Randomised controlled trial of the effects of fish oil emulsion in total parenteral nutrition upon tumour vascularity in patients with hepatic colorectal metastases

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by
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Abstract

Tumour growth is dependent on angiogenesis. Angiogenesis often predicts a poorer prognosis in cancer patients and is also associated with the increased development of tumour metastases. Angiogenesis is stimulated by vascular endothelial growth factor, which increases vascular permeability, induces endothelial cell proliferation and promotes endothelial cell survival, which, are pivotal in tumour genesis. As such angiogenesis and vascular endothelial growth factor inhibitors are now commonplace in the armoury of anti-cancer treatments.

In vitro, in-vivo and epidemiological studies have demonstrated that omega-3 fatty acids, principally eicosapentaenoic acid and docosahexaenoic acid have profound anti-inflammatory, anti-apoptotic, anti-proliferative and anti-angiogenic properties. Anti-angiogenic actions include a reduction vascular endothelial growth factor concentration and alteration of vascular endothelial growth factor receptor signalling response. Unfortunately only negligible amounts of EPA and DHA can be produced by human metabolism. Omega-3 fatty acids are however synthesised in abundance by algae and plankton and as a consequence fish are the main dietary source of essential omega-3 fatty acids. However even with a balanced diet rich in fish, human plasma concentrations are minimal. In contrast parenteral administration of a fish-oil lipid emulsion leads to a significant and rapid increase in eicosapentaenoic acid and docosahexaenoic acid concentrations in plasma, and platelet and leukocyte membrane phospholipids within hours.

There is a large body of work in the scientific literature that supports the theory that omega-3 fish oils have marked anti-angiogenic properties and that plasma concentrations of omega-3 fatty acids can be quickly and safely manipulated with intravenous infusion. I present the findings from a randomised controlled pilot study in patients with hepatic colorectal metastases to assess the response of biomarkers of angiogenesis to intravenous administration of omega-3 fatty acids compared to controls.
Acknowledgements

I would like to dedicate this thesis to my-step father Michael. He has been and continues to be an inspirational force to me. He made me strive to better myself and not accept mediocrity.

A major thank you to Ashley and Bruno my supervisors. Ashley your direction developed me into a clinician with an analytic, but levelheaded approach to research. Wednesday morning coffee with Bruno was great and I learnt all there is to know about MRI from an expert.

Omer my clinical trial partner in crime. We worked well as a team, had a laugh, kept each other sane and developed a friendship for life. Sister Pollard, the day to day running of clinical trial would have been for more laborious without your assistance and helpful tips.

To my long-suffering wife, whom without, this work would not have been completed. Your motivation and subtle kicks up the backside kept me going!

Finally and most importantly I need to thank the volunteers who made a decision to take part in this clinical trial and make this MD thesis possible.
Declarations

The written thesis is my own work in its entirety.

The clinical trial was supervised by myself and Dr. Omer Al-Taan. We took over the running of the trial after it had been started by Dr Laura Spencer, seven patients had been enrolled by this point. Trial supervision involved trial logistics, patient enrolment, patient monitoring, scan supervision, blood sample processing. All TPN related issues and, trial research and development/ethics were coordinated by me. I performed all the tissue sampling.

The trial had two arms; 1: angiogenesis, and 2: inflammation. I produced this MD thesis on angiogenesis, Dr Omer Al-Taan wrote a thesis on inflammation.

Both Omer and I performed the fatty acid analysis using gas chromatography at the Institute of Human Nutrition at Southampton University. We both used the plasma phospholipid data for proof of uptake, however we performed our own statistical analysis. The tissue data was used in this thesis only.

The circulating angiogenic cytokine analysis was performed by me for this MD only. I sought help with statistical analysis for this data from a medical statistician Paul Bassett.

Other than MRI scan supervision, all other MRI related work and data was performed by me for this thesis only.

All work related to the thematic analysis was performed by me.
Published Work

Peer Reviewed Publications

At the time of submitting this thesis the following manuscripts had been published:


Published Abstracts and Meeting Presentations

At the time of submitting this thesis the following abstracts had been presented at scientific meetings and published:


Oral presentation for the Patey Prize at the Society of Academic and Research Society, Dublin 2011


Poster presentation at the British Association of Surgical Oncology Meeting, London 2010.


Prize poster presentation at the European Society of Nutrition Conference, Nice, Sept 2010.


Poster presentation at the European Society of Nutrition Conference, Nice, Sept 2010

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AIF</td>
<td>Arterial input function</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis gene</td>
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<tr>
<td>ANG</td>
<td>Angiopoietins</td>
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<tr>
<td>BOLD</td>
<td>Blood oxygen level dependent</td>
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<tr>
<td>CE</td>
<td>Cholesterol esters</td>
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<td>CML</td>
<td>Chronic myelogenous leukaemia</td>
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<td>COX</td>
<td>Cycloxygenases</td>
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<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>DCE</td>
<td>Dynamic contrast enhanced</td>
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<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<td>DW-MRI</td>
<td>Diffusion-weighted magnetic resonance imaging</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGF-R</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EES or Ve</td>
<td>Extra-vascular, extra-cellular space</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FID</td>
<td>Flame ionisation detector</td>
</tr>
<tr>
<td>FO</td>
<td>Fish oil</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td>GEE</td>
<td>Generalised estimating equation</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumour</td>
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<td>Gd</td>
<td>Gadolinium</td>
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<td>H &amp; N</td>
<td>Head and neck cancer</td>
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<td>HER2</td>
<td>Human Epidermal growth factor receptor-2</td>
</tr>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>H-MSI</td>
<td>High frequency microsatellite instability</td>
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<tr>
<td>IAUC</td>
<td>Initial area under the enhancement curve</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>K^{trans}</td>
<td>Leak rate constant</td>
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<td>mCRC</td>
<td>Metastatic colorectal cancer</td>
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<td>MCT</td>
<td>Medium chain triglycerides</td>
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<td>MLH1</td>
<td>MutL Homolog 1</td>
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<td>Multiple myeloma</td>
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<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<td>MRI</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MutS Homolog 2</td>
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<td>Omega-3</td>
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<td>NHL</td>
<td>Non-Hodgkin’s lymphoma</td>
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<td>OA</td>
<td>Oleic acid</td>
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<td>PACS</td>
<td>Picture archiving and communication system</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PDGF-R</td>
<td>Platelet derived growth factor receptor</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>PL</td>
<td>Phospholipid</td>
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<td>PPL</td>
<td>Plasma phospholipid</td>
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<tr>
<td>PMS2</td>
<td>Post-meiotic increased 2</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during transfection</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>s-HRP</td>
<td>Streptavidin-horseradish peroxidase</td>
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<tr>
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<td>Solid phase extraction</td>
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<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
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<tr>
<td>TE</td>
<td>Time to echo</td>
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<td>TGF</td>
<td>Transforming Growth Factor</td>
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<td>TKI</td>
<td>Tyrosine kinase inhibitors</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor metalloproteinases</td>
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<tr>
<td>TR</td>
<td>Time to repeat</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
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<td>USS</td>
<td>Ultrasound scan</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGF-R</td>
<td>Vascular endothelial growth factor receptor</td>
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<tr>
<td>Vp</td>
<td>Plasma volume</td>
</tr>
<tr>
<td>VPF</td>
<td>Vascular permeability factor</td>
</tr>
<tr>
<td>wsCOV</td>
<td>Within subject coefficient of variation</td>
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Chapter 1

Introduction
1.1.0 The History of Angiogenesis

There are more than 100 distinct types of malignancy with marked heterogeneity within each tumour type, but there exists a remarkable similarity in the cellular processes that promote cancer growth across most malignancies [Hanahan & Weinberg 2000]. In their seminal paper “The Hallmarks of Cancer” Hanahan and Weinberg proposed six acquired capabilities by which normal cells become cancer cells.

![Figure 1.1: The six hallmarks of cancer growth. From Hanahan 2000.](image)

These traits include self-sufficiency in growth signals, insensitivity to growth inhibiting signals, evasion of apoptosis, tissue invasion and metastasis, limitless reproductive potential and sustained angiogenesis.

1.1.1 Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vessels and is a normal and highly regulated physiological process throughout
the body. Since physiological angiogenesis is associated only with tissue growth, tissue repair and the reproductive cycle, angiogenesis in normal adult tissues is a very rare event, and the endothelium of most tissues is therefore an extremely stable population of cells with very low mitotic activity [Hall 2005]. Angiogenesis also occurs after implantation of the blastocyst of the embryo to the uterus. This produces the placenta [Torry 2007, Rogers 1992], and was first observed in pregnant monkeys by Hertig in 1935 [Hertig 1935]. In the developing embryo both angiogenesis and the earlier distinct process of vasculogenesis are required for foetal growth and viability [Breier 2000].

1.1.2 The Beginnings of Angiogenesis; From Hunter to Folkman, a Slow Beginning

The founder of ‘scientific surgery’ John Hunter (1728-1793), who sought to provide an experimental basis to surgical practice, first used the term angiogenesis in 1787 [Hall 2005]. He stated that vessels “would appear to have more powers of perfecting themselves, when injured, than any other part of the body; for their use is almost immediate and constant, and it is they which perform the operation of restoration on the other parts, therefore they themselves must first be perfect” [Hunter 1861a]. He noted the early appearance of new blood vessels to supply the growing antlers from the Fallow Deer’s skull, observing “a soft membranous pulp shooting out from this knob which is extremely vascular” [Hunter 1861b].

Despite this early and accurate observation from Hunter, reports of tumour angiogenesis in the next 100 years were very few and nearly all were from anatomical studies.
In the mid Nineteenth Century, Karl Thiersch (1822-1895), working in Leipzig Germany, demonstrated a vigorous formation of new vessels in the stroma of carcinomas. Using vessel injection techniques, he discovered that these new vessels originated from pre-existing capillaries [Thiersch 1865]. Rudolf Virchow (1821-1902) studied the vascular morphology of tumours in great detail [Virchow 1863] and at about the same time Theodor Bilroth (1829-1894), the founding father of modern abdominal surgery, noted that in the youngest parts of tumours there were numerous fine vessels and vascular networks, whereas the older areas of these tumours were associated with wider thrombosed and atrophied vessels [Bilroth 1871].

Bashford and Goldmann confirmed the findings of both Hunter and Bilroth in the early Twentieth Century [Goldmann 1907, Bashford1904]. Goldmann visualised the vascular networks in human and animal tumours by injecting India ink and bismuth-in-oil into the vasculature, which enabled him to study the number, form and width of blood vessels. At a lecture to the Royal Society of Medicine in 1907 he noted that the regular distribution of blood vessels is disturbed by the invading growth of a cancer, and that at the tumour periphery there was extensive new vessel formation.

Lewis used Goldmann's experimental methods in 1927. He wrongly concluded that blood vessels did not determine the growth of the tumour; but rather that the tumour determined the growth and pattern of vessels, a misconception that continued until late in the 20th century and hindered the acceptance of Folkmans conclusions. He did however state that, different tumours had different vascular patterns, which may be useful in diagnosis [Lewis 1927].
The early 1930's saw the emergence of the field of vascular biology, when Clark and Clark placed glass-windowed chambers in the rabbit ear and made exquisite drawings of the branching patterns of the blood vessels that entered the wound in the rabbit ear [Clark 1932]. Later in the 1930’s the theories of Goldmann were supported by work carried out by Ide and Algire. Using a rabbit tumour model, Ide inserted fragments of epithelioma into a rabbit ear. He observed that tumour growth was accompanied by infiltration of newly formed blood vessels and that neovascularisation occurred in the implanted tumours and not the controls [Ide 1939]. Algire first demonstrated the observation that tumours actively attract new blood vessels in the 1940’s using wound chambers [Algire 1943a, 1943b, 1945].

From 1940 to 1960 Harry S. N. Green (1905-1969) was working at the forefront of the field of tissue transplantation. Greene’s study on heterologous transplantation of mammalian tumours showed that rabbit adenocarcinomas transplanted into the anterior chamber of guinea pig eyes would grow if these tumours became vascularised. In those tumours that grew, he described a pinkish colouration of the tumour substance two weeks after transplantation, which represented neovascularisation. If tumour growth was delayed he observed a lack of vascularisation [Greene 1941]. Greene moved to Yale University in 1943 and became the Anthony N. Brady Professor and Chairman of the Department of Pathology in 1950. His continued work at Yale, later showed that human tumours became vascularised and then grew following implantation into the anterior chamber of a guinea pig eye [Greene 1952]. Using the same model, small tumour fragments often failed to gain a blood supply but when these fragments were transplanted into the vessel rich environment of muscle they increased in size after undergoing vascularisation [Greene 1951]. Interestingly, Greene gained marked public prominence, not for his scientific work on
transplantation and tumour growth, but for his outspoken and sceptical views in
the late 1950’s concerning the theory that smoking was connected to lung cancer
on. He continued to smoke and died happy at the relatively young age of 64.

Over this period of twenty years numerous authors published data of
neovascularisation of tumours implanted subcutaneously, in transplant chambers
or in hamster cheek pouches. [Cowdry 1946, Lutz 1950, Bierman 1951, Urbach
1962, Rubin 1966]. These studies attributed the cause of tumour
neovascularisation to various factors including inflammation, vasodilatation, and
increased tumour metabolism or over-production of specific metabolites such as
lactic acid.

Thus, at this early stage the concept of tumour neovascularisation was
well established but hypotheses as to its causes and implications were
misleading because prevailing opinion was that tumour growth did not depend on
angiogenesis, but that tumour vascularity was non-specific inflammation
[Folkman 2007, Van Epps 2005].

A key change came in 1962 when Judah Folkman (1933-2008) and
Frederick Becker studied tumour growth in organ culture and first suggested that
neovascularisation was due to hypoxia from “tumours outgrowing their blood
supply” [Folkman 1962].

1.1.3 Judah Folkman; the ‘Father of Angiogenesis’

Moses Judah Folkman was born in Cleveland, US on February 24, 1933
to Rabbi Jerome Folkman and wife Bessie. Shortly after his birth the family
moved to Grand Rapids, Michigan, US. As a child Folkman was encouraged to
seek knowledge, particularly in the field of science and one of the first books he read was a biography of Madame Marie Curie [Cooke 2001]!

![Moses Judah Folkman](image)

**Figure 1.2: Moses Judah Folkman, the father of angiogenesis.**

Folkman graduated from Harvard Medical School in 1957, and was a third year surgical resident at Massachusetts General Hospital, Boston, USA, when he was drafted by the navy in 1960 to work in the laboratory of the Naval Medical Research Institute in Bethesda, Maryland, US. While at the Naval Medical Research Institute, Folkman worked with David Long, a surgical resident from the University of Minnesota and the above-mentioned Fred Becker, a pathology resident from New York University. One of the projects they were charged with was testing freeze-dried haemoglobin solutions as an alternative to fresh blood [Folkman 2007, Van Epps 2005]. The haemoglobin preparations were tested for the ability to sustain the viability of dog thyroid glands and later, the growth of mouse tumour cells implanted within these dog thyroid glands within organ culture. The implanted tumours stopped growing after reaching a modest size, but grew explosively if transplanted back into a mouse. Examining the tumours
Folkman found a network of tiny blood vessels inside the re-transplanted tumour cells, whereas there were no vessels in the original thyroid tumours [Folkman 1962]. In 1962 Folkman returned to his surgical residency position post in Boston inspired by what he had observed, and concurrently continued experimental work to these observations and his developing theories on neovascularisation in tumours [Folkman 2007]. Fascinatingly, while at the Naval Medical Research Institute, Long and Folkman also discovered that sustained drug release could be achieved from silicone rubber.

On discharge from the Navy, they gifted the patent ‘royalty-free’ to the Population Council of the Rockefeller Foundation, who used the technology to develop an implantable sustained release contraceptive – Norplant. This technology is still used today for estradiol sustained release vaginal rings [Folkman 1964 & 2007].

After completing his residency in 1965, Folkman joined Harvard’s Department of Surgery at the Boston City Hospital. In 1966, under the chairman of the department, Professor William McDermott, Folkman and colleagues developed an isolated organ perfusion system to provide a vascular bed for the study of tumour growth [Folkman 1971]. He continued research alongside his clinical and teaching role. In 1968, Folkman spent six months at The Philadelphia Children’s Hospital as chief surgical resident prior to his appointment as ‘Julia Dyckman Andrus’ Professor of Pediatric Surgery and surgeon-in-chief at the Children’s Hospital in Boston, where he directed a laboratory that focused on angiogenesis research [Folkman 2007, Cooke 2001]. In 1969, Folkman’s hypothesis was fuelled clinically when he saw a child with a retinoblastoma. The child had a large neovascularised tumour (> 1 cm³), which protruded from the retina into the vitreous, with numerous tiny metastases from
primary tumour cells that had shed into the vitreous and aqueous humour. The metastases averaged 1.25 mm diameter, with a thin rim of viable tissue surrounding a necrotic core. He correctly assumed they could not become neovascularised as they were too far removed from the vascular bed and thus could not grow further [Folkman 2008].

This lab work and clinical observations helped Folkman develop the hypothesis that “tumour growth is angiogenesis-dependent”. He appreciated that for a tumour to receive enough oxygen and nutrients to promote growth beyond 2 mm, simple diffusion was not enough and new vessels must be recruited. Along with this hypothesis he also suggested other concepts:

(1) Virtually all tumours would be restricted to a microscopic size in the absence of angiogenesis

(2) Tumours would be found to secrete diffusible angiogenic molecules

(3) Tumour dormancy would result from blocked angiogenesis

He also proposed the term anti-angiogenesis to mean prevention of new capillary sprouts from being recruited into an early tumour implant and predicted the future discovery of angiogenesis inhibitors, suggesting that an antibody to a tumour angiogenic factor (TAF), could be an anticancer drug [Folkman 1971].

These ideas were widely criticised, as the prevailing opinion was that tumour growth did not depend on angiogenesis, but that tumour vascularity was non-specific inflammation [Folkman 2007, Van Epps 2005]. Sceptics challenged Folkman’s hypothesis and journal reviewers complained that the conclusions of his experiments reached beyond the data [Folkman 2007].
Due to the scepticism Folkman’s conclusions received, it wasn’t until 1971 that his seminal paper ‘Tumour angiogenesis: therapeutic implications’ was published [Folkman 1971]. Throughout the 1970’s general belief in the scientific community was that tumours grew around established capillary blood vessels and that new vessel recruitment was not possible. This was despite the published findings discussed previously from Greene’s work in the 1940s. His report of new blood vessels at the border of tumours was interpreted as an “inflammatory reactions of the host to dying tumour cells in the necrotic centre of the tumour” [Folkman 2007].

Figure 1.3: Original depiction of how angiogenesis affects tumour growth. When the tumour is small it obtains nutrients and oxygen by diffusion. The tumour induces neovascularisation at which point it can grow larger than 2 mm and spread. From Folkman 1971.

His hypothesis gained more credibility when it was backed up by work published by Folkman’s post-doctoral fellow Michael Gimbrone in 1972. Utilising the eye transplant model he first demonstrated that epithelioma tumour fragments implanted into the eyes of male rabbits would not grow beyond 1mm² if placed away from blood vessels on the avascular anterior chamber of the eye and histology confirmed these tumours to be avascular. Secondly he showed that
tumour fragments implanted directly onto the iris grew nearly 16,000 times their original size in only two weeks. The growth pattern was observed in three phases described by a sigmoid curve; an early pre-vascular phase rate with slow growth, then a rapid growth observed in the vascular phase leading to a slower late growth phase once the tumour filled the anterior chamber [Gimbrone 1972a & 1972b].

