, continued

2. Are there any **OTHER** foods which you ate more than once a week? Yes ☐ No ☐
If **YES,** please list below

Food	Usual serving size	Number of times eaten each week

3. What type of milk did you most often use?
Select one only
Full cream/whole ☐ Semi-skimmed ☐
Skimmed ☐ Channel Islands, gold ☐
Dried milk ☐ Soya ☐
Other, specify ☐ None ☐

4. How much milk did you drink each day, including milk with tea, coffee, cereals etc?
None ☐ Three quarters of a pint ☐
Quarter of a pint ☐ One pint ☐
Half a pint ☐ More than one pint ☐

5. Did you usually eat breakfast cereal (excluding porridge and Ready Brek mentioned earlier)? Yes ☐ No ☐

If **YES,** which brand and type of breakfast cereal, including muesli, did you usually eat?
List the one or two types most often used

Brand e.g. Kellogg's	Type e.g. cornflakes

6. What kind of fat did you most often use for frying, roasting, grilling etc?
Select one only
Butter ☐ Solid vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐
If you used vegetable oil, please give type eg. corn, sunflower ☐

7. What kind of fat did you most often use for baking cakes etc?
Select one only
Butter ☐ Solid vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐
If you used margarine, please give name or type eg. Flora, Stork ☐

8. How often did you eat food that was fried at home?
   Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
   Less than once a week ☐ Never ☐

9. How often did you eat fried food away from home?
   Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
   Less than once a week ☐ Never ☐

10. What did you do with the visible fat on your meat?
    Ate most of the fat ☐ Ate as little as possible ☐
    Ate some of the fat ☐ Did not eat meat ☐

11. How often did you eat grilled or roast meat? ☐☐ times a week

12. How well cooked did you usually have grilled or roast meat?
    Well done /dark brown ☐ Lightly cooked/rare ☐
    Medium ☐ Did not eat meat ☐

13. How often did you add salt to food while cooking?
    Always ☐ Rarely ☐
    Usually ☐ Never ☐
    Sometimes ☐

14. How often did you add salt to any food at the table?
    Always ☐ Rarely ☐
    Usually ☐ Never ☐
    Sometimes ☐

15. Did you regularly use a salt substitute (eg LoSalt)? Yes ☐ No ☐
    If **YES**, which brand? _____

16. During the course of last year, on average, how many times a week did you eat the following foods?

Food type	**Times/week**	**Portion size**
Vegetables (not including potatoes)	☐☐	medium serving
Salads	☐☐	medium serving
Fruit and fruit products (not including fruit juice)	☐☐	medium serving or 1 fruit
Fish and fish products	☐☐	medium serving
Meat, meat products and meat dishes (including bacon, ham and chicken)	☐☐	medium serving

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17. Have you taken any vitamins, minerals, fish oils, fibre or other food supplements during the past year?

- ☐ Yes
- ☐ No
- ☐ Sometimes
- ☐ Don't know

If **YES or SOMETIMES,** please complete the table below.

If you have taken more than 8 types of supplement please put the most frequently consumed brands first.

Example: If you take one tablet of vitamin C two times a day, please write '2' in the amount-column and tick (✓) the 'once a day' box. Most supplements mention a strength value (in our example 500mg), please write this information in the table.

Supplements				Average frequency for the past year Tick (✓) **ONE** box per line to show how often on average you took the amount consumed as mentioned in 'amount' column.					
Brand	Name	Strength (strength of the supplement for each tablet or capsule)	Amount (number of tablets, capsules or teaspoons taken in one day)	Never or less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day
Boots	High strength vitamin C	500mg	2 tablets	EXAMPLE					✓

Thank you for your help

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