The model was then used to hypothesise that cellular contact was not required between host vessels and tumour cells for neovascularisation to occur. Tumour fragments were implanted on the cornea either in the central avascular area (>3mm from the limbus) or on the periphery 2mm from the limbal edge. The peripherally placed fragments vascularised as new capillary blood vessels grew from the limbus, invading the stroma of the avascular cornea and reached the edge of the tumour within 8-10 days. There was no sign that the neovascularisation occurred due to inflammation as the cornea did not become opaque or oedematous. The tumours grew exponentially in three-dimensions and protruded from the cornea with 2-3 weeks. The tumours in the centre of the cornea remained non-vascularised and grew slowly in two dimensions, as thin flat, translucent, intracorneal lesions until one edge extended to within ~2 mm of the limbus, when they recruited new blood vessels and subsequently grew in three dimensions. It was suggested that a diffusible mediator or ‘angiogenic factor’ existed that stimulated vascularisation [Gimbrone 1972b, 1973, 1974a and 1974b]. This supported work produced by Greenblatt and Ehrmann, which had shown that tumour-stimulated vessel growth did not require direct contact between tumour and host tissue [Greenblatt 1968, Ehrmann 1968].

Knighton, working with Folkman in 1977 saw similar results in different animal models. An implant of Walker 256 carcinoma was injected into the...
chorioallantoic membrane of a chicken egg through a shell window, subsequently using the window to observe vessel growth. As previously the tumours exhibited pre-vascular and vascular growth phases. Unlike the previous studies this experiment placed the tumours in an area surrounded by healthy vessels. Even so, tumour growth still did not occur until host vessels penetrated the tumour. Neovascularisation always occurred by 72 hours [Knighton 1977].

Despite the impressive initial results from Folkman’s group, his ideas were still widely criticised throughout the 1970s. The 1971 New England Journal of Medicine paper is said to have initiated the field of angiogenesis, but it was really a decade before the international scientific community woke up to implications of these discoveries. Of the 33 angiogenesis publications between 1971 and 1976, Folkman was co-author of more than half! From 1970 to 1980 there were only 65 publications with angiogenesis in the title and merely 135 with angiogenesis as a keyword.

The lack of bioassays for angiogenesis, the inability to culture endothelial cells in-vitro, and the absence of angiogenesis regulatory molecules confounded development [Folkman 2008]. Throughout the 1970’s Folkman’s group set out to develop reagents and methods that would allow investigators to isolate pro-angiogenic and anti-angiogenic factors and provide supporting evidence for their struggling hypothesis [Folkman 2007].

Having suggested that a diffusible mediator or ‘angiogenic factor’ existed that stimulated vascularisation Folkman and his colleagues had to identify it. They struggled to assess potential isolated angiogenic molecules from tumour extracts due to inadequate experimental techniques available [27]. This problem was eventually solved by Robert Langer, another of Folkman’s post-doctoral
fellows, who along with a group lead by Robert Auerbach developed a polymer, polyhydroxy ethylmethacrylate (polyhema). This allowed implantation of the proteins of interest onto the mouse cornea in polymer pellets to detect angiogenic effects [Langer 1976, Muthukkaruppan 1979].

One of the major steps in allowing scientific appreciation of the role of angiogenesis in tumour formation and demonstrating angiogenesis in-vitro was developing techniques that allowed long term passage of endothelial cells. This was first reported in 1973, independently by both Folkman’s laboratory [Gimbrone 1973, 1974a, 1074b] and by Eric Jaffe at Cornell University, Itheca, New York, US [Jaffe 1973]. However, it was not until 1979 that long-term passage of cloned endothelial cells was reported [Folkman 1979] and a year later angiogenesis in-vitro was demonstrated [Folkman 1980].

Later in 1979 they made an important step in confirming the hypothesis that removal of an angiogenic stimulus lead to neovascularisation regression [Ausprunk 1978]. From these observations it was possible to elucidate that angiogenesis occurred in a continuum but could be divided into discrete stages for explanation. At this point the field of angiogenesis research bloomed, but 10 years later than Folkman may have expected in 1971.
Numerous growth factors and cytokines are now known to play an important role in the regulation of angiogenesis. Many factors that promote angiogenesis in bioassays were identified from 1980s onwards, including EGF, TGF-alpha, aFGF, bFGF and angiogenin. Unfortunately, linking these factors to angiogenesis in tumour models using neutralising antibodies or expression analyses yielded negative results [49]. However this changed from 1983, with the next major discovery in angiogenesis.
1.1.4 Vascular Permeability Factor, or is it Vascular Endothelial Growth Factor

After the developments of the late 1970’s, many other scientists entered the field of angiogenesis and Folkman’s sceptics ‘became his competitors’ [Van Epps 2005]. In 1983 Harold Dvorak and colleagues at Harvard medical school discovered a potent angiogenic factor [Segner 1983]. The authors purified what they called ‘vascular permeability factor’ (VPF) from a guinea pig tumour cell line that promoted blood-vessel leakage and subsequent ascites. They proposed that this VPF may be a mediator of tumour blood vessel permeability. However they could not completely purify this VPF at the time, so it was not fully identified [Ferrara 2009]. In 1989, this VPF was unknowingly revisited by three independent lines of research. Napoleone Ferrara purified a novel angiogenic protein from cow pituitary cells, which he termed “vascular endothelial growth factor”.

Figure 1.5: Mediators involved in the angiogenic cascade. From Spencer 2009.
This “VEGF” displayed growth-promoting activity towards only vascular endothelial cells [Ferrara 1981]. At the same time in Folkman’s laboratory, Rosenthal had isolated and purified to homogeneity an angiogenic protein that, when compared with the sequenced protein from Ferrara’s research, was identical [Folkman 2007]. By 1990 it had become apparent that VEGF and VPF were in fact identical, as a team lead by Daniel Connolly from Monsanto Company had followed up on the work by Dvorak and, purified and sequenced VPF [Keck 1989]. The first reported VEGF ligand identified from a tumour was published in a paper co-authored by Folkman and Ferrera in 1990 [Rosenthal 1990].

1.1.5 Vascular Endothelial Growth Factor; The big step from bench to bedside

We now know the VEGF family consists of the following members: VEGF-A (commonly known as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor [Liekins 2001, Holmes 2005].

Figure 1.6: Vascular Endothelial Growth Factor.
The VEGF family are heparin-binding peptides, which bind selectively to 5 distinct receptors. The three primary receptors belong to the super family of receptor tyrosine kinase and are VEGF receptor-1 (VEGFR-1, Flt-1) VEGFR-2 (kinase insert-domain containing receptor, Flk-1) and VEGFR-3 (Flt-4). The fourth and fifth receptors are neuropiloin-1 and -2.

Of the primary receptors, VEGR-2 and to a lesser extent VEGFR-1 are associated with angiogenesis and VEGFR-2 mediates the majority of VEGF angiogenic effects [Dvorak 2002, Ferrara 2004, Hicklin 2005, Kawasaki 1999]. The endothelial cell specificity of VEGF is the result of the expression of VEGFR-2, almost exclusively by endothelial cells [Veikkola 2000], whereas VEGFR-1 and VEGFR-3 are selectively expressed in distinct vascular beds [Ferrara 2004, Hicklin 2005, Kawasaki 1999].

Figure 1.7: The VEGF family and receptor affinity. From Hicklin 2005.
As discussed neovascularisation must occur to provide oxygen and nutrients to tumour cells [Poon 2003]. Furthermore the immature neovessels enhance tumour cell entry into the circulation [Folkman 1990]. Over expression of the VEGF ligand has been observed across a range of tumour types, including colon, lung, breast, glioblastoma, renal, ovarian and prostate, and is widely correlated with tumour development and prognosis [Ferrara 2004, Hicklin 2005, Margolin 2002].

VEGF is the central mediator of tumour angiogenesis stimulated by hypoxia and certain oncogenes [Shweiki 1992, 1995, Rak 2000]. It is the only angiogenic factor known to be present through the entire tumour life cycle. As the tumour develops it begins to activate secondary pathways such as basic FGF, TGF beta and platelet derived growth factor (PDGF). Even as these secondary pathways emerge VEGF continues to be over expressed and remains one of the critical mediators [Folkman 2005, Jain 2006, Bergers 2000, Inoue 2002]. The observation that tumours are highly dependent on VEGF throughout their life cycle is reflected in pre-clinical research where VEGF inhibition has significant anti-tumour effects throughout the tumour cycle [Gerber 2005, Shojaei 2008].

The first successful treatment of an angiogenesis-dependent tumour was in 1988 and reported in 1989 by Carl White a pulmonary specialist at the National Jewish Medical Centre in Denver, Colorado, US [Van Epps 2005]. He had contacted Folkman for advice in the treatment of a teenager who had progressive bilateral pulmonary haemangiomatosis. All treatment avenues had failed and the condition was rapidly becoming fatal. Folkman suggested a trial of low dose interferon-alpha, based on its anti-angiogenic experimental properties. The patient had a complete response and was still alive 18 years later [Folkman
2007]. Some years later in 2002 at a keynote lecture to the American Society of Law, Folkman expressed his gratitude to the Institutional Review Board at Boston’s Children’s Hospital for approving the use of interferon-alpha in the treatment of life threatening haemangiomas. He stated that ‘from 1990 to 2000, 83 infants were treated with 72 complete and durable regressions; 87 percent now off therapy up to 9 years. Eleven hemangiomas failed to regress, and six infants died’. Explaining that ‘there have been no deaths in the past 3 years’ (1999-2002) and that ‘prior to 1990, the mortality had been 30 to 50 percent in infants who failed conventional therapy. The addition of interferon alpha has reduced the overall mortality to less than 3 percent, and that's pretty much held up in other centres around the world’.

Eleven angiogenesis inhibitors were discovered in Folkman’s laboratory [Folkman 2007] and there are numerous new anti-angiogenic molecules continuously being developed that fall into two distinct types, firstly antibody directed towards angiogenic factors such as VEGF, e.g. Avastin (Bevacizumab, Genentech) and secondly small molecule tyrosine kinase inhibitors that bind to VEGF receptors e.g Sutent (Sunitinib, Pfizer) and Nexavar (Sorafenib, Bayer and Onyx Pharmaceuticals).

Currently there are hundreds of on going clinical trials of anti-angiogenic treatments within oncology and beyond with numerous therapeutic agents licensed for treatment. However despite important and sometimes dramatic results, the overall clinical benefits of anti-VEGF therapy are relatively modest, and not all cancer patients respond to anti-VEGF treatments, and when they do increased survival may only be measured in weeks or months. Many trials are also underway in the adjuvant setting where it is hoped that these agents may slow or prevent disease remission.
<table>
<thead>
<tr>
<th>Category</th>
<th>Drug name</th>
<th>Target/mechanism of action</th>
<th>Cancer indication</th>
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<tbody>
<tr>
<td>Monoclonal antibodies</td>
<td>Bevacizumab (Avastin)</td>
<td>VEGF</td>
<td>mCRC, NSCLC</td>
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<td>Cetuximab (Erbitux)</td>
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<td>Panitumumab (Vectibix)</td>
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<td>Trastuzumab (Herceptin)</td>
<td>EGFR, HER-2</td>
<td>Breast Cancer</td>
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<td>Small-molecule TKI</td>
<td>Erlotinib (Tarceva)</td>
<td>EGFR</td>
<td>NSCLC, Pancreatic Cancer</td>
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<td>Gefitinib (Iressa)</td>
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<td>Imatinib (Gleevec)</td>
<td>bcr/abl, c-kit, PDGFR</td>
<td>CML, GIST</td>
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<td></td>
<td>Lapatinib (Tykerb)</td>
<td>EGFR, HER-2</td>
<td>Breast Cancer</td>
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<td></td>
<td>Sorafenib (Nexavar)</td>
<td>VEGF-2, -3, PDGFR-β, Raf-1,</td>
<td>RCC</td>
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<tr>
<td></td>
<td>Sunitinib (Sutent)</td>
<td>GFR-2, PDGFR-β</td>
<td>GIST, RCC</td>
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<tr>
<td>Other agents</td>
<td>Bortezomib (Velcade)</td>
<td>Proteosome Inhibitor</td>
<td>MM, Mantle Cell Lymphoma</td>
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<td>Interferon Alfa-2b (Roferon A, Intron A)</td>
<td>Inhibits angiogenic factor expression</td>
<td>Hairy cell leukemia, Melanoma, Kaposi’s Sarcoma, CML, NHL</td>
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<td>mTOR inhibitor</td>
<td>RCC</td>
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<td>Thalidomide (Thalomid)</td>
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<td>MM</td>
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<td></td>
<td>Lenalidomide (Revlamid)</td>
<td>Unknown</td>
<td>MM</td>
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There is no doubt however that angiogenesis is of paramount importance in the development of cancer and is an accepted target for anti-cancer therapy thanks to the dedicated, meticulous and persistent nature of Folkman and his colleagues who overcame the scepticism of the scientific community towards the fledgling study of angiogenesis in the 1960s and 1970s.
Moses Judah Folkman died in 2008, having given many keynote lectures, publishing 463 scientific papers and numerous book chapters, and being highly decorated and recognised for his achievements. He has also inspired a generation of clinicians and researchers around the globe to believe in their theories, and strive to make that break-through against the odds. To enable quantitative assessment of the potential effects of novel anti-angiogenic therapies uncomplicated surrogate markers of angiogenesis are of paramount importance.

1.1.6.1 Measurement of circulating markers of angiogenesis

The most commonly used surrogate markers of anti-angiogenic effect are soluble angiogenic factors in serum, plasma and urine [Davis 2008]. These include growth factors and cytokines, endothelial cell surface molecules and circulating endothelial and endothelial precursor cells [Brown 2008].

There are numerous growth factors and cytokines that may be measured as markers, which are involved in the complex milieu surrounding angiogenesis. VEGF, FGF, Hepatocyte Growth Factor (HGF) and EGF have all been associated with stage, progression and prognosis in various malignancies including carcinomas of the colon, stomach, pancreas, breast, prostate and lung [Secord 2004, Braybrooke 2000, Chan 2004, Coskun 2003, Kaya 2004, Krzystek-Korpacka 2007, Li 2004, Poon 2001, 2003, 2004, Shariat 2004, Sliutz 1995, Tamura 2004, Kuroi 2001, Dvorak 2002]. Of these mediators VEGF is thought to play the most important role in angiogenesis and has been measured most frequently [Davis 2008]. VEGF levels have been shown to increase in relation to disease progression in a study looking at the effect of Razoxane, an anti-angiogenic topoisomerase II inhibitor, in renal cell carcinoma [Braybrooke 2000]. VEGF and MMPs have been shown to be strong predictors of 1-year progression free survival [Chan 2004]. MMPs in their own right have been
investigated as biomarkers of angiogenesis as have their regulating protein
tissue inhibitor metalloproteinases (TIMPs) [Liotta 1990, Gomez 1997, Levitt
2001]. Other investigated cytokines include angiopoietins (ANG) and cellular
adhesion molecules – E-selectin, VCAM-1.

Specific circulating markers of angiogenesis will be discussed in more
detail in the results chapter corresponding to each measured cytokine.

1.1.6.2 Radiological biomarkers of angiogenesis

The use of radiological imaging techniques to assess tumour micro-
environment in response to treatment is advantageous as the methods are non-
invasive and serially repeatable without disrupting tumour tissue [Brown 2008].

Radiological techniques utilised for measurement of anti-angiogenic
therapy include positron emission tomography (PET), dynamic contrast
enhanced magnetic resonance imaging (DCE-MRI), functional computed
Dynamic contrast enhanced-MRI involves acquisition of images before, during
and after rapid delivery of a contrast agent. It has been used in many clinical
trails to assess tumour vasculature in response to radiation and anti-angiogenic
therapy [Brown 2008, Morgan 2008, 201, 211, 212]. DCE-MRI has shown
decreases in tumour enhancement and thus vascular permeability and flow within
2 days of anti-angiogenic treatment [Morgan 2003, Thomas 2005].

The use of MRI techniques in assessment of tumour micro-environment
will be discussed in more detail in the results chapter corresponding to each
utilised method.
**1.2.0 Colorectal Cancer**

Fundamental advances are taking place in the understanding of colorectal cancer development, patient risk stratification, diagnosis and management. Over the past decade numerous new chemotherapeutic and biological agents such as oxaloplatin, irinotecan, bevacixumab and cetuximab have increased our armoury in the medical fight against colorectal cancer [Sanoff 2008; Chu 2008]. This along with improvements in surgical resection, radiation therapy and liver directed therapies such as local tumour ablative techniques should inevitably lead to improved prognosis [Dragovich 2010].

**1.2.1 Pathophysiology of Colorectal Cancer**

More than 90% of bowel cancers are adenocarcinomas, with the majority arising from adenomatous polyps. These common benign tumours develop from normal colonic mucosa and are present in about 1/3 of European and US populations [Midgley 1999].

Flat adenomas account for 10% of all lesions and not only are these more difficult to detect they have a greater propensity to malignant change [Hardy, 2000; O’Brien 2000]. Only a small proportion of polyps (1-10%) develop into invasive bowel cancer. [Schofield 2000]

Genetically, colorectal cancer represents a complex disease, and genetic alterations are often associated with progression from premalignant lesions (adenoma) to invasive adenocarcinoma.

Vogelstein characterised the sequence of genetic and molecular events that lead to transformation from normal colonic mucosa to adenomatous polyps.
and then to malignant neoplasms [Vogelstein 1988].

It is well reported that the early molecular event in the colorectal cancer development pathway is the adenomatous polyposis gene (APC) mutation. APC protein encoding leads to activation of oncogenes cyclin D1 and c-myc, which drives the progression to malignant phenotype [Kinzler 2002]. The APC gene was first discovered in familial adenomatous polyposis coli, a rare hereditary syndrome, which accounts for 1% of colorectal cancer cases [Goss 2000; Kinzler 2002; Lynch 2008].

Malignant transformation also occurs due to epigenetic events such as DNA methylation, which may cause activation of oncogenes and silencing of tumour suppressor genes. This includes the KRAS oncogene [Bos 1987; Downward 1998] and chromosome 18 loss of heterozygosity [Watanabe 2001] that subsequently leads to inactivation of SMAD4 and SMAD2 [Thiagalingam 1996; Takagi 1996].

Chromosome arm 17p deletion and mutations affecting p53 tumour suppressor gene confers resistance to apoptosis, which is thought to be a late event in colorectal carcinogenesis [Baker 1989; Baker 1990; Malkin 1990].

Mutations of genes such as MutS Homolog 2 (MSH2), MutL Homolog 1 (MLH1), and postmeiotic increased 2 (PMS2) are associated with deficiencies in DNA mismatch repair. These mutations result in high-frequency microsatellite instability (H-MSI). H-MSI is a hallmark of hereditary non-polyposis colon cancer syndrome (Lynch syndrome), which accounts for about 6% of all colon cancers. However, H-MSI is also found in about 20% of sporadic colon cancers [Leach 1993; Ionov 1993; Fishel 1993; Papadopoulos 1994; Bronner 1994; Lynch 2008; Boland 2008]
1.2.2 Incidence of Colorectal Cancer

Colorectal cancer (ICD9 153-154 and ICD10 C18-21) is a disease predominantly of the developed world with almost 60% of all colorectal cancers occurring in more developed regions. This is related to cultural practices and western diets with high dietary fat and low fibre. This combined with sedentary lifestyles has been shown in population-based studies to account for this discrepancy between developed and developing countries [Willillett 1990; Giovannucci 1996; Boyle 2000]. Epidemiological studies show a rapid increase in risk from colorectal cancer in migrants moving from low to high risk countries [Boyle 2000] and the rates for second generation migrants can be double that of first [Flood 2000]

Worldwide an estimated 1.23 million new cases of colorectal cancer were diagnosed in 2008 [Ferlay 2010]. The incidence in Europe is increasing
particularly in Southern and Eastern Europe where rates were originally much lower than Western Europe [Coleman 1993, Bray 2004]

![Figure 1.9: 2008 estimated age-standardised (World) incidence rates for colorectal cancer by sex and world regions. From Ferlay 2010.](image)

There are around 106 new case of colorectal cancer diagnosed in the UK each day. In 2007 there were 38,608 new cases registered in the UK with 24,274 in the colon and 14,334 in the rectum. [Office for National Statistics 2010, ISD Online 2010, Welsh Cancer Intelligence and Surveillance Unit 2010, Northern Ireland Cancer Registry 2010]

The occurrence is strongly related to age with 84% of cases arising in people over the age of 60 years. Until the age of 50 men and women have a similar incidence of bowel cancer but as age increase the incidence in men compared to women giving an overall male to female ratio of 6% [Office for
It is the third most common malignancy in the UK. Using the UK incidence and mortality data for 2001-2005 the lifetime risk of developing colorectal adenocarcinoma is 1 in 16 for men and 1 in 20 for women [Cancer Research UK 2009]. This makes it the second most common cancer in females after breast cancer and the third most common in men after prostate and lung cancer in the UK. Between 1979 and 1999 incidence rates in males increased by 1% each year, however since there has been a slight decrease. Rates in women have been relatively static [Cancer Research UK 2009].

The prevalence is high due to survival rates, which have doubled over the past 30 years. An estimated 250,000 people are alive in the UK having received a diagnosis of colorectal cancer [Cancer Research UK 2009].
The development of colorectal adenocarcinoma has been largely associated with socioeconomic status and the incidence is 11% higher in the most deprived groups compared to affluent groups [Boyle 2000].

**1.2.3 Mortality Rates from Colorectal Cancer**

In 2008 there were 16,259 deaths from colorectal cancer, which makes it the second most common cause of death from cancer in the UK after lung cancer. Over 80% of deaths occurred in people over the age of 65 and approximately 40% in over 80 year-olds [Office for National Statistics 2010, ISD Online 2010, Welsh Cancer Intelligence and Surveillance Unit 2010, Northern Ireland Cancer Registry 2010].
Figure 1.12: Number of colorectal cancer deaths and age specific mortality rates by sex in the UK 2008. From CRUK.

Despite incidence increasing between 1970 and 1999 mortality rates have been falling

Figure 1.13: Age standardised mortality rates for colorectal cancer in the UK from 1971 - 2008. CRUK

Between 1999 and 2008, the bowel cancer age-standardised mortality rates in the UK fell by 13% [Cancer Research UK 2009].
1.2.4 Survival

The five-year survival rates for both male and female colon and rectal cancer have doubled between the early 1970s and mid 2000s [Coleman 1999; Office for National Statistics 2007; Rachet 2009; Richard 2009]

Over this period five-year survival rates for male colon cancer rose from 25% to 50% and for females from 23% to 51%. For rectal cancer, male rates rose from 25% to 51% and from 27% to 55% in women. Ten-year survival rates are only slightly lower than five-year rates suggesting that patients who survive for five years are cured from the disease [Coleman 1999; Rachet 2009; Richard 2009].
Survival is directly related to stage of disease at presentation. Over 93% of patients with Dukes A survived 5 years compared with only 7% of patients with Dukes D [Woodman 2001; National Cancer Intelligence Network 2009]. There is also an increase in survival of between 5% and 9% in affluent groups compared to deprived groups. [Coleman 2004]

1.2.5 Colorectal liver metastases

Colorectal cancer principally metastasises to loco-regional lymph nodes and via the portal venous system to the liver. It is recognized that surgical resection of isolated hepatic metastases from colorectal cancer may be curative [Wiess 1986, Fong 1997]

Of new cases of colorectal cancer, 20-25% will have clinically detectable liver metastases at diagnosis and approximately another 40% of patients will develop liver metastases after resection of the primary colonic tumour [Hughes 1986; Scheele 1990; Sugarbaker 1990; Stangle 1994; Scheele 1995]. This most commonly occurs in the first 3 years of follow-up and it has been reported that
38% of patients who die from colorectal adenocarcinoma have liver metastases as the only site of distant spread [Scheele 1990; Sugarbaker 1990; Stangl 1994; Scheele 1995; Fong 1997]. However only 20-30% of patient's with liver involvement are suitable for hepatic resection [Stangl 1994; Geoghegan 1999].

Without resection, survival for patients with hepatic metastases at five years is poor [Goslin 1982] with overall median survival for patients with liver metastases being less than eight months [Lahr 1983]. Prognosis is improved in patients with isolated metastases and those with disease confined to one lobe [Lahr 1983; Stangl 1994; Scheele 1995; Hughes 1998].

With hepatic resection large published case series have suggested that five-year survival ranges from 25% to 44% with an operative mortality of 0 to 6.6% [Cady 1991; Scheele 1995; Nordlinger 1996; Fong 1999; Choti 1999]. The series by Fong from Memorial Sloan-Kettering Cancer Centre and the multicentre series from Nordlinger in France also suggest an aggressive surgical approach is acceptable with extensive resections being performed [Nordlinger 1996; Fong 1999].

Recurrence may occur in up to 60% of patients following liver resection. Of these, 90% are detected in the first two years following liver resection [Wanebo 1996; Topal 2003].

Patients who undergo hepatic resection generally die from recurrence of the disease, however chemotherapy has been shown to improve survival in both the group who are amenable to repeat hepatic resection and patients who are not [Simmonds 2000; Jonker 2000; Nordlinger 2007]. Despite this, the search for
other agents to augment the success of best surgical management and current recommended chemotherapeutic regimes continues.

Recent advances in chemotherapy have improved survival, but stage IV disease is usually incurable (Libutti 2008; Compton 2008).

1.2.6 Angiogenesis in Colorectal Cancer

In colorectal cancer, increased VEGF expression correlates with invasiveness, vascular density, metastases, recurrence and prognosis [Kabbinavar 2003]. VEGF is expressed early in the progression of colorectal cancer [Kabbinavar 2003] and is more highly expressed in tumours than distant metastases [Kuniyasu 2000].

Because of the well-defined steps in the development of colorectal adenocarcinoma it has been possible to study the effects of angiogenesis throughout the tumour development pathway [Takahashi 2003] in an effort to try and delineate at which point the ‘angiogenic-switch’ occurs to allow neovascularisation. Neovascularisation occurs when the ‘switch’ is turned on at a point when angiogenesis activators (VEGF in particular) outweigh inhibitors [Bergers 2003].

Preclinical models have also confirmed the importance of VEGF and angiogenesis in the progression of colorectal adenocarcinoma. Kondo and colleagues demonstrated that VEGF-expressing tumours had increased vascularity and amplified metastatic potential in colorectal cancer cell lines compared with control tumours in which VEGF was not up-regulated [Kondo 2000].
Results from Takahashi and colleagues suggest the angiogenic switch occurs early in the development of colorectal adenocarcinoma and that VEGF plays a vital role. They evaluated the intensity of VEGF staining in resected adenomas, mucosal in-situ (Tis) cancers and T1 and T2 cancers VEGF levels were elevated in all specimens and stages of disease but there was a significantly higher level in T1 samples compared to Tis tumours [Takahashi 2003].

Hanrahan investigated the gene expression of various VEGF ligands and receptors by colorectal carcinomas correlated tumour stage. They showed that VEGF-A was the most predominant and commonly expressed of the VEGF ligands. They also observed that VEGF-A and VEGF-B were the most abundant ligands in adenomas and importantly that not only VEGF-A but also VEGF-C was increased within carcinomas [Hanrahan 2003].
Combing the results from Kondo, Takahashi and Hanrahan allows us to hypothesize that the angiogenic switch occurs early in the course of colorectal cancer development and progression, and that VEGF is associated with initiation and maintenance of angiogenesis in colorectal cancer.

Angiogenesis and VEGF may also play a role in reducing tumour cell apoptosis. Aotake and colleagues observed that there was a significant correlation between tumour cell apoptosis and angiogenesis. They demonstrated that there was an inverse relationship between microvessel density and apoptotic index [Aotake 1999].

Evidence from various studies suggests that the VEGF ligand and angiogenesis are prognostic markers in colorectal cancer. VEGF over expression in patients with colorectal carcinoma appears to be correlated with poor prognosis and overall survival [Tanigawa 1997; Ishigami 1998; Des Guetz 2006; Kuramochi 2006; Ottaiano 2006; Saad 2006].
Disease-free survival and overall survival is affected by lymph node involvement. Saad and colleagues investigated VEGF expression and correlated it with lymph node metastases in 90 patients. They found a significant correlation between increased VEGF expression and lymph node metastases [Saad 2006]. A significant correlation has also been reported by Ottaiano [Ottaiano 2006].

In a meta-analysis performed by Des Guetz, VEGF ligand overexpression in colorectal cancer was strongly predictive for poor relapse-free survival, with a risk ratio of 2.84 [Des Guetz 2006].

This is backed up by work from Tanigawa who observed that microvessel counts were directly related to haematogenous spread of colorectal liver metastases [Tanigawa 1997].

Figure 1.18: Bar chart showing the percentage of patients with colorectal metastases at varying vessel counts. From Tanigawa 1997.
Similarly, Ishigami and colleagues showed that VEGF ligand over expression was significantly correlated with depth of tumour infiltration ($p = 0.046$), liver metastases ($p < 0.0001$) and lymph node metastases ($p = 0.036$), concluding that VEGF is associated with progression, invasion and metastasis of colorectal cancer [Ishigami 1998].

In the same 1998 paper Ishigami also suggested a strong correlation between VEGF ligand expression and mortality. They demonstrated that patients with a higher ratio of VEGF mRNA in tumour tissue compared to non-tumour tissue had a significantly poorer survival [Ishigami 1998].

![Figure 1.19: Comparison of survival after surgery for colorectal cancer in patient's with high level VEGF expression (VEGF T/N ratio >4.8) versus patients with low level VEGF expression (VEGF T/N ratio <4.8). From Ishigami 1998](image)

This relationship to survival was also shown in the meta-analysis performed by Des Guetz. It demonstrated a risk ratio of 1.65 (95% CI=1.27-2.14; $p < 0.001$) [Des Guetz 2006].

This avenue of research was furthered by Kuramochi. His group demonstrated that patients with higher metastatic disease burden (multiple versus solitary liver metastasis) had significantly higher levels of VEGF...
messenger ribonucleic acid (mRNA) in both the primary tumour and corresponding liver metastases [Kuramochi 2006].

Figure 1.20: Comparison of VEGF mRNA levels in patients with solitary liver metastases compared to multiple liver metastases. From Kuramochi 2006.

Recently it has been suggested that VEGF levels predict response to conventional colorectal cancer systemic chemotherapies or local radiotherapies [Zlobec 2005; Ogata 2006]. Zlobec used pre-radiation tumour biopsies to determine VEGF expression and correlated this to subsequent neoadjuvant radiotherapy response in rectal carcinoma. VEGF over-expression correlated significantly with lack of response to preoperative radiotherapy [Zlobec 2005].

Many other pro-angiogenic molecules have been found to have an association with colorectal cancer. These include FGF [New 1992], PDGF [Anzano 1989], PDGF-R [Craven 1995; Takahashi 1996], ANG-2 [Ahmad 2001], interleukins (IL) 8 [Brew 1996] and 15 [Kuniyasu 2003] and cyclooxygenases (COX) [Tsujii 1998]. EGF is also shown to have a putative role. Treatment of colorectal cancers with EGF-R inhibitor leads to reduced VEGF expression and tumour microvessel counts [Ciardiello 2000 and 2001].
Though surgery remains the definitive treatment modality for colorectal cancer the search for new agents continues to improve cure rates for patients with early stage disease and prolonged survival for those with advanced disease. Further advances are likely to come from the development of new-targeted agents and integration of these with current treatments. One such naturally occurring compound that has been investigated extensively in laboratory-based studies is fish oil (the omega-3 components principally).
1.3.0 Omega-3 fatty acids

Fatty acids (FAs) are a diverse group of molecules. The fatty acyl structure represents the major building block of complex lipids and FAs should be regarded as one of the most fundamental categories of biological lipids [Ratnayake 2009].

<table>
<thead>
<tr>
<th>Category</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td>oleic acid</td>
</tr>
<tr>
<td>Glycerolipids</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>Glycerophospholipids</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>Sphingolipids</td>
<td>sphingosine</td>
</tr>
<tr>
<td>Sterol lipids</td>
<td>cholesterol</td>
</tr>
<tr>
<td>Prenol lipids</td>
<td>farnesol</td>
</tr>
<tr>
<td>Saccharolipids</td>
<td>UDP-3-O-(3-hydroxy-tetradecanoyl)-N-acetylglucosamine</td>
</tr>
<tr>
<td>Polyketides</td>
<td>aflatoxin</td>
</tr>
</tbody>
</table>

Table 1.3: Lipid categories and typical examples. Adapted from Fahy et al. [2005].

Fatty acids are key nutrients that affect early growth and development, as well as chronic disease in later life. The benefits and potential risks of FAs go well beyond their defined role as fuel [Burlingame 2009].

A FA containing more than one carbon double bond it is described as a “polyunsaturated fatty acid” (PUFA). The most important families in human metabolism are omega-6 (n-6) and omega-3 (n-3) PUFAs. Specific n-6 and n-3 PUFAs are essential nutrients, while the eicosanoids and docosanoids they produce following metabolism have distinct biological activities affecting the prevalence and severity of cardiovascular disease, diabetes, inflammation, cancer and age-related functional decline [Burlingame 2009, Ratnayake 2009].
Important n-3 PUFAs involved in human nutrition are α-linolenic acid (ALA or 18:3n-3), eicosapentaenoic acid (EPA or 20:5n-3) docosapentaenoic acid (n-3 DPA or 20:5n-3) and docosahexaenoic acid (DHA or 22:6n-3).

ALA is the parent FA of the n-3 PUFA family. ALA is mainly found in the plant kingdom with high concentrations in flaxseed oil and perilla oil. It is also found in canola oil, soybean oil and vegetable oils from where humans derive it in their diet. The human body is unable to readily synthesize ALA, which makes ALA, like linoleic acid (LA or 18:2n-6), the parent of the n-6 PUFA family, an “essential fatty acid” [Ratnayake 2009].

LA and ALA are converted to their respective n-6 and n-3 PUFA families by a series of independent reactions. However both pathways require the same enzymes for desaturation and elongation. This leads to competition between n-6 and n-3 PUFA for this metabolic conversion. The first step in the pathway requires Δ6 Desaturase [Moore 1995, Sprecher 2002] which has a higher affinity for ALA than LA [Rose 1999] but due to the typically higher intake and concentration of LA [Burdge 2005, Lands 2008] there is greater conversion of n-6 PUFA producing the predominant product of the n-6 pathway, Arachadonic acid (AA or 20:4n-6) [Ackman 2008, Ratnayake 2009]. Thus the capacity of human metabolism to derive EPA and DHA by the desaturation of ALA is negligible [Ratnayake 2009]). The efficiency of conversion is particularly poor in relation to DHA [Li 1999, Burdge 2005, Brenna 2008]. Therefore the concentration of EPA and DHA in tissues is enhanced when they are directly ingested either in oily fish or as a fish oil (FO) supplement [Brenna 2002] or when competing amounts of n-6 PUFAs are relatively small [Sinclair 2002, Goyens 2006].
The n-3 PUFAs are synthesised in abundance by marine lipids in algae and plankton. By consuming these small organisms, fish build up large concentrations of n-3 PUFA in their tissues and therefore are the main dietary source of essential n-3 PUFAs in humans. In particular cold-water oily fish such as mackerel, salmon, herring, anchovies, sardines and smelt provide relatively large amounts of EPA and DHA [Ackman 2008].

1.3.1 Physiological effects of omega-6 and omega-3 polyunsaturated fatty acids

The n-6 and n-3 PUFAs have a number of vital functions in the human body [Eberhart 1995, Corridan 1998]. As structural phospholipids in the cell membrane, they modulate cellular signaling, cellular interaction and membrane fluidity [Roynette 2004].
They regulate the immune system by acting as precursors for eicosanoids which are potent immunoregulatory metabolites. Eicosanoids are synthesised from the n-6 PUFA arachadonic acid (AA, 20:4n-6) and the n-3 PUFA, EPA. Arachadonic acid and EPA are metabolised by cyclooxygenase (COX) or lipoxygenase (LOX) enzymes into immunoregulatory metabolites prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) [Roynette 2004]. As cell membrane phospholipids generally contain significantly higher levels of AA than EPA (Yaqoob 2000), AA is the most common eicosanoid precursor and gives rise to 2-series PGs and TXs and 4-series LTs. EPA gives rise to 3-series PGs and TXs, 5-series LTs and E-series resolvins [Li 2002, Serhan 2002, Roynette 2004].

![Diagram of eicosanoid metabolism]

Figure 1.22: The wide range of compounds formed from metabolism of omega-3 and omega-6 fatty acids and their general effects. From Gleissman 2010.

DHA is a poor substrate for COX and LOX and it was thought that DHA did not produce bioactive COX and LOX mediators. However, Serhan and others

AA and EPA also compete for the COX and LOX enzymes. Again, n-3 PUFAs are preferentially used, so supplementation with n-3 PUFAs will have a considerable impact on the production of eicosanoids and docosanoids. Thus, increased intake of n-3 PUFAs results in decreased generation of AA-derived eicosanoids and increased EPA derived eicosanoids and DHA docosanoids [Sperling 1993, Caughey 1996, Calder 2006].

It is considered that the eicosanoids and docosanoids produced from EPA and DHA have less biological activity and are less pro-inflammatory in their action than the potent pro-inflammatory AA-derived mediators [Calder 2002, Roynette 2004, Lee 2008] and it also suggested they have anti-inflammatory properties [Serhan 2002, Bazan 2007, Lee 2008].


1.3.2 The importance of n-6 to n-3 polyunsaturated fatty acid ratio

The balance of eicosanoid and docosanoid production from the n-6 PUFA, AA and the n-3 PUFAs, EPA and DHA is dependent upon n-3 PUFA
levels in tissues. It is generally accepted that increasing n-3 PUFA concentration or decreasing n-6 PUFA levels has beneficial health effects on inflammatory diseases, coronary artery disease, metabolic disease and cancer.

It is suggested that this is over simplified and that it is the balance between n-6 and n-3 PUFA rather than the individual amount of each class of PUFA, which may influence health outcomes [Chajes 2003].

This can be traced back to the diet of our ancestors were the ratio of n-6:n-3 PUFA was 2/3:1, due to higher intake of EPA and DHA [Simopoulos 2002]. The ratio in today’s Western society ranges from 10:1 to 20:1 indicating that Western diets are depleted in n-3 PUFAs [Simopoulos 2001]. This high ratio is the consequence of excessive production of vegetable oils, increased intake of processed food, red meats and decreased intake of oily fish. The substitution of saturated fat and butter with oils high in omega-6 PUFA to lower serum cholesterol also affects the ratio [Simopoulos 2001, Simopoulos 2002]. Food producers have realised this and also understand the beneficial effects of n-3 PUFAs. This can be seen in the increased number of products on our supermarket shelves containing increased levels of n-3 PUFAs.

The benefits of n-3 PUFAs and their ratio with n-6 PUFAs was discovered by the fathers of n-3 PUFA research Dyerberg and Bang in the early 1970s from their studies into the diet, blood lipid profile and disease incidence rates in Inuits from Greenland [Bang 1971, 1972, 1976 & 1980, Dyerberg 1975 & 1978]. Their initial research focused on how Inuit’s who consumed diets high in fat, conversely had low rates of cardiovascular disease. It was discovered that their diets were high in marine fish, which contained DHA and EPA and that they had beneficial n-6:n-3 PUFA ratios of 2:1.
Studies have shown that n-6 AA derived eicosanoids can accelerate cancer growth. AA has also been found in abundance in malignant tissue. Also enzymes responsible for the conversion of FAs to eicosanoids are up regulated in malignant tissues compared to normal [Krishnamoorthy 2008]. This is especially true for COX-2 [Koki 2002]. In addition, PGE2- and LTB4-receptors are often up regulated [Hull 2004, Massoumi 2007]. However, not only is the amount of AA higher in cancer tissue, but the concentration of the DHA and EPA is also lower compared to the corresponding normal tissue [Martin 1996, Kokoglu 1998, Reynolds 2001]. The accelerated cancer growth could therefore be an effect of both increased n-6 levels and decreased n-3 levels leading to a detrimental n-6:n-3 ratio [Gleissman 2010].

1.3.3 The role of polyunsaturated fatty acids in tumourgenesis

Hanahan and Weinberg in their landmark review ‘The Hallmarks of Cancer’ and the subsequent ‘Hallmarks of the Cancer: the next generation’ suggested that the vast catalogue of cancer cell genotypes is a manifestation of essential alterations in cell physiology that collectively dictate malignant growth (Hanahan 2000 & 2011).

The original six essential alterations described are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. This results in the cancerous cell having the predatory properties that allow it to survive, invade and multiply where it should not. Recently the addition of reprogramming of energy metabolism and evading immune destruction has been suggested. Each of these physiologic changes (novel capabilities acquired during tumour
development) represents the successful breaching of anticancer defence mechanisms. They proposed that these capabilities are shared in common by most and perhaps all types of human tumours and must be satisfied for tumour growth to occur within the tumour microenvironment (Hanahan 2000 & 2011).

EPA and DHA have been shown to have multiple anti-tumour actions that affect all of the original six essential alterations that dictate malignant growth [Stephenson 2013]. This is a result of various pathways including inhibition of AA metabolism and independent effects on various cytokines involved in tumourgenesis. Also n-6 PUFA derived eicosanoids have potential promoting effects in cancer cell growth [Abou-el-Ela 1989, Rose1990], angiogenesis [Rose 1990] and invasion [Brown 2006].

1.3.4 Effects of omega-3 polyunsaturated fatty acids on sustained angiogenesis

As previously discussed, for a tumour to grow beyond 2 mm, angiogenesis and neovascular formation is required. The ability to induce and sustain angiogenesis from vascular quiescence is controlled by the ‘angiogenic switch’. Tumours appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors (Hanahan 1996). This is seen with increased production, expression and signal transduction of pro-angiogenic factors such as VEGF. The n-3 PUFAs have been shown to have a profound effect on angiogenesis [Spencer 2009].

1.3.4.1 In vitro evidence

Omega-3 PUFAs have been shown to decrease sprouting angiogenesis by suppressing VEGF-stimulated endothelial cell proliferation, migration and tube formation [Tsuzuki 2007, Tsuji 2003, Yang 1998]. Tsuzuki and colleagues
treated human umbilical vein endothelial cells with conjugated EPA and demonstrated a reduction in sprouting angiogenesis tube formation and endothelial cell migration [Tszuki 2007] was also seen in bovine aortic endothelial cells pre-treated with DPA. VEGF-R2 expression was also found to be suppressed [Tsuji 2003]. The reduction in endothelial cell proliferation in response to EPA was shown to be dose dependent in bovine carotid artery endothelial cells [Yang 1998]. The study by Yang also elicited a dose dependent decrease in VEGFR-1 (FlK-1) expression [126]. A reduction in VEGF/VEGF-R binding has also been demonstrated by Yuan using an n-3 PUFA rich shark oil [Yuan 2006]

The n-3 PUFAs also have stark effects on numerous other mediators involved in angiogenesis. PDGFs play an important role in angiogenesis by stimulating fibroblast and vascular smooth muscle cell motility and acting as a chemo-attractant [Ross 1974, Heldin 1999]. As early as 1988 Fox and DiCorleto demonstrated that in vitro production of PDGF was inhibited by n-3 PUFAs [Fox 1988]. Investigating the effects of EPA and DHA on PDGF signal transduction Terano and colleagues demonstrated that EPA inhibited PDGF was able to bind to its receptor and suppressed c-fos mRNA expression, a gene involved in receptor signal transduction. These effects lead to inhibition of smooth muscle proliferation a prerequisite for angiogenesis [Terano 1996].

As previously discussed PGE2 is formed from AA, catalysed by COX-2. There is well-defined link between E series prostaglandins and carcinogenesis [Stein-Werblowski 1974]. Decreased levels of VEGF, COX-2 and PGE2 have been demonstrated in HT-29 colon cancer cell lines when cultured in vitro with EPA and DHA [Calviello 2004] and an synergistic inhibitory effect of n-3 PUFAs
and COX-2 inhibitors on growth of human colon cancer cell lines has been shown [Narayanan 2005, Reddy 2005].


DHA has been demonstrated to inhibit NO production and iNOS expression in murine macrophages [Ohata 1997, Komatsu 2003, Jeyarajah 1999, Boutard 1994] and down-regulate NO and nuclear factor kappa beta (NFKB) in human colon cancer cell lines [Narayanan 2003].

In the study previously discussed by Tsuzuki, they also demonstrated that production of matrix metalloproteinase’s (MMP) 2 and 9 - proteases which play a role in basement membrane proteolysis in the 3rd stage of sprouting angiogenesis in human endothelial cells was inhibited by EPA [Tsuzuki 2007].

It has also been demonstrated that DHA inhibits Beta-catenin, a transcriptional regulator of angiogenesis production in colon cancer cells [Narayanan 2004]

1.3.4.2 In vivo evidence

In a study where Fischer 344 rats were implanted with fibrosarcomas, the group with diets supplemented with EPA had tumours with significantly lower tumour volume and decreased VEGF-alpha mRNA levels [Tevar 2002].
A study in nude mice supplemented with n-3 PUFA undergoing implantation of human colorectal carcinomas showed that tumour expression of VEGF, COX-2 and PGE2 was decreased compared to control [Calviello 2004]. Benefits were also seen in nude mice transplanted with breast carcinoma. Breast tumours in mice feed diets high in EPA and DHA had lower tumour microvessel density and VEGF levels compared to controls [Rose 1999, Mukutmoni-Norris 2000].

Induction of vascular smooth muscle cell migration by PDGF, required for angiogenesis is inhibited by EPA and DHA in vivo [Terano 1996]. Several other small animal models have demonstrated that n-3 PUFA enriched diets inhibit COX-2 and PGE2 production [Bommareddy 2006] and reduced HT-29 colon cancer cell tumour growth and microvessel density after implantation into nude mice [Calviello 2004].

Factors such as PGE2, NO, COX-2 and NFkB have well-documented roles in both the inflammatory and angiogenic cascades with significant cross-relation in both pathways. This demonstrates the potential for n-3 FAs as anti-angiogenic agents via inhibition of these factors and others including VEGF and PDGF.
There is a large and growing body of evidence from laboratory-based studies that n-3 PUFAs have a marked beneficial effect on the hallmarks of cancer. However do these mechanistic studies translate into a clinical benefit?

1.3.5 Effects of omega-3 polyunsaturated fatty acids on tumourgenesis in humans

As well as the cellular mechanisms described in the in-vitro and -vivo studies above, epidemiological observations also appeared to suggest a benefit of n-3 PUFA in cancer prevention in humans. An example is an observational study in Inuit’s. Inuit’s have DHA levels several fold higher than Caucasians [Lucas 2004] which has demonstrated significantly lower levels of childhood cancer occurrence, particularly neuroblastoma (tenfold decrease), and Hodgkin lymphoma [Gleissman 2010, Lanier 2003]

However, a systematic review of the ‘Effects of Omega-3 Fatty Acids on Cancer Risk’ by MacLean et al reviewed 38 articles published from 1966 to 2005 which included 65 estimates of association calculated over 20 differing cohorts
for 11 different cancer types, concluded that only 10 were statistically significant and that the body of literature does not provide evidence to suggest a significant association between n-3 PUFA and cancer incidence. They also stated that dietary supplementation with n-3 PUFAs is unlikely to prevent cancer [MacLean 2006a]. Chen et al raised concerns with the systematic review [Chen 2006]. The studies included in this systematic review did not formally measure FA consumption. Food frequency questionnaires and dietary records were used which correlate poorly with direct PUFA measurement [Hunter 1992] or differentiate between the source of FO consumption; farm raised fish are poor sources of n-3 PUFAs [Chen 2006].

Animal data, some of which has been discussed above, is an invaluable tool for mechanistic studies and the models can closely mimic the clinical course of cancer progressions [Chen 2006, Ward 2004, Maddison]. However the translation of animal data into the clinical arena is difficult due to the higher amounts of n-3 PUFAs used in relation to fat intake and percentage weight [MacLean 2006b]. Inherent to the majority of animal studies is the use of high levels of dietary constituents [Dommels 2002] with n-3 PUFA intake between 18-48% of daily energy compared to 4-10% in human population based studies [Zock 1998]. This is likely to be one of the reasons that only weak associations of PUFA intake and cancer are found in population-based studies [Dommels 2002]. Extrapolation of findings is also confounded by poor descriptions of experimental conditions and, dose and purity of n-3 PUFA supplementation [MacLean 2005]

However, a role is potentially being developed for n-3 PUFA in combination with current chemotherapeutic agents to augment their action [Pardini 2006]. Animal models have shown that the efficiency of doxorubicin
[Hardman 2001] and mitomycin C [Shao 1995] in inhibiting tumour growth and the inhibitory effect of tamoxifen in estrogen-dependent xenografts [Chen 2006] are enhanced when combined with n-3 PUFA-enriched diets.

DHA supplementation with concurrent cytotoxic drug treatment is a potential approach to enable the clinical use of DHA in cancer treatment. DHA in combination with doxorubicin, irinotecan, cisplatin, melphalan, and vincristine on neuroblastoma cell survival shows additive or synergistic interactions [Lindskog 2006, Gleissman 2010].

A therapeutic study in breast cancer patients where DHA was combined with the chemotherapeutic drugs epirubicine, cyclophosphamide, and 5-fluorouracil showed delayed time to tumour progression and longer overall survival. However, these findings were only observed when patients were stratified into 2 groups of either high or low incorporation of DHA into plasma and erythrocytes. Patients who had high incorporation of DHA into plasma and erythrocytes had a positive response compared to those whom had low level DHA incorporation [Bougnoux 2009]. This observation correlates well with other studies showing that DHA incorporation differs between individuals due to different rates of metabolism, enzymatic activity, background diet, age, and sex [Rusca 2009, Childs 2008, Arterburn 2006]. This is likely to be a recurrent problem in studies using oral n-3 PUFA supplementation.

There are currently several ongoing clinical trials to assess this, where n-3 PUFAs are being tested for cancer prevention, support, or therapy [Berquin 2008] but initial evidence suggests that researchers do not seem to be translating the profoundly beneficial results seen in the laboratory to the bedside. This is potentially due to the way in which n-3 PUFAs are being supplemented and we
need to think about novel ways of overcoming the difficulties faced with FO supplementation to assess the true benefit of n-3 PUFAs in the fight against cancer.

1.3.6 Supplementation of n-3 polyunsaturated fatty acids

The evolution of dietary habits in the western world has lead to dramatic decreases in consumption of n-3 PUFAs and significantly increased consumption of n-6 PUFAs. As a result Western populations show an imbalance in the n-6:n-3 PUFA ratio with low plasma and cell phospholipid (PL) concentration of n-3 PUFAs, particularly EPA and DHA. At the same time scientific interest in the benefits of n-3 PUFA has amplified [Carpentier 2007].

To regulate and participate in cellular metabolism and exert indirect effects via eicosanoids and docosanoids, PUFAs must be incorporated into membrane PLs. It is suggested that to maintain general good health an intake of at least 2 portions of fish a week (including 1 portion of oily fish) is required [Scientific Advisory Committee on Nutrition 2004]. However, individuals not regularly consuming oily fish are unlikely to meet the recommended intake of EPA and DHA and will subsequently have low concentrations of EPA and DHA in membrane PLs [Calder 2009].

The demand for FO supplementation containing EPA and DHA has grown. There are numerous products on the market containing varying amounts of EPA and DHA. The highest strength oral preparation is currently 1.5g of EPA and 0.75g of DHA per 5ml serving (Rx omega 3 factors®). However the oral bioavailability of EPA and DHA is unknown, but modification of a persons lipid pools, cells and tissues by oral supplementation with EPA and DHA has been widely reported. Incorporation of DHA and EPA occurs in a dose response
fashion, with studies reporting near linear relationships between EPA and DHA intake and the EPA and DHA content of plasma phospholipids (PPL) [Blonk 1990, Harris 1991, Marsen 1992]. The response also depends upon which lipid profile is measured [Calder 2009]; EPA and DHA incorporation into PPLs reaches maximal concentration after 3-4 weeks supplementation [Calder 2009]. Near maximal incorporation into blood mononuclear cells occurred after 4 weeks of oral supplementation [Yaqoob 2000], after 30 days in serum cholesterol esters and between 56 – 182 days in erythrocytes [Katan 1997]. This can be problematic, because to cause a significant rise in EPA and DHA, numerous tablets or a large volume of liquid must be consumed to reach an adequate dose. A number of studies have reported potential benefits with oral supplementation of FO but patient compliance has hampered the significance of the result [Wigmore 1996 & 2000, Barber 1999a, 1999b, 2000, 2001, Burns 1999, Fearon 2003].

Cessation of FO supplementation leads to cell membrane levels of DHA and EPA returning to pre-supplementation baseline levels. Return to baseline levels occurs in a fashion where by cells which undergo rapid incorporation of EPA and DHA have a brisk return to pre-supplementation levels, but cells in which incorporation is slow resolution to baseline levels is also measured [Katan 1997, Calder 2009].

Another limiting factor in FO cell membrane incorporation is that n-3 containing triglycerides found on oral FO preparations are not efficiently hydrolysed by pancreatic lipase leading to slow intestinal absorption [Carpentier 2007]. Slow absorption and incorporation of FO to maximal levels into cell membranes after oral ingestion [Christensen 1995, Oliveira FL 1997] is adequate in use for chronic diseases but for many acute conditions such as sepsis or time limiting illness such as cancer, this delay to maximal incorporation limits the
efficacy of FO supplementation. Shorter periods of incorporation are required and can be achieved using intravenous FO supplementation.

Our knowledge of the modification of cellular mechanisms by n-3 PUFAs is predominantly derived from enteral supplementation and there is no data comparing oral and intravenous bioavailability. An increasing number of studies reporting the benefits of intravenous n-3 PUFA supplementation either within total parenteral nutrition (TPN) or as a lipid emulsion have been published.

Studies investigating the incorporation of intravenously (IV) administered n-3 PUFAs within TPN into tissue total lipids, PPL and leukocyte membrane PLs have shown rapid enrichment with maximal incorporation observed within 3-5 days [Nau 1993, Morlion 1996, Wachtler 1997, Dupont 1999] without any side effects [Morlion 1996]. Comparing this data to previously published data on incorporation post oral supplementation would suggest that IV supplementation results in maximal enrichment far quicker.

Trials are now beginning to be reported infusing n-3 PUFA lipid emulsions (Lipidem®, B Braun Melsungen and Omegaven®, Fresenius Kabi) independently from TPN. Torrinhas and colleagues infused an n-3 PUFA lipid emulsion in pre-operative patients over a six-hour period. This was found to be safe and well tolerated [Torrinhas 2010]. Simoens investigated the response of blood cell phospholipids to four repeated 5 hourly infusions of lipid emulsion containing 10% n-3 PUFA over a four-day period. Each infusion raised the EPA concentration in blood cell PLs to reach a 7-fold enrichment in platelets and a >2-fold enrichment in leukocytes was reached after 4 infusions [Simoens 2008]. A study by Carpentier infused a 50 ml n-3 PUFA enriched lipid emulsion over 5 minutes. Rapid enrichment of cells was reported with the EPA content of
leukocyte and platelet phospholipids being increased at 60 minutes and 24 hours [Carpentier 2009].

Heller demonstrated that n-3 PUFA infusion in patients with abdominal sepsis and poly-trauma decreased demand for antibiotics and shortened Intensive Care Unit (ICU) and hospital stay [55 Heller 2006]. He also showed a survival advantage in patients with severe head injuries and poly-trauma. A study of 25 patients in the ICU with sepsis by Barbosa looked at the benefits of of n-3 PUFA infusion. Inclusion of FO in TPN significantly increased plasma EPA concentration, modified inflammatory cytokine concentrations and improved gas exchange. These changes are associated with a tendency towards shorter length of hospital stay and a decrease in 28-day mortality [Barbosa 2010]. A study in acute pancreatitis reported improved gas exchange and reduced requirements for renal replacement therapy when patients received n-3 PUFA supplementation [Wang 2008]. Conversely, no differences were observed in inflammatory markers, bleeding, ventilation requirement, infection rate, ICU stay and mortality in a study of critically ill medical patients who were supplemented with n-3 PUFAs [Friesecke 2008].

Papers looking at the use of intravenous n-3 PUFA supplementation in the critically ill appear to suggest a benefit. However, definitive conclusions are difficult to reach due to the studies incorporating a low volume of heterogeneous patients with differing clinical courses. Further studies are awaited with larger numbers to show clear-cut beneficial clinical outcomes. However results of studies looking at n-3 PUFA supplementation in the peri-operative period appear more convincing.
Several studies in patients who have received an intravenous infusion of lipid emulsions containing EPA and DHA in the peri-operative period have shown that the production of AA derived eicosanoids is decreased compared to controls. It has also shown that there is up regulation of EPA-derived eicosanoids [Morlion 1996, Wachtler 1997, Koller 2003, Grimm 2006]. At a cellular level studies have shown decreased levels of pro-inflammatory cytokines TNFα [Wachtler 1997] and IL-6 [Wachtler 1997, Weiss 2002] in the n-3 PUFA supplemented groups. Clinically, studies in patients undergoing intra-abdominal surgery supplemented with n-3 PUFA have shown significant decreases in mean post-operative intensive care stay [Weiss 2002, Heller 2004, Berger 2008], requirement for mechanical ventilation [Tsekos 2004] readmission rates to the intensive care unit [Tsekos 2004, Heller 2004], infection rates, complication rates, mean total hospital stay [Koch 2005, Grimm 2006, Wichmann 2007, Weiss 2002, Tsekos 2004, Berger 2008] overall in hospital mortality [Tsekos 2004] in comparison to patients receiving no EPA or DHA. The greater benefit appeared to be derived from additional infusion of n-3 PUFA pre-surgery [Tsekos 2004]. Significantly, these studies did not report any detrimental effects of n-3 PUFA infusion. A recent meta-analysis of randomised controlled trials evaluating the safety and efficacy of FO-enriched PN in patients undergoing major abdominal surgery was carried out by Chen and colleagues. They concluded that FO-supplemented PN was safe, improved clinical outcomes, and altered the fatty acid pattern as well as leukotriene synthesis [Chen 2009].

The effect of n-3 PUFAs on immunomodulation is predominantly due to production of EPA eicosanoids and DHA docosanoids, and antagonism of AA. However, n-3 PUFAs in particular DHA and to a lesser extent EPA appear to have significant beneficial effects on cytokines involved in the systemic inflammatory cascade.
The potential benefits of n-3 PUFAs in cancer protection continue to be examined. The results from in-vivo and in-vitro studies are extremely convincing but translation into clinical practice has been difficult to prove due to the majority of studies supplementing FO orally with poor bioavailability and patient compliance. Maybe it is time to consider IV n-3 PUFA supplementation in the fight against cancer.
Chapter 2

Materials and Methods
2.1.0 Trial Materials and methods

We carried out a double-blind randomised controlled pilot clinical study to test the effect of EPA and DHA on tumour angiogenesis in humans, and assert whether the profound anti-angiogenic effects of n-3 PUFAs demonstrated in the pre-clinical arena have the potential to bear fruit in clinical medicine.

A model that had previously been utilised within the department assessing the effect of an angiogenesis inhibitor on hepatic colorectal metastases was adapted [Morgan 2003]. This study used MRI techniques as a biomarker to determine vascular change in the tumour microenvironment in response to angiogenesis inhibitor treatment in a phase II trial.

This trial had 3 distinct outcome areas.
1. Verification of n-3 PUFA uptake
   - Gas Chromotographical analysis of plasma phospholipids and, tumour and normal liver tissue
2. Effect of EPA and DHA on circulating angiogenic cytokines
   - Enzyme linked immunosorbent assay (ELISA) analysis of circulating serum pro- and anti-angiogenic cytokines
3. Effect of EPA and DHA on tumour vasculature and microenvironment
   - MRI biomarker analysis
     - DCE-MRI

2.1.1 Aims and hypothesis

The aim was to assess the effect of parenteral n-3 PUFA upon markers of angiogenesis in patients with hepatic colorectal metastases using TPN containing
either n-3 PUFA containing lipid emulsion (Lipidem®, B Braun, Melsungen) or control substance without n-3 PUFA (Lipofundin® MCT 20%, B Braun, Melsungen).

The null hypothesis for this pilot investigational study is that the administration of parenteral nutrition containing n-3 PUFA for 72 hours will not alter the outcome measures of angiogenesis detailed below in study subjects compared to controls without n-3 PUFA infusion.

2.1.2 Ethical approval

Regional ethical approval was granted (REC number: 06/Q2501/16) along with approval from the Medicines and Healthcare Products Regulatory Agency (EudraCT number: 2006-000044-71) prior to study commencement. The trial ‘Randomised Controlled Trial on the Effect of Fish Oils on Human Hepatic Colorectal Metastases’ was registered on the U.S. Institutes of Health clinical trials registry (ClinicalTrials.gov identifier NCT00942292).

2.1.3 Trail design

The study was designed as a blinded Phase II, single centre, 2-arm placebo controlled randomised trial with 10 patients in each arm (n=20). Sample size was calculated pragmatically in relation to the previously noted effect of a TKI investigated at this institution upon DCE-MRI outcomes and financial constraints. A 20% reduction in tumour vascularity, with an estimated 15% standard error of the mean was perceived to be a positive change in angiogenesis and this had been noted previously. With this in mind a sample size of 9 in each arm would be sufficient to demonstrate an alpha error of 0.05
and a power of 80%. Thus, 10 patients were enrolled into each arm to allow for a 10% drop out rate.

The study design also allowed patients to act as their own control, this is discussed later.

2.1.3.1 Trial Protocol

For full trial protocol see appendix 1. Patients were randomised to receive Nutriflex based TPN containing either Lipidem® (active substance) or Lipofundin® MCT 20% (control).

The patients followed the following trial pathway:

- Pre-trial serum lipids, full blood count, liver function tests, renal function, glucose and coagulation screen were performed on all patients and a thorough history and physical examination performed to screen for exclusion criteria. Baseline blood samples were taken for storage and subsequent analysis (secondary outcome measures).
- Patients fulfilling all inclusion criteria and who had no exclusion criteria giving written informed consent were recruited into the study.
- They were randomised to receive TPN with (Lipidem 200mg/ml, B Braun, Melsungen) or without (Lipofundin MCT 20%, B Braun, Melsungen) n-3 PUFA by sealed envelope system from random number tables.
- Patients underwent an initial MRI at this stage.
- Within the following 2 weeks, subjects were admitted to Leicester General Hospital.
- This is the normal clinical management of these patients, and staging laparoscopy and curative intent surgery was not delayed by the study for any subject.
• On the day of admission, the day before staging laparoscopy, a peripheral inserted central cannula was sited in the arm of subjects for administration of TPN.

• A second MRI was performed, comparison with the first scan permitted calculation of a rate of change of vascularity and vascular permeability prior to the intervention of the study.

• TPN will be commenced.

• The TPN bags were made up according to patient prescription at Sheffield Teaching Hospitals/Royal Free Hospital and blinded to the study and surgical teams caring for the patient, with regard to the presence or absence of n-3 PUFA. A sealed envelope with a code break informing the trial team of which product the study participant was receiving was kept in pharmacy in the event of a serious adverse reaction.

• Routine haematological and biochemical blood tests were monitored every 24 hours while the patient was receiving the TPN infusion.

• Patients were permitted only free clear fluids orally whilst on TPN to reduce the interference of diet on the PUFA uptake results.

• Participants underwent a staging laparoscopy with an intra-operative ultrasound scan, which is routinely used in staging of colorectal metastases.

• Patients remained inpatients for 48 hours after the laparoscopy, during this time they continued on the TPN regimen.

• During the admission, parallel to the trial, standard clinical pre-operative preparation and investigation proceeded as normal.

• Throughout the TPN infusion at 7 time-points, blood samples were taken for storage and subsequent analysis of secondary outcome measures (see below re timings and method of preparation).
• After 72 hours of TPN, a third and final MRI scan was performed, to allow comparison with the second scan which will provide a new rate of change in the vascular characteristics of the lesion of interest to assess the impact of the interventions.

• Post TPN trial blood samples were taken for storage and subsequent assay.

• The patients proceeded to surgical resection of metastases when this was not contra-indicated by laparoscopy findings. At the time of resection a further set of routine clinical blood tests and trial blood samples were taken. Tumour samples and normal liver tissue was taken from the excised histological specimen to allow analysis PUFA composition and histological examination of the vasculature.

• After the resection the patient exited the trial, and proceeded with standard clinical management of their disease.

Figure 2.1: Schematic of the patient trial pathway
2.1.4 MRI protocol

Patients underwent three identical MRI scan sequences (for prescriptive description see appendix 2) for trial purposes at the time-points described above.

All scans were supervised by the study investigator and performed by a MRI radiographer. The initial phase of scanning involved standard MRI sequences (T1-, T2- and VIBE- weighted) to allow tumour characterisation and localisation of the region of interest (ROI (tumour to be studied)). After the ROI was defined a T2* study and a diffusion weighted sequence was run without contrast. A DCE sequence (150 image acquisitions) was then performed with infusion of gadolinium (Gd) chelate contrast (Magnevist, Schering AG) after the first 6 image acquisitions. All scans were transferred and stored on the radiology department picture archiving and communication system (PACS). They were then transferred anonymously to a separate image analysis system in the Department of Cancer Imaging at the University of Leicester to allow formal data generation and statistical analysis.

2.1.5 Preparation and storage of blood samples

During the study blood samples were taken at baseline, throughout the infusion period and post-infusion for subsequent investigational analysis of PUFA and cytokine levels. Nine separate time-points were used (table 2.1) and two
separate blood samples were taken at each time-point (table 2.2) for the study into angiogenic factors (other samples were taken for analysis in parallel studies but these will not be discussed here).

<table>
<thead>
<tr>
<th>Time-point</th>
<th>Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baseline</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>44</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
</tr>
<tr>
<td>8</td>
<td>74 (2 hrs post infusion cessation)</td>
</tr>
<tr>
<td>9</td>
<td>Liver resection (LR)</td>
</tr>
</tbody>
</table>

*Table 2.3: Times at which samples were taken and stored for future analysis*

<table>
<thead>
<tr>
<th>Sample collection</th>
<th>Volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin Plasma</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>3 ml</td>
</tr>
</tbody>
</table>

*Table 2.4: Study samples for collection at each time-point*

Routine biochemical and haematological samples were taken in parallel to trial samples, for patient monitoring at baseline, 20, 44, 66 and 74 hours, and at liver resection (LR).
2.1.5.1 Preparation and storage of Heparin Plasma samples

3 ml of blood was collected in a heparin plasma sample bottle. The sample was immediately transferred to ice awaiting preparation. The sample was subsequently centrifuged in the sample tube used for collection at 1000 rpm for 15 minutes at 4 °c with no brake. The upper plasma layer was then transferred into four epindorphs in aliquots of 250 µl using a manual micropipette. The four plasma samples were labelled and transferred into storage boxes and stored at -80 °c.

2.1.5.2 Preparation and storage of serum samples

3 ml of blood was collected in a serum sample bottle. The sample was clotted at room 21 °c (room temperature) for 30 minutes. The sample was subsequently centrifuged in the sample tube used for collection at 1000 rpm for 15 minutes at 4 °c with no brake. The upper serum layer was then transferred into four epindorphs in aliquots of 250 µl using a manual micropipette. The four serum samples were labelled and transferred into storage boxes and stored at 80°C.

2.1.6 Preparation and storage of tissue samples

At liver resection samples of tumour and associated normal liver tissue were banked for subsequent analysis. After surgical resection of the tumour 1g of tumour and 1g of normal liver tissue were excised from the histological specimen. To excise the tumour tissue the lesion was transected and a sample was taken from near the edge of the tumour, as the central areas were often necrotic. The tissue samples were then sited in labelled cryotubes and placed in liquid nitrogen prior to transfer to the laboratory. They were subsequently stored at -80 °c for future use.
2.1.7 Gas Chromatographical analysis of n-3 PUFA uptake

For prescriptive text see appendix 3. Gas chromatography (GC) is a well-established technique for analysis of lipids which are either volatile or can be made volatile by modification of chemical groups. The major limitation of GC is that complex lipids, such as triacylglycerol (TAG) and PLs cannot be studied intact and so potentially important information regarding the combinations of fatty acids in these molecules is lost. However, provided the data are interpreted carefully, this technique is sufficient for most nutritional studies.

The main principle underlying the separation of FAs by GC is that they differ in the temperature at which they become volatile. This depends upon carbon chain length, number and position of double bonds. Increasing chain length increases the temperature at which fatty acids enter the vapour phase. In contrast, the greater the number of double bonds, the lower the boiling point. In addition to the effects of temperature, differences in the interaction between fatty acids and the lining of the GC column are also used to separate FAs. Typically, a methyl group is added to the carboxylic end of the fatty acid to form a fatty acid methyl ester (FAME) by reaction with a methyl donor such as methanol, in the presence of a catalyst, for example sulphuric acid, sodium methoxide or boron trifluoride. This lowers the boiling point that, in turn, allows separations of a wide range of fatty acid at moderate temperatures. In addition, the methylation reaction releases fatty acids from phospholipids, TAG and cholesterol esters (CE).

The GC consists of a heated injection port, a fused silica capillary column located within a high efficiency oven and a detector (figure x). FAMEs injected into the injection port of the GC are rapidly heated to 250 – 300°C and so become volatile. The FAMEs are carried into the capillary column by a stream of
helium (or hydrogen in some applications).

Figure 2.5: Schematic diagram of gas chromatograph

The column is held at a lower temperature than the injection port, and FAMEs rapidly condense on the column lining. Very volatile substances, such as the hexane used to dissolve the FAMEs prior to injection, do not condense. The column is then heated and FAMEs dissociated from the column lining as their boiling point is reached. After entering the vapour phase FAMEs then transiently interact with the column lining. The strength of this interaction is determined by chemical properties of the FAME including the number of double bonds. The longer the column, the more interactions occur and the greater the resolution of FAMEs. Different column linings will cause differences in the order in which fatty acids elute.

The end of the column is located within the flame ionisation detector (FID), held at about 250 °C. The hydrogen flame causes combustion of the FAMEs and thus generates an ion current proportional to the amount of FAME in the sample.
However, a correction needs to be made for the number of carbons in FAME, which is determined experimentally. The resulting chromatogram contains a series of peaks, each corresponding to a FAME (figure 7). The area under each peak is proportional to the mass of the FAME injected onto the column.

![Figure 2.6: Example of a gas chromatogram](image)

2.1.7.1 Principles of lipid extraction and purification by solid phase extraction

Before analysis by GC, lipids have to be extracted from their biological matrix, purified into single classes (TAG, CE, phospholipid) and methylated. There are several methods for extraction of total lipid from both tissues and plasma, all of which use the solubility of lipids in mixtures of chloroform and methanol. The most widely used are those by Folch et al. (Folch 1957) and Bligh and Dyer (Bligh 1959). As with all biochemical extraction the first step is homogenisation of the sample. Tissues are ground in ice-cold saline and plasma is vortexed vigorously in a chloroform/methanol mixture. Addition of 1M NaCl promotes portioning of lipids into the organic phase and facilitates separation of the chloroform and aqueous layers. The chloroform phase contains the lipid extract. Water-soluble metabolites, such as choline, can be isolated form the
aqueous phase.

Separation of lipid classes has been traditionally carried out by thin layer chromatography. However, this method produces low yields (about 30%) and is labour intensive. Solid phase extraction (SPE) using cartridges containing silica to which functional groups have been bonded overcomes both of these limitations. Yields of 95% are readily achieved and up to 20 samples can be separated in a batch. The method was described by Burdge et al (Burdge 2000). This initially separates polar lipids (phospholipids and non-esterified fatty acids (NEFA)) from non-polar lipids (CE and TAG). Phosphatidylcholine, phosphatidylethanolamine and NEFA are eluted sequentially by increasing the polarity of the eluting solvent. CE and TAG are then separated by a second SPE cartridge under conditions, which facilitate binding of TAG but not CE. TAG is eluted by increasing the polarity of the eluting solvent.

Altering fatty acids to form FAMEs involves transfer of a methyl group from methanol in the presence of a catalyst and heating. This also breaks the bond between fatty acids and glycerol (in TAG and phospholipids) and cholesterol (in CE). Fatty acids which are attached to phospholipids by ether bonds form dimethylacetal derivatives rather than FAMEs.

2.1.7.2 Preparation of total lipid extract from plasma and tissue

For a prescriptive description of methods see appendix 3. Plasma membrane samples were thawed at room temperature and mixed by vortexing and spun in a centrifuge at 13000 rpm for 5 minutes to remove denatured protein. To assist optimal extraction and aide phase separation the sample was made up to 0.8 ml using 0.9% NaCl. Tumour and healthy liver tissue (0.5 g) was thawed, gently homogenised and then mixed with 0.9% NaCl to 0.8 ml. Total lipid was
extracted with chloroform/methanol (2:1 vol/vol); butylated hydroxytoluene was added to the extraction as antioxidant.

Purified lipid was dissolved in toluene and fatty acid methyl esters generated by reaction with methanol containing 2% (vol/vol) sulphuric acid at 50°C for 2 hours. After cooling and neutralisation, FAMEs were extracted into hexane. FAMEs were separated by chromatography on a BPX-70 column (30 m x 220 µm; film thickness 0.25 µm) fitted to a Hewlett-Packard HP6890 gas chromatograph. Front inlet temperature was 300°C; initial column temperature was 115°C and was programmed to hold this temperature for 2 min, then to increase temperature at 10°C/minute to 200°C, to hold at 200°C for 10 min, to increase temperature at 10°C/min to 240°C, and then to hold this temperature for 2 min. Helium was used as the running gas and fatty acid methyl esters were detected by flame ionisation. FAMEs were identified by comparison with retention times of standards run previously and they were quantified using ChemStation software. Data are expressed as percentage contribution to the total fatty acid pool [Ratnayake 2009].
2.1.8 Analysis of circulating angiogenic cytokines

Analysis of circulating angiogenic cytokines was performed on stored serum samples from each time-point for every patient. Analysis was performed using ELISA. The ELISA assay was performed by a third party (Aushon Biosystems, Boston, USA) under our guidance to introduce another layer of blinding and reduce the risk of any bias in the results. Samples were couriered to Aushon Biosystems on dry ice and thawed on arrival to allow ELISA analysis. A technique called SearchLight® was used. SearchLight® is a multiplex sandwich ELISA in a planar, plate-based array format, for the quantitative measurement of secreted proteins in serum; EDTA, heparin and sodium citrate plasma; tissue culture supernatants; and other matrices. Each well of the microplate is pre-spotted with analyte-specific antibodies. These antibodies capture specific proteins in the standards and samples added to the plate. After unbound proteins are washed away, biotinylated detection antibodies are added that bind to a second site on the target proteins. After washing away excess detection antibody, streptavidin-horseradish peroxidase (s-HRP) is added. The s-HRP enzyme subsequently reacts with the substrate, SuperSignal® ELISA Femto Chemiluminescent Substrate (Patent 6,432,662), to produce a luminescent signal that is detected using the SearchLight Imaging and Analysis System. The amount of signal produced is proportional to the amount of each protein in the original standard or sample. Customized Array Analyst software uses a weighted four-parameter curve fit to back calculate unknowns using results extrapolated from the corresponding standard curve.

Each assay consisted of a 96-well plate custom arrayed with target protein-specific anti-human antibodies, a lyophilised recombinant standard for each assay, sample diluent containing 0.1% sodium azide, biotinylated antibody reagent, SA-HRP reagent, SuperSignal stable peroxide solution, and
SuperSignal luminol/enhancer solution and wash buffer. Plates were read using the SearchLight Black Ice Cooled Camera System. Images were analyzed using Array Analyst software, and data analysis was completed and summarised using Microsoft Excel.

Diluted samples and controls were incubated for one hour on the arrayed plates. All incubations were performed at room temperature with shaking at 200 rpm. Plates were decanted and washed six times before adding a cocktail of biotinylated detection antibodies to each well. After incubating with detection antibodies for 30 minutes, plates were washed three times and incubated for 30 minutes with streptavidin-horseradish peroxidase. Plates were again washed before adding SuperSignal Femto Chemiluminescent substrate. The plates were immediately imaged using the SearchLight Black Ice imaging system, and data was analyzed using Array Analyst software. Concentrations of all unknown samples were back calculated using results extrapolated from the corresponding standard curve and multiplied by the dilution factor. The assay range was determined during development and was selected to optimize sensitivity and linearity.
2.2.0 Statistical analysis

Various statistical tests were employed to analyse various different parts of the data set. Statistical analysis was performed using SPSS for windows v 18.0 software (SPSS Inc., Chicago, US).

Observed differences between 2 groups or time points were assessed for significance using the unpaired non-parametric Mann-Whitney test. This was used instead of a students t-test, as a Mann-Whitney test is less likely to spuriously indicate significance because of the presence of outliers.

Trends were analysed for correlation using the non-parametric spearman correlation.

A significant feature of the study was that variables were measured on 9 different occasions. With such data, there are a number of different analyses that can be performed, such as comparisons of specific time points, or producing a single summary of all data points for analysis. Of principle interest was how the two groups varied over time, and whether the profile of values varied between groups. Therefore, with advice from a medical statistician it was decided to perform a single analysis of all time points combined. The statistician modelled a technique called generalised estimating equations (GEEs).

As noted, each subject was measured on more than one time point. It is likely that values measured at any specific time point will be dependent on the previous time point for that subject, and thus this should be factored into the analysis. To allow for this, all analyses were performed using GEEs.
One option would be to use time as a categorical measurement, so as 9 distinct categories in the analysis. However, such an approach would not take account of the order of the categories. Therefore time was considered as a continuous measure in the analyses.

In relation to the cytokine results LR values were some considerable time after the other measurements. These would be very influential in the analyses and were not directly related to the infusion of n-3 PUFAs. As a result of this, along with the wide time spread of LR values, it was chosen to omit the LR values from the cytokine analyses.

The main objective of the analysis was to examine if the profile of the values over time varied between groups. This can be done statistically by fitting the interaction between time and group. A significant interaction would imply that the change in values over time would vary between the control and study -n-3 PUFA- groups.

GEEs were used to examine if there was a trend over time for any of the pathways in each group, and also whether these trends varied between the two groups. GEE methods, along with a lot of statistical methods, have some assumptions that have to be satisfied for the methods to give a good fit to the data. These assumptions are not always satisfied when the outcome variables have a very skewed distribution. Some outcomes were found to have a highly skewed distribution. For these outcomes, a log transformation of the outcome variable was taken, and the analysis was performed with the outcome on the log scale.
When outcomes were performed on the log scale, the regression coefficients are less meaningful. However, these can be converted into ratios. These ratios represent the ratio of the change in outcome values for an increase of 10 hours in time. A ratio above 1 would suggest an increase in values over time, whilst a ratio of below 1 would suggest a decrease in values over time.

Corresponding confidence intervals are presented with these estimated values. The first set of p-values indicates, for each group, if there was a significant change in outcome values over time. The last interaction p-value indicates if the change over time varies between control and study groups. A significant interaction would suggest that the change over time varied between groups.
Chapter 3

Results
3.1.0 Results Introduction

In the following chapter I will discuss the results for each outcome measure stated in chapter 2, in discrete sections.

3.1.1 Recruitment

Trial recruitment commenced in May 2007 and the clinical arm of the trail ceased after the twentieth patient had completed the active part of the trial in September 2009 (28 months).

Pre trial sample size calculations indicated that 20 patients (n=20) were required with 10 patients in each arm. Forty-nine patients were considered for trial entry. Five were not approached as they had received chemotherapy within the past three months. Nine patients could not be included as they had been...
taking oral FO supplementation. Three patients declined to enter the trial and one patient was too heavy to safely lie on the MRI scanner. Four patients could not be approached despite fulfilling study criteria when recruitment ceased for a period of five months in early 2009 (see below). Twenty-seven patients gave consent to join the trial but seven were excluded prior to receiving the investigated medicinal product. Two patients had metal clips from previous surgery in close proximity to the current liver lesion, which distorted the MRI images, liver lesions were too small for characterization in two patients, one patient could not lie still for the MRI scan due to a known hemiballismus from a previous cerebro-vascular accident. I was unable to get venous access (PICC line) in two patients to administer the TPN. Of these seven patients four had been formally randomised for TPN compounding. As such final group numbers in the active group and control group were not exact due to pre-defined random number tables for randomization (FO group = 9, control group = 11).

3.1.1.1 Difficulties faced with recruitment

The trial recruitment period was longer than provisionally expected. Difficulties to recruitment included patients already taking oral FO supplementation and who had received chemotherapy within the preceding three months prior to the trial.

After 12 patients had been recruited a decision was made to amend the inclusion criteria of the study to include patients who were taking oral FO supplementation. Ethical review granted the change, however none of the subsequent 8 patients who were part of the final study cohort were taking oral FO supplementation.

Another delay to recruitment came when Sheffield Teaching Hospitals Pharmacy Trails Department relinquished their Medicine Health Regulatory
Authority (MHRA) approval to produce medicinal products for investigation – thus compound and randomise the TPN for this trial- in January 2008. The trial had recruited 11 patients at this time. The Royal Free Hospital, London, Clinical Trials Department took over the production and randomisation for subsequent patients. To gain ethical and MHRA approval for this move along with organising associated paperwork and logistics took a period of 5 months and recruitment recommenced in June 2008.

3.1.2 Demographics

Twenty patients were randomised to either receive active substance (Lipidem® 20%, B Braun, UK (FO group)) or control (Lipofundin® MCT 20%, B Braun, UK). Nine patients received FO and 11 received the control.

Patients were well matched for sex (FO group m=4, f=5, control group m=5, f=6), age (total median = 66 years (range 44-79 years), FO group median = 63 years (range 45-78 years), control group median = 66 years (range 44-79)), weight (total median = 69 kg (range 45-90 kg) FO group median = 69 kg (range 48-80 kg), control group median = 68 kg (range 45-90 kg)) and all had colorectal liver metastases > 3 cm in size deemed operable on the basis of radiological and laparoscopic staging, were not taking oral FO supplementation and had not received chemotherapy within three months of enrolling in the trial. All patients were Caucasian.

3.1.3 Safety and toxicity

There were no safety concerns with the FA infusion in either the control group or the FO group. No patients experience fat overload syndrome and, routine bloods and basic physiological observations were within normal limits throughout the trial period.
One patient developed an area of thrombophlebitis around the PICC line insertion site on day 2 of infusion. The line was removed and a new PICC line inserted in the contra-lateral ante-cubital fossa. The thrombophlebitis settled with no further intervention necessary. As precaution this was reported as an Adverse Event (AR) to the trial sponsor and MHRA. No further action was taken.
3.2.0 Analysis of the incorporation of unsaturated fatty acids into plasma phospholipids in response to intravenous eicosapentaenoic acid and docosahexaenoic acid infusion

3.2.1 Introduction

Tissue levels of n-3 PUFA reflect dietary intake. To regulate and participate in cellular metabolism and exert indirect effects via eicosanoids and docosanoids, PUFAs must be incorporated into membrane PLs. As previously discussed in chapter 1.3, the majority of studies in the literature use oral FO supplementation, but the oral bioavailability of EPA and DHA is unknown, but thought to be poor. n-3 PUFA containing triglycerides found in oral FO preparations are not efficiently hydrolysed by pancreatic lipase leading to slow intestinal absorption [Carpentier 2007] in turn leading to slow incorporation of FO to maximal levels into cell membranes after oral ingestion [Christensen 1995, Oliveira FL 1997]. A study by Visioli showed that increments in plasma EPA and DHA after regular salmon intake, 100 g/day providing ~383 mg EPA and 544 mg DHA, were significantly higher than patients who had FO capsules of similar or higher doses; groups with intake ranging from 450 mg of EPA and 318 mg DHA up to 2580 mg EPA and 1920 mg DHA per day [Visioli 2003].

However, modification of lipid pools, cells and tissues by oral supplementation with EPA and DHA has been widely reported. Incorporation of DHA and EPA occurs in a dose response fashion, with studies reporting near linear relationships between EPA and DHA intake and the EPA and DHA content of PPLs and mononuclear cells [Blonk 1990, Harris 1991, Marsen 1992, Rees 2006].
Response also depends upon which lipid profile is measured [Calder 2009]. EPA and DHA incorporation into PPLs reaches maximal concentration after 3-4 weeks supplementation [Calder 2009]. Near maximal incorporation into blood mononuclear cells occurred after 4 weeks oral supplementation [Yaqoob 2000], after 30 days in serum cholesterol esters and between 56 – 182 days in erythrocytes [Katan 1997]. Katan also showed that incorporation was dose dependent and that different (n-3) FAs were incorporated with different efficiencies. Changes in DHA were small despite long-term supplementation. The changes in EPA were more pronounced and EPA levels in cholesteryl esters reflect intake over the past week or two, erythrocytes over the past month or two, and adipose tissue over a period of years. Typically the increase in content of n-3 PUFAs occurs at the expense of n-6 PUFAs, particularly AA [Calder 2007].
Figure 3.2: Time course of incorporation of EPA and DHA into human mononuclear cells. Healthy subjects supplemented their diet with FO capsules providing 2.1 g EPA plus 1.1 g DHA per day for a period of 12 weeks (indicated by bar). Blood mononuclear cell PLs were isolated and their fatty acid composition determined by gas chromatography. Data are mean and SEM from 8 subjects. From Yaqoob 2000.

The difficulty faced by investigators with this slow incorporation and subsequent lag to peak concentration, is as previously discussed that numerous tablets or a large volume of liquid must be consumed over a long period of time to effect a significant change that will effect cellular processes.

Our knowledge of the modification of cellular mechanisms by n-3 PUFAs is predominantly derived from enteral supplementation and there is no data comparing oral and intravenous bioavailability. An increasing number of studies are reporting benefits of intravenous FO supplementation. However many of these investigational studies are looking at the downstream cellular and clinical effects of intravenous n-3 PUFAs supplementation and do not routinely measure fatty acid profile and incorporation of PUFAs into membrane PLs. One study, which investigated clinical and cellular outcomes in patients with pancreatitis did also measure change in EPA from baseline after five days of TPN supplemented with or without FO. They found a significantly higher level of plasma EPA in the FO supplemented group [Wang 2008].
There are studies solely investigating the incorporation of intravenously administered n-3 PUFAs into membrane PLs, which have shown similar findings for both EPA and DHA. PPLs and leukocyte membrane PLs have shown rapid enrichment of EPA and DHA with maximal incorporation observed within 3-5 days [Nau 1993, Morlion 1996, Wachtler P 1997, Dupont 1999] with an associated decrease in the AA/EPA ratio and without any side effects [Morlion 1996]. A more recent paper from Mayer et al showed that volunteers receiving two 12 hour infusions of a 350 ml FO based lipid emulsion in a 48 hour period had significant rises of total n-3 PUFA, EPA and DHA compared to baseline [Mayer 2003].

Data suggests that these changes may be apparent after a five-hour infusion. A trial in eight healthy volunteers receiving an infusion of a FO emulsion over a five-hour period showed a significant increase of EPA in platelets and white blood cell phospholipids at the end of the infusion [Siderova 1997]

Simoens et al investigated the response of blood cell phospholipids to four repeated 5 hourly infusions of a mixed MCT-LCT lipid emulsion containing...
10% n-3 PUFA over a four-day period. Each infusion raised the EPA concentration in blood cell phospholipids to reach a 7-fold enrichment in platelets and a >2-fold enrichment in leukocytes was reached after 4 infusions [Simoens 2008], however DHA levels remained unchanged in blood cell phospholipids. A study by Carpentier infused a 50 ml n-3 PUFA enriched lipid emulsion over 5 minutes. Rapid enrichment of cells was reported with the EPA content of leukocyte and PPLs being increased at 60 minutes and 24 hours respectively [Carpentier 2010].

Comparing this data to previously published data on incorporation post oral supplementation would suggest that intravenous supplementation of TPN containing FO or lone FO emulsion results in maximal enrichment far quicker.

3.2.2 Methods

We quantified the n-3 and n-6 FA levels within PPLs from patients with hepatic colorectal liver metastases and demonstrated whether a 72 hours intravenous infusion of n-3 PUFAs EPA and DHA altered these levels. As previously discussed plasma samples were taken from patients at nine time points throughout the infusion to quantify change in response to infusion. Heparin plasma samples were used for collection of plasma, for analysis of PUFA levels within PPLs.

Three millilitres of blood was collected in a heparin plasma sample bottle. The sample was immediately transferred to ice awaiting preparation. The sample was subsequently centrifuged in the sample tube used for collection at 1000 rpm for 15 minutes at 4 °c with no brake.
The upper plasma layer was then transferred into four epindorphs in aliquots of 250 µl using a manual micropipette. The four plasma samples were labelled and transferred into storage boxes and stored at -80 °c.

Subsequent FA analysis was performed as previously described (Chapter 2 and Appendix 3) using GC by myself at the Institute of Human Nutrition, University Hospitals of Southampton under the guidance of Prof. Philip Calder a world authority on FA extraction and quantification.
3.2.3 Results

As previously discussed twenty patients were randomised to either receive active substance (Lipidem® 20%, B Braun, UK) or control (Lipofundin® MCT 20%, B Braun, UK). Nine patients received n-3 PUFAs and 11 received the control. For analysis of PPL FA composition zero patients were excluded from analysis.

The 20 patients were well matched for sex (n-3 PUFA group m=4, f=5, control group m=5, f=6), age (total median = 66 years (range 44-79 years), n-3 PUFA group median = 63 years (range 45-78 years), control group median = 68 years (range 44-79)) and weight (total median = 69 kg (range 45-90 kg), n-3 PUFA group median = 69 kg (range 48-80 kg), control group median = 68 kg (range 45-90 kg)).

A summary of the results of the outcome for the two groups is shown in the next table. The figures are the mean percentage concentration and standard deviation of EPA, DHA and AA as a product of the total PPL FA profile for each time point.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Mean (SD)</strong></td>
<td><strong>Mean (SD)</strong></td>
</tr>
<tr>
<td>EPA</td>
<td>Baseline</td>
<td>1.52 (0.44)</td>
<td>1.32 (0.65)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>1.47 (0.50)</td>
<td>1.27 (0.53)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>1.32 (0.40)</td>
<td>1.25 (0.52)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>1.35 (0.32)</td>
<td>1.33 (0.53)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>1.10 (0.21)</td>
<td>1.69 (0.42)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>1.10 (0.36)</td>
<td>2.84 (0.51)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>0.98 (0.16)</td>
<td>3.91 (0.74)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>1.00 (0.21)</td>
<td>3.96 (0.81)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>1.57 (0.64)</td>
<td>1.61 (0.43)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>LR</strong></td>
<td><strong>LR</strong></td>
</tr>
<tr>
<td>DHA</td>
<td>Baseline</td>
<td>3.70 (1.29)</td>
<td>3.33 (1.48)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>3.81 (1.20)</td>
<td>3.44 (1.30)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>3.84 (1.20)</td>
<td>3.35 (1.26)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>3.80 (1.14)</td>
<td>3.53 (1.34)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>3.71 (1.18)</td>
<td>3.67 (1.18)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>3.54 (0.99)</td>
<td>4.10 (1.19)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>3.30 (0.80)</td>
<td>4.58 (1.24)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>3.46 (0.95)</td>
<td>4.54 (1.30)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>3.58 (1.11)</td>
<td>4.17 (1.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>LR</strong></td>
<td><strong>LR</strong></td>
</tr>
<tr>
<td>AA</td>
<td>Baseline</td>
<td>9.28 (2.46)</td>
<td>9.67 (2.50)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>9.58 (2.48)</td>
<td>9.95 (2.56)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>9.55 (2.44)</td>
<td>9.90 (2.53)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>9.59 (2.42)</td>
<td>10.00 (2.48)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>9.22 (2.18)</td>
<td>9.67 (2.09)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>8.73 (1.71)</td>
<td>9.09 (1.68)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>8.30 (1.47)</td>
<td>8.56 (1.51)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>8.59 (1.63)</td>
<td>8.88 (1.67)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>9.11 (2.38)</td>
<td>9.36 (2.63)</td>
</tr>
</tbody>
</table>

Table 3.1: Table showing the mean percentage concentration and standard deviation of EPA, DHA and AA as a product of the total PPL FA profile for each time point.

The variations in results over time are displayed graphically in the next plots for each of the three outcomes. These first set of graphs are for the outcomes on the original scale of measurement.
Figure 3.4: Graph showing mean percentage concentration of EPA over time in the fish oil and control group.

Figure 3.5: Graph showing mean percentage concentration of DHA over time in the fish oil and control group.
Figure 3.6: Graph showing mean percentage concentration of AA over time in the fish oil and control group.

With regards to EPA concentration in the study group, the first signs of increased PPL EPA concentration occurred 20 hours into the infusion (baseline mean percentage concentration and SD 1.32 +/-0.65 vs 20 hours 1.69 +/-0.42 p = >0.05), however the first statistically significant increase was seen at 44 hours (baseline 1.32 +/-0.650 vs 44 hours 2.840 +/- 0.510 p = <0.0001). Maximal levels were reached by 72 hours (Baseline 1.32 +/-0.65 vs 72 hours 3.96 +/- 0.81 p = <0.0001), however the data suggests that a steady state was reached by 68 hours. There was a 200% increase in the level of EPA from baseline to 72 hours. Levels were still elevated compared to baseline at liver resection (baseline 1.32 +/-0.65 vs liver resection 1.61 +/-0.43 p = >0.05) however this was non-significant and levels were returning towards baseline.

Similar trends were seen with PPL DHA concentration. The first increase in concentration occurred at 6 hours (3.33 +/- 1.48 vs 3.53 +/-1.34), however the first statistically significant increase occurred at 68 hours (baseline 3.33 +/- 1.48 vs 68 hours 4.58 +/- 1.24 p = 0.05). There was a 25% increase in the level of
EPA from baseline to 72 hours. Levels were still elevated compared to baseline at liver resection (baseline 3.33 +/- 1.48 vs liver resection 4.17 +/- 1.30 p = >0.05) however this was not significant and levels were returning towards baseline.

With regards to AA the maximal decrease was seen at 68 hours and represented a 13% decrease, however this was not statistically significant when taken in isolation (baseline 9.67 +/- 2.50 vs 68 hours 8.56 +/- 1.51). However a significant cohort decrease was observed as discussed below.

Results for the control group showed decreases in EPA, DHA and AA concentration. The maximal percentage decreases in EPA, DHA and AA were 35.5%, 10.2% and 10.5% respectively. All three-outcome variables were skewed in their distribution, and thus were analysed on the log scale. Similar graphs with the outcomes on the log scale are shown below.

![Graph showing the log scale of mean percentage concentration of EPA over time in the fish oil and control group.](image-url)
GEEs were used to examine if there was a trend over time in each group, and also whether these trends varied between the two groups. Again the LR time point was omitted from these analyses.
A summary of the analysis results is given in the next table. As all three outcomes were analysed on the log scale, the results are presented in the form of ratios. These ratios represent the ratio of the change in outcome values for each increase of 10 hours of infusion time. Corresponding confidence intervals are presented with these ratios.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA</td>
<td>Control</td>
<td>0.96 (0.93, 0.98)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>1.19 (1.15, 1.22)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DHA</td>
<td>Control</td>
<td>0.98 (0.97, 0.99)</td>
<td>0.008</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>1.05 (1.03, 1.06)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AA</td>
<td>Control</td>
<td>0.98 (0.97, 0.99)</td>
<td>&lt;0.001</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.98 (0.97, 0.99)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.2: A summary of the analysis results of PPL FA concentration.*

The results suggested that for both EPA and DHA, there was a significant decrease in values over time in the placebo group, but a significant increase in values for the fish oil group. In the fish oil group, each 10-hour increase in time was associated with a 19% increase in EPA, whilst each 10-hour increase in time was associated with a 5% increase in DHA. Maximal concentrations of EPA and DHA were reached by 64 hours when a steady state was reached. This change over time was highly significantly different between the two groups (p<0.001 for both outcomes).

The liver resection time-point was excluded from the above analysis but results suggest that levels of EPA and DHA were returning to pre-treatment levels by the time of liver resection (median 6 days (range 4-12)).
For AA there was a significant decrease in values over time for both treatment groups. There was no significant interaction between group and time, suggesting that the changes in time did not vary significantly between groups.

3.2.4 Discussion

As anticipated a 72-hour infusion of EPA plus DHA in the study group significantly altered the concentrations of EPA, DHA and AA in PPLs. Concentrations of EPA, DHA and AA within PPLs were also significantly altered in the group who received the control product Lipofundin MCT/LCT 20%. This is to be expected when taking into account the contents of Lipofundin MCT/LCT 20% (see appendix).

Intravenous supplementation of EPA and DHA altered PPL concentration of these FAs far quicker than quoted figures for oral FO supplementation. Intravenous infusion as part of TPN lead to a maximal concentration being achieved by 68 hours compared with 3-4 weeks quoted by Calder et al for oral supplementation [Calder 2009].

The differences in EPA and DHA incorporation compared to those published by Katan et al [Katan 1997]. Both this study and Katans’ study which used oral supplementation showed that different n-3 FAs were incorporated with differing efficacies. Changes in EPA were more pronounced compared to DHA. The maximal percentage increase from baseline for EPA was 200% compared to 25% for DHA. This was also reflected in the cohort difference, with each 10-hour increase in time associated with a 19% increase in EPA, whilst each 10-hour increase in time was associated with a 5% increase in DHA.
These results also mimic data from studies using oral supplementation when considering alteration of AA levels in response to increased PPL EPA and DHA levels [Calder 2007]. As discussed in chapter AA is replaced in the PPL membrane by EPA and DHA when EPA and DHA concentrations are increased.

When comparing our results to published data on intravenous FO supplementation there are numerous similarities. We demonstrated that levels started to rise significantly by 44 hours, which correlates to data published by Mayer [Mayer 2003]. Peak levels/steady state levels were reached by 66 – 72 hours, which is similar to published figures of 3-5 days [Nau 1993, Morlion 1996, Wachtler P 1997, Dupont 1999]

Our results support data already published that intravenous FO infusion leads to rapid PL enrichment compared to oral supplementation. They also suggest that any significant changes in the later analysis of surrogate markers of angiogenesis in this thesis are likely to be related to change increases EPA and DHA levels along with an associated decrease in AA levels.

However, further studies incorporating an increased number of time-points for PPL sampling and measurement specifically around the six to 20 hour mark to determine the inflection point of the concentrations of EPA and DHA in response to EPA and DHA infusion, the 60- 72 hour point to determine the exact point that a steady state and maximal concentrations are reached and in the post infusion period to determine the rate at which levels return to baseline are needed.

Also studies with direct comparison of change in PL levels of EPA, DHA and AA in response to intravenous supplementation versus oral supplementation
are required as there is a large gap in the scientific literature. Further work is also needed to work out the optimum concentration of FO infusion and over what time course to cause maximal change in PL concentration.

If FO supplementation is found to have profound beneficial effects then investigation is required into whether levels reached quickly by intravenous infusion can be maintained by oral supplementation.
3.2.0 Analysis of unsaturated fatty acids in normal healthy liver tissue and hepatic colorectal metastases in response to intravenous eicosapentaenoic acid and docosahexaenoic acid infusion.

3.3.1 Introduction

As discussed in chapter 1, studies have shown that eicosanoids can accelerate tumourgenesis and that enzymes responsible for conversion of FAs to eicosanoids are up regulated in malignant tissue [Krsihnamoorthy 2008]. AA, which is found in abundance in many cancer tissues [Koki 2002], is the precursor of these pro-tumourigenic eicosanoids. EPA and DHA decrease AA concentrations and inhibit AA metabolism to eicosanoids. The concentrations of EPA and DHA have been found to be in lower in malignant tissue compared to the corresponding normal tissue [Martin 1996, Kokoglu 1998, Reynolds 2001]. Accelerated cancer growth could therefore be an effect of both increased n-6 PUFA and decreased n-3 PUFA levels.

Endogenous synthesis of EPA and DHA is poor and so diet is the major factor determining their status and the ratio of n-6 to n-3 PUFAs in body compartments. An increased n-6/n-3 PUFA ratio has been suggested to play a role in cancer growth. This is seen in the Japanese population, whose traditional diet included large amounts of oily fish with associated high n-3 PUFA intake. Recently the incidence of certain cancers in Japan, such as breast cancer, has increased in line with a change to a diet containing a larger proportion of western food and n-6 PUFAs [Marugame 2006]. A recent large prospective cohort study showed a clear correlation between total fat intake and colorectal cancer [Norat 2005]. Interestingly, consumption of fish, the major dietary source of n-3 PUFAs, was associated with reduced risk for colorectal cancer. Several other studies
have postulated that n-3 PUFA consumption is associated with decreased risk of breast, prostate, colon, and renal cancer [Simth-Warner 2006, Wolk 2006, Courtney 2007, Fradet 2009, Thiebaut 2009].

This epidemiological data is supported by a large number of in-vitro experiments showing profound multimodal anti-tumour effects of n-3 PUFAs by suppressing neoplastic transformation, angiogenesis, and tumour cell growth and promoting tumour cell apoptosis [Roynette 2003, Hardman 2004, Dupertuis 2007]. Early in-vitro and animal feeding studies demonstrated the potential to alter the fatty acid profile of tumour cells and to increase their content of n-3 PUFAs by supplementation of exogenous n-3 PUFAs [Spector 1967, Hyman 1981, Burns 1987, Petersen 1992, Atkinson 1997, Vartak 1997, Shao 1997]. This increase in n-3 PUFA content, also markedly affected tumour cell sensitivity towards cytotoxic drugs. Lipid emulsions containing n-3 PUFA have been shown to have growth inhibitory effects on human colon adenocarcinoma cell lines [Sala-Vila 2010]. Dietary EPA has also been shown to decrease tumour uptake of LA, the precursor of AA in-vivo [Sauer 2001].

Despite the large body of scientific evidence outlining the effects n-3 and n-6 PUFAs on tumourgenesis there is very little data on the effect of omega-9 (n-9) FAs. However there is evidence from epidemiological studies of a low incidence of certain malignancies in the Mediterranean basin where n-9 oleic acid (OA) intake is high: consumption of olive oil, a rich source of OA was associated with lower the risk of breast, stomach, ovarian, colon and endometrial cancer [Simopoulos 2001, Visioli 2001, Stark, 2002, La Vecchia 2004, Wahle 2004, Perez-Jimenez 2005, Serra-Majem 2006, Colomer 2006, Menendez 2007]. Menendez et al. have shown in-vitro that exogenous supplementation of cultured cancer cells with OA significantly suppressed Human Epidermal growth factor
Receptor-2 (HER2) expression and activity [Menendez 2004, 2005, 2006a, 2006b, 2007]. HER2 plays a pivotal role in oncogenic transformation leading to tumourigenesis and metastasis [Neve 2001, Yarden 2001, Rubin 2001, Menard 2003] thus suggesting a putative protective mechanism of n-9 FAs in carcinogenesis. There is currently very little scientific evidence to support the protective mechanism of n-9 FAs in non-HER2 related malignancies. Takeshita et al. showed that OA supplementation does not enhance colon carcinogenesis in ICR mice [Takeshita 1997] while Bartoli et al. showed that rats fed an isocaloric diet containing OA had a lower incidence of colonic carcinoma than rats fed an isocaloric n-6 PUFA rich diet [Bartoli 2000].

We used intravenous EPA and DHA supplementation to rapidly alter the n-6/n-3 PUFA ratio in patients with known operable hepatic colorectal metastases and measured the effect on the fatty acid composition of normal hepatic parenchyma and tumour tissue. We quantified n-3, n-6, and n-9 FAs within normal liver tissue and tissue from hepatic colorectal liver metastases.

3.3.2 Methods

We quantified the n-3, n-6, and n-9 fatty acid levels within normal liver tissue and tissue from hepatic colorectal liver metastases and demonstrated whether a 72 hours intravenous infusion of EPA and DHA altered these levels.

Patients subsequently underwent a formal curative hepatic tumour resection within 14 days of the end of the infusion. Samples were taken from the resected specimen for future analysis. Sections were excised from the edge of the tumour (avoiding frank areas of necrosis) and from distant healthy liver tissue in the resected specimen. As previously explained samples were snap frozen in sealed containers placed in liquid nitrogen and transferred to a -80°C freezer.
Fatty acid analysis was performed as previously described (Chapter 2 and Appendix 3) using gas chromatography by myself at the Institute of Human Nutrition, University Hospitals of Southampton.

3.3.3 Results

As discussed twenty patients were randomised to either receive active (i.e. n-3 PUFA containing) substance (Lipidem® 20%, B Braun, UK) or control (Lipofundin® MCT 20%, B Braun, UK). Nine patients received n-3 PUFAs and 11 received the control. Five patients were excluded from analysis (2 from the n-3 PUFA group and 3 from the control group). Two of these patients (1 from the n-3 PUFA group and 1 from the control group) were excluded secondary to prolonged time to liver resection (33 and 40 days to resection respectively) due to medical reasons not related to the study. The other three excluded patients were found to be inoperable at formal laparotomy. The remaining 15 patients (n = 7 in the n-3 PUFA group and n = 8 in the control group) were well matched for sex, age and weight (see table 3.3). Paired tumour and healthy liver tissue samples were available for all 15 patients. The median time to liver resection was 8 days: 11 days (range 5-12 days) in the n-3 PUFA group and 6 days (range 4-12 days) in the control group.

There were no significant differences in n-3, n-6 or n-9 fatty acids as a percentage of total of tissue fatty acids between the two groups for healthy liver tissue and tumour tissue (table 3.4). This shows that a 72 hour EPA and DHA infusion had not altered the tissue or tumour fatty acid levels significantly when measured between 4-12 days after finishing the infusion.
<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Included patients</th>
<th>n-3 group</th>
<th>Control group</th>
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<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>15</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
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<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Age* (y)</td>
<td>66 (44-79)</td>
<td>68 (44-79)</td>
<td>64 (45-78)</td>
<td>71 (44-79)</td>
</tr>
<tr>
<td>Weight* (kg)</td>
<td>68 (45-90)</td>
<td>69 (45-90)</td>
<td>69 (48-80)</td>
<td>68 (45-90)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>23 (17-28)</td>
<td>22 (17-28)</td>
<td>21 (18-25)</td>
<td>23 (17-28)</td>
</tr>
<tr>
<td>Time to liver resection* (d)</td>
<td>8 (4-40)</td>
<td>7 (4-12)</td>
<td>10 (4-12)</td>
<td>6 (4-12)</td>
</tr>
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</table>

*Data are median (range)

<table>
<thead>
<tr>
<th></th>
<th>Liver tissue without tumour</th>
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<tbody>
<tr>
<td></td>
<td>n-3 group</td>
</tr>
<tr>
<td>OA</td>
<td>11.2 ± 0.4</td>
</tr>
<tr>
<td>LA</td>
<td>18.2 ± 0.8</td>
</tr>
<tr>
<td>AA</td>
<td>9.6 ± 0.6</td>
</tr>
<tr>
<td>EPA</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>DHA</td>
<td>6.1 ± 0.6</td>
</tr>
<tr>
<td>Total n-9 fatty acids</td>
<td>13.0 ± 0.5</td>
</tr>
<tr>
<td>Total n-6 fatty acids</td>
<td>31.1 ± 0.7</td>
</tr>
<tr>
<td>Total n-3 fatty acids</td>
<td>9.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Liver tissue without tumour</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td></td>
<td>n-3 group</td>
</tr>
<tr>
<td>OA</td>
<td>$19.5 \pm 2.7^*$</td>
</tr>
<tr>
<td>LA</td>
<td>$12.3 \pm 2.4$</td>
</tr>
<tr>
<td>AA</td>
<td>$10.8 \pm 1.8$</td>
</tr>
<tr>
<td>EPA</td>
<td>$1.1 \pm 0.3^*$</td>
</tr>
<tr>
<td>DHA</td>
<td>$2.8 \pm 1.3^*$</td>
</tr>
<tr>
<td>Total n-9 fatty acids</td>
<td>$23.8 \pm 1.4^*$</td>
</tr>
<tr>
<td>Total n-6 fatty acids</td>
<td>$27.8 \pm 1.0^*$</td>
</tr>
<tr>
<td>Total n-3 fatty acids</td>
<td>$5.6 \pm 0.5^*$</td>
</tr>
</tbody>
</table>

Table 3.4 A & B. Fatty acid composition of liver tissue without tumour and hepatic colorectal metastases in the n-3 and control groups. Data are mean ± SEM percentage of total fatty acids. *Significantly different from liver tissue without tumour.

However, in the n-3 PUFA group there was an inverse association of n-3 PUFA content with time between stopping the lipid infusion and hepatic resection - thus tissue sampling (figure 3.10). In other words n-3 PUFAs were higher in both tumour tissue and tissue without tumour in patients who had most recently received EPA plus DHA.
For patients who underwent resection between 4-7 days, mean percentage concentration of n-3 PUFAs in normal liver tissue was 10.46 vs. 8.13 in patients who underwent resection between 10-12 days (p = 0.081). In tumour tissue mean percentage concentration was 6.82 at 4-7 days vs. 4.66 at 10-12 days (p = 0.0126). Similarly, there was a trend for the n-6/n-3 PUFA ratio to be lower in those patients who had an earlier resection (4-7 days normal liver tissue 2.96 vs. 3.98 at 10-12 days (p = 0.081) and 4-7 days tumour tissue 4.08 vs. 6.06 at 10-12 days (p = 0.0064)) (Figure 3.11).
Figure 3.11. Relationship between days to liver resection and ratio of n-6 to n-3 PUFAs in healthy normal liver tissue and hepatic colorectal metastases with line of best fit extrapolated back to time point 0 for patients who received EPA and DHA infusion (cessation of n-3 PUFA infusion)

Table 3.5 gives the contents (% of total fatty acids) of individual and total n-9, n-6 and n-3 fatty acids in hepatic colorectal liver metastases and liver tissue without tumour in both groups combined. The levels of EPA were 50% higher in the liver tissue without tumour compared to tumour tissue (p = 0.003), while DHA levels were 125% higher (p<0.0001). There was no significant difference in AA concentration, but there was a 42% lower level of LA in tumour tissue (p<0.0001). Tumour tissue had a 40% lower content of total n-3 PUFAs (p<0.0001) and a 12% lower content of total n-6 PUFAs (p=0.0002) compared with normal liver tissue. Tumour tissue also had a 47% higher ratio of n-6/n-3 PUFAs compared to normal liver tissue (p<0.0001).

The percentage of n-9 fatty acids in the tumour tissue was significantly higher than in normal liver tissue (p<0.0001). OA comprised the largest proportion of the n-9 fatty acids, contributing over 85% in the normal liver and
over 81% in the tumour tissue. Thus, there was a significantly higher proportion of OA in tumour tissue compared to normal liver tissue (p < 0.0001).

<table>
<thead>
<tr>
<th></th>
<th>Liver tissue without tumour</th>
<th>Hepatic colorectal metastases</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>10.9 ± 0.3</td>
<td>18.6 ± 0.7</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LA</td>
<td>17.5 ± 0.6</td>
<td>12.3 ± 0.7</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AA</td>
<td>10.2 ± 0.4</td>
<td>10.5 ± 0.4</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>EPA</td>
<td>1.6 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.003</td>
</tr>
<tr>
<td>DHA</td>
<td>6.3 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>n-9 fatty acids</td>
<td>12.6 ± 0.3</td>
<td>22.9 ± 0.9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>n-6 fatty acids</td>
<td>31.6 ± 0.7</td>
<td>27.9 ± 0.6</td>
<td>0.0002</td>
</tr>
<tr>
<td>n-3 fatty acids</td>
<td>9.3 ± 0.5</td>
<td>5.6 ± 0.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>3.6 ± 0.3</td>
<td>5.2 ± 0.3</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Table 3.5. Fatty acid composition of liver tissue without tumour and hepatic colorectal metastases in the n-3 and control groups combined. Data are mean ± SEM percentage of total fatty acids

3.3.4 Discussion

It was anticipated that a 72 hours infusion of EPA plus DHA would elevate the concentrations of those PUFAs in liver tissue but this was not observed here. This may be because the tissue samples were collected several days (5 to 12; mean 10) after ceasing the infusion. Thus any EPA and DHA incorporated during the infusion may have been lost by the time the samples were collected. There was a statistically significant trend for n-3 PUFA concentration to be higher in tumour tissue of the patients who received EPA plus DHA and underwent hepatic resection (thus tissue sampling) earlier. Likewise there was a significant trend for the n-6/n-3 PUFA ratio to be lower in tumour tissue in those patients who had an earlier resection. This same pattern was observed as a trend in normal liver tissue, however the lack of significance reflects the small sample size in the subgroups. However this trend is indicative that the infusion of EPA and DHA did elevate the concentrations of these fatty acids in both normal and tumour tissue during the infusion period. It is known that providing EPA and DHA orally to rats
increases the amounts of those FAs in liver tissue [Yaqoob 1995, Lamaziere 2013].

Oral FO supplementation enriches cells and tissues with EPA and DHA, but upon cessation of the supplementation membrane levels of n-3 PUFA returning to pre-supplementation levels. The rate of return to baseline reflects the original rate of incorporation in that cells which undergo rapid incorporation of EPA and DHA have a brisk return to pre-supplementation levels, but cells in which incorporation is slow, resolution to baseline levels is also sluggish [Katan 1997, Calder 2009]. Extrapolation of the lines in Figure 3.10 back to day zero (the day infusion ceased) suggests the maximal percentage content of total n-3 PUFAs reached after 72 hours infusion. Values would be approximately 13.8% in normal liver tissue and 8.7% in tumour tissue in the n-3 PUFA group compared to 9.5% in normal liver tissue and 5.5% in tumour tissue in the control group. This would indicate that the fish oil infusion increased n-3 PUFA levels by 45% in normal liver tissue and 56% in tumour tissue, but that these levels returned rapidly back to baseline after cessation of the n-3 PUFA infusion.

The differences in n-3 and n-6 PUFA levels in normal liver tissue without tumour compared to colorectal liver metastases tumour tissue are similar to those published by Kokoglu et al. [Kokoglu 1998a, 1998b] and Martin et al. [Martin 1996] for glioma and meningioma tissue compared to normal brain tissue. Martin et al. showed a decreased amount of DHA and an increased amount of LA in tumour tissue compared to control [Martin 1996]. Kokoglu et al. in two separate studies showed that AA, LA and total n-6 PUFA levels were increased in tumour tissue [Kokoglu 1998a, 1998b]. They also found that DHA was significantly lower in the tumour tissue compared to controls [Kokoglu 1998a]. We showed that the levels of total n-3 PUFAs and DHA were significantly lower in tumour tissue.
There was a significantly higher level of LA but not AA in tumour tissue compared to control. Interestingly converse to the findings from Kologlu et al., total n-6 PUFAs concentration was significantly lower in tumour tissue in our study. Szachowic et al. examined PUFA levels in pT3 colonic cancer and found that malignant transformation was accompanied by an increase in AA [Szachowic 2007].

Despite the unexpected lower level of n-6 PUFAs in the tumour tissue, when we analysed the n-6/n-3 PUFA ratio, which may be more important than total concentration of n-6 and n-3 PUFAs in determining tumourgenesis, there was both a statistically and biologically significant higher ratio (approx 46%) in the tumour tissue compared to normal liver tissue. This was due to an even greater decrease in n-3 PUFAs than n-6 PUFAs in the tumour tissue, which is consistent with the idea that a higher ratio of n-6/n-3 PUFA is beneficial to tumour growth. Berstad et al. reported that colonic tumour tissue had a higher n-6 to n-3 PUFA ratio compared to normal mucosa [Berstad 2012].

Limited evidence suggests that n-9 FAs, specifically OA, are potentially protective against carcinogenesis [Simopoulos 2001, Visioli 2001, Stark, 2002, La Vecchia 2004, Wahle 2004, Perez-Jimenez 2005, Serra-Majem 2006, Colomer 2006, Menendez 2007]. Kokoglu et al. demonstrated no difference in total n-9 FA concentration between brain tumour tissue and control brain tissue [Kokoglu 1998a] but Szachowicz et al. showed an increased level of OA was associated with colonic mucosal malignant transformation [Szachowicz 2007]. Our data mirrors that of Szachowicz et al. with a higher level of not only OA, but also total n-9 FAs in the tumour specimens compared to control. This increase in n-9 FAs was at the expense of n-3 PUFAs leading to a potentially detrimental increased n-6/n-3 PUFA ratio in tumour tissue. The reason for higher OA in
tumour tissue is not clear. However, many tumours over-express fatty acid synthetase [Baron 2004, Swinnen 2006, Flavin 2010] the enzyme responsible for synthesis of saturated fatty acids. This could supply substrate for synthesis of OA, which requires the delta-9 desaturase enzyme. This enzyme, also known as stearoyl Co Adesaturase 1, or SCD-1, is increasingly recognised to play a role in tumourgenesis [Igal 2010]. Thus, tumours may possess the metabolic machinery to synthesise OA, which could result in a higher content of this fatty acid.

In a study looking at the effect of n-9 FAs based TPN on liver regeneration following hemi-hepatectomy in rats, Ok et al. showed a beneficial effect on liver regeneration [Ok 2003]. This effect may result in an increased generation of malignant cells in the tumourgenesis cycle as well the effect seen in normal liver tissue and needs further exploration. A previous study from Calder et al. determining the effect of dietary fatty acid intake on growth of human colon cancer tumour growth in athymic mice inoculated with HT29 cells, showed that diets high in olive oil (thus OA), promoted human colon cancer tumour growth [Calder 1998] and gives weight to this proposed hypothesis.

This study confirms and supports existing data on decreased levels of n-3 PUFAs in tumours. It is considered that a low n-3 PUFA content favours the development of malignancy. Data suggesting that n-9 FAs and OA are potentially protective against cancer needs to be treated with caution due to the significantly higher amounts of these FAs in tumour tissue compared to healthy tissue. More in-vitro and in-vivo work is required to assess the role of OA and total n-9 FAs in tumourgenesis before they can be promoted as beneficial.
3.4.0 Analysis of circulating cytokine levels associated with angiogenesis in response to intravenous eicosapentaenoic acid and docosahexaenoic acid infusion

In the following chapter each of the investigated cytokines will be discussed discretely with an associated introduction explaining the role that cytokine plays in the angiogenesis pathway. The investigated cytokines were:

- VEGF-A, -C, -D
- VEGFR-R1, -R2
- EGF
- HGF
- TGF-B1
- PDGF-aa, -ab, -bb
- MMP-2, -9
- TIMP-1, -2

3.4.1 Methods

We quantified the levels of the cytokines named above in serum samples using ELISA from patients with hepatic colorectal liver metastases and demonstrated whether a 72 hours intravenous infusion of n-3 PUFAs EPA and DHA altered these levels. As previously discussed plasma samples were taken from patients at nine time points throughout the infusion to quantify change in response to infusion.

3 ml of blood was collected in a serum sample bottle at each of the nine timepoints. The sample was clotted at room 21 °c (room temperature) for 30 minutes. The sample was subsequently centrifuged in the sample tube used for
collection at 1000 rpm for 15 minutes at 4 °c with no brake. The upper serum layer was then transferred into four epindorps in aliquots of 250 µl using a manual micropipette. The four serum samples were labelled and transferred into storage boxes and stored at -80 °c.

As discussed ELISA was performed using Aushon Biosystems SearchLight® analysis for the quantitative measurement of secreted proteins in serum.

### 3.4.2 Result demographics

As previously discussed twenty patients were randomised to either receive active substance (Lipidem® 20%, B Braun, UK) or control (Lipofundin® MCT 20%, B Braun, UK). Nine patients received n-3 PUFAs and 11 received the control. For analysis of circulating cytokine levels zero patients were excluded from analysis.

The 20 patients were well matched for sex (n-3 PUFA group m=4, f=5, control group m=5, f=6), age (total median = 66 years (range 44-79 years), n-3 PUFA group median = 63 years (range 45-78 years), control group median = 68 years (range 44-79)) and weight (total median = 69 kg (range 45-90 kg), n-3 PUFA group median = 69 kg (range 48-80 kg), control group median = 68 kg (range 45-90 kg).

The LR timepoint is included in the tables and graphical representations for each measured cytokine, however this timepoint was excluded from the statistical analysis due to the variation in length of time from the infusion ending to surgery.
3.4.3 Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor

As previously discussed in chapter 1 the VEGF family and its receptors are central mediators in tumour angiogenesis and the only known angiogenic factor present throughout the entire tumour life cycle [Bergers 2000, Inoue 2002, Folkman 2005, Jain 2006]. VEGF inhibition is a primary strategy at the fore of anti-cancer research.

VEGF measurement has been associated with stage, progression and prognosis in many malignancies and it has been the most frequently measured circulating cytokine in studies monitoring effects of on angiogenesis [Davis 2008].

In studies looking at the effect of n-3 PUFAs on angiogenesis it has been shown that VEGF levels, VEGF-stimulated endothelial cell proliferation and VEGF/VEGF-R binding amongst other anti-tumour effects, have decreased in response to EPA and DHA [Spencer 2009].

3.4.3.1 Vascular Endothelial Growth Factor Results

A summary of the analysis results is given in the next table. The figures are the mean and standard deviation in each group measured in pg/ml.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Baseline</td>
<td>540 (407)</td>
<td>467 (174)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>597 (387)</td>
<td>439 (215)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>600 (487)</td>
<td>397 (209)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>547 (406)</td>
<td>388 (206)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>612 (476)</td>
<td>438 (193)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>634 (462)</td>
<td>449 (158)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>572 (447)</td>
<td>375 (161)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>610 (455)</td>
<td>421 (161)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>539 (399)</td>
<td>359 (167)</td>
</tr>
</tbody>
</table>

Table 3.6: Change in VEGF concentration (pg/ml) over time course of the infusion for the control and fish oil group.

A graphical illustration of the mean values in the two groups at each time is shown in the next table. The first graph gives the original VEGF values, whilst the second gives the values on the log-transformed scale.

![Graph showing VEGF concentration over time](image)

Figure 3.12: Mean change of VEGF concentration in the control and FO group over time.
A formal analysis examined the change in VEGF values over time, and how this varied between groups was examined. The analysis results are summarised below. As the outcome was log transformed, the effect of time is given as a ratio, indicating the ratio of change in VEGF values for a 10-hour increase in time.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Control</td>
<td>1.00 (0.94, 1.06)</td>
<td>1.00</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>1.00 (0.94, 1.06)</td>
<td>0.94</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7: Statistical analysis of the change in VEGF concentration over time for the control and FO group, and the interaction between the two groups.

The results suggested that the VEGF values did not significantly vary over time for either the placebo or fish oil group. Additionally the non-significant interaction suggests that the change in values over time did not vary between the two groups.
3.4.3.2 Vascular Endothelial Growth Factor-C Results

A summary of the values at each time in each group is reported in the next table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF C</td>
<td>Baseline</td>
<td>124 (81)</td>
<td>132 (127)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>132 (81)</td>
<td>127 (51)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>145 (126)</td>
<td>127 (72)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>162 (131)</td>
<td>132 (84)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>211 (168)</td>
<td>95 (34)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>166 (146)</td>
<td>75 (50)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>157 (117)</td>
<td>78 (36)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>187 (158)</td>
<td>118 (66)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>157 (119)</td>
<td>135 (79)</td>
</tr>
</tbody>
</table>

Table 3.8: Change in VEGF-C concentration (pg/ml) over time course of the infusion for the control and fish oil group.

Graphs were produced to show the mean values over time in the two groups. These are shown in the next graphs, firstly with the VEGF-C values on the original scale of measurement, and secondly on the transformed log scale.
Figure 3.14: Mean change of VEGF-C concentration in the control and FO group over time.

Figure 3.15: Log-transformed mean change of VEGF-C concentration in the control and FO group over time.

GEEs were used to examine the change in values over time, and how this varied by group. The results are summarised in the next table.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-C</td>
<td>Control</td>
<td>1.01 (0.95, 1.07)</td>
<td>0.78</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.97 (0.91, 1.03)</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.9: Statistical analysis of the change in VEGF-C concentration over time for the control and FO group, and the interaction between the two groups.

The analysis of results suggested that there was no significant change over time in VEGF-C measurements for either the placebo or fish oil group. Additionally, the change over time did not significant vary between the two groups.

3.4.3.3 *Vascular Endothelial Growth Factor-D Results*

A summary of the outcome values in the two groups is given in the next table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF D</td>
<td>Baseline</td>
<td>612 (381)</td>
<td>586 (244)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>518 (286)</td>
<td>551 (239)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>546 (353)</td>
<td>613 (363)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>547 (300)</td>
<td>612 (407)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>479 (307)</td>
<td>487 (201)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>401 (206)</td>
<td>354 (213)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>444 (312)</td>
<td>389 (254)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>515 (434)</td>
<td>372 (176)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>565 (404)</td>
<td>647 (281)</td>
</tr>
</tbody>
</table>

Table 3.10: Change in VEGF-D concentration (pg/ml) over time course of the infusion for the control and fish oil group.
The results are illustrated graphically in the next plots, on both the original scale of measurement, and on the log transformed scale.

*Figure 3.16: Mean change of VEGF-D concentration in the control and FO group over time*

*Figure 3.17: Log-transformed mean change of VEGF-D concentration in the control and FO group over time.*

GEEs were used to examine the change in values over time, and how this varied by group. The results are summarised in the next table.
Table 3.11: Statistical analysis of the change in VEGF-D concentration over time for the control and FO group, and the interaction between the two groups.

The results suggested that there was a highly statistically significant effect of time upon the VEGF-D values for both groups. As the relationship between time and VEGF-D was found to be non-linear, it is more difficult to picture the relationship from the ratios alone, so the fitted relationship for each group is shown in the next graph.

Figure 3.18: Fitted relationship for change in VEGF-D over time for the control and FO groups.
The graph suggests an initial decrease in VEGF-D values for the first 40 hours, after which there was little change in values for the subsequent time period.

There was no significant interaction between time and group, suggesting similar changes over time for both groups.

3.4.3.4 Vascular Endothelial Growth Factor Receptor-1 Results

A summary of the results in the two groups at each time point is given in the next table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-R1</td>
<td>Baseline</td>
<td>68 (74)</td>
<td>56 (35)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>46 (49)</td>
<td>66 (47)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>39 (24)</td>
<td>53 (42)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>207 (204)</td>
<td>234 (177)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>47 (30)</td>
<td>119 (81)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>45 (34)</td>
<td>64 (37)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>66 (53)</td>
<td>60 (37)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>69 (95)</td>
<td>62 (30)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>64 (82)</td>
<td>69 (38)</td>
</tr>
</tbody>
</table>

Table 3.12: Change in VEGF-R1 concentration (pg/ml) over time course of the infusion for the control and fish oil group.

Graphical illustrations of the mean values in each group at each time are shown in the next scatter plots. The first graph is for the VEGF-R1 values on the original measurement scale, and the second graph is the log-transformed values.
Figure 3.19: Mean change of VEGF-R1 concentration in the control and FO group over time.

Figure 3.20: Log-transformed mean change of VEGF-R1 concentration in the control and FO group over time.

The results are summarised in the next table.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-R1</td>
<td>Control</td>
<td>0.99 (0.90, 1.07)</td>
<td>0.73</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.96 (0.86, 1.07)</td>
<td>0.43</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.13: Statistical analysis of the change in VEGF-R1 concentration over time for the control and FO group, and the interaction between the two groups.*

VEGF-R1 was not found to significantly vary over the course of the study in either group. The results also suggested that there was no significant time by group interaction. This result suggests that the change in VEGF-R1 values over time did not vary by group.

### 3.4.3.5 Vascular Endothelial Growth Factor Receptor-2 Results

A summary of the mean and standard deviation value in each group at each time is shown below.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-R2</td>
<td>Baseline</td>
<td>7210 (2511)</td>
<td>9953 (11084)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>7079 (2187)</td>
<td>7276 (4981)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>7180 (2566)</td>
<td>8211 (8230)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>6417 (2110)</td>
<td>10023 (13142)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>6356 (2277)</td>
<td>9498 (10738)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>6126 (2091)</td>
<td>7719 (6855)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>7095 (3412)</td>
<td>7453 (5468)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>7458 (3365)</td>
<td>9512 (9924)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>7979 (4093)</td>
<td>13769 (20491)</td>
</tr>
</tbody>
</table>

*Table 3.14: Change in VEGF-R2 concentration (pg/ml) over time course of the infusion for the control and fish oil group.*
A graphical illustration of the mean VEGF-R2 value at each time point is shown in the next graph, with equivalent values on the log transformed scale in the second graph.

**Figure 3.21:** Mean change of VEGF-R2 concentration in the control and FO group over time

**Figure 3.22:** Log-transformed mean change of VEGF-R2 concentration in the control and FO group over time.
GEEs were used to examine how the VEGF-R2 values varied over time. As with previous outcomes, the effect of time is given as a ratio, which represents the change in the ratio of VEGF-R2 values for a 10-hour increase in time for each treatment group.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-R2</td>
<td>Control</td>
<td>1.00 (0.97, 1.03)</td>
<td>0.96</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.99 (0.96, 1.02)</td>
<td>0.51</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.15: Statistical analysis of the change in VEGF-R2 concentration over time for the control and FO group, and the interaction between the two groups.*

The analysis suggests that there was no significant change in VEGF-R2 values over time for either the placebo or fish oil group. The analysis also indicated that the change in values over time was not significantly different between the two groups.

### 3.4.4 Epidermal growth factor

Epidermal growth factor is a growth factor that plays a role in cell growth, proliferation and differentiation via binding to EGFR [Wong 1999, Stortelers 2002]. It acts via protein-tyrosine kinase activity leading to a rise in intracellular calcium levels, increased glycolysis and protein synthesis which leads to DNA synthesis and cell proliferation [Fallon 1984]. For the receptor to be activated it must be bound to EGF.

Activation of the EGF receptor results in autophosphorylation of key tyrosine residues. These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains and leads to the activation of downstream signalling cascades including the RAS/extracellular signal regulated
kinase (ERK) pathway, the phosphatidylinositol 3-kinase (PI3) pathway and the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway. These pathways act in a coordinated manner to promote cell survival.

Many tumours have elevated production of EGF and overactive signalling through the EGF:EGFR pathway [Yarden 2001, Goodsell 2003]. This leads to uncontrolled cell growth and evasion of apoptosis leading to cell survival [Sastry 2006].

EGF also plays an important role in angiogenesis [Pepper 1996]. Petit et al. reported that the oncogenicity of the EGF and its receptor might partially be mediated through promotion of angiogenesis by up regulating VEGF [Petit 1997]. Since then there has been growing evidence that stimulation or inhibition of EGFR also has significant consequences for tumour-induced angiogenesis [Petit 1997, Yen 2002, Ellis 2004].

The n-3 PUFA’s have been shown to have an effect on EGFR but there are no studies looking at the effect of n-3 PUFA on EGF. Studies in the Syrian hamster embryo have shown that n-6 PUFAs play a role in up regulation of the EGFR receptor via LA interaction with EGF [Glasgow 1992].

In vitro studies of EGF signalling via EGFR have also demonstrated that EGFR protein levels are decreased as a result of combination EPA/DHA treatment or lone DHA treatment in MDA-MB-321 breast carcinoma cell lines, EPA/DHA and lone DHA, A549 lung adenocarcinoma, lone DHA and WiDr colon carcinoma, lone DHA [Schley 2007, Rogers 2010]. DHA/EPA combination is associated with decreased tumour growth by 48-62% [Schley 2007] and lone DHA treatment enhanced the efficacy of EGFR inhibitors. [Rogers 2010].
3.4.4.1 Epidermal growth factor Results

Summaries of the results of the outcome for the two groups are shown in the next table. The figures are the mean and standard deviation values at each time point.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>Baseline</td>
<td>28.5 (23.5)</td>
<td>25.5 (22.9)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>30.3 (23.0)</td>
<td>16.7 (18.2)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>43.4 (52.1)</td>
<td>19.2 (15.6)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>28.1 (22.3)</td>
<td>20.2 (24.5)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>39.1 (36.9)</td>
<td>25.7 (20.4)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>53.0 (44.3)</td>
<td>28.6 (23.6)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>57.2 (64.1)</td>
<td>22.2 (12.6)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>35.1 (28.6)</td>
<td>17.1 (11.9)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>26.9 (28.7)</td>
<td>24.4 (16.8)</td>
</tr>
</tbody>
</table>

Table 3.16: Change in EGF concentration (pg/ml) over time course of the infusion for the control and fish oil group.

The variation in results is displayed graphically in the next plot.

Figure 3.23: Mean change of EGF concentration in the control and FO group over time.
GEEs were used to examine if there was a trend over time for any of the pathways in each group, and also whether these trends varied between the two groups. Due to highly skewed distribution of the outcome values, it was necessary to analyse this data on the log scale.

A summary of the analysis results is given in the next table. The first set of p-values indicates if there was a significant change in outcome values over time. The last interaction p-value indicates if the change over time varies between control and study groups. A significant interaction would suggest that the change over time varied between groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Term</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Linear</td>
<td>1.35 (1.09, 1.67)</td>
<td><strong>0.02</strong></td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>Squared</td>
<td>0.97 (0.94, 0.99)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish Oil</td>
<td>Linear</td>
<td>1.33 (1.07, 1.65)</td>
<td><strong>0.03</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Squared</td>
<td>0.97 (0.94, 0.99)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.17: Statistical analysis of the change in EGF concentration over time for the control and FO group, and the interaction between the two groups.*

The data suggested a non-linear relationship between time and EGF hence a more complicated relationship. It is difficult to picture the relationship from the regression coefficients alone, so the fitted relationship for each group is shown in the following graph.
Figure 3.24: Fitted relationship for change in EGF over time for the control and FO groups.

There was a significant effect of time upon EGF for both groups. The graph suggests that the nature of this effect is that there was an increase in values up to about 40 hours after which there was a decrease in EGF values.

There was no significant interaction between time and treatment group, suggesting that the changes over time did not vary between groups.

3.4.5 Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a paracrine cellular growth factor. It acts primarily on epithelial and endothelial cells via the tyrosine kinase-signaling cascade after binding to the proto-oncogenic c-MET receptor. It has a central role in angiogenesis, tumourgenesis and tissue regeneration [Bussolino 1992]. HGF has been shown to confer a growth advantage to human breast cancer xenotransplants, linked with higher microvessel density [Lamszus 1997]. Studies have also shown that EGF acts in synergy with VEGF for the induction and amplification of angiogenesis [Van Belle 1998, Xin 2001]. It had also been...
shown to increase VEGF expression [Wojta 1999] and act independently of VEGF in angiogenesis induction [Sengupta 2003].

There are no studies investigating the effect of n-3 PUFAs on HGF.

3.3.5.1 Hepatocyte growth factor Results

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>Baseline</td>
<td>12295 (9632)</td>
<td>8822 (5196)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>11368 (5878)</td>
<td>8289 (3461)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>11434 (8947)</td>
<td>10485 (9652)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>24594 (21757)</td>
<td>27138 (14196)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>13115 (9187)</td>
<td>9078 (3109)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>13764 (5508)</td>
<td>9087 (1682)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>13339 (6909)</td>
<td>7618 (2578)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>16068 (8129)</td>
<td>8283 (3891)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>11518 (5534)</td>
<td>7577 (1660)</td>
</tr>
</tbody>
</table>

Table 3.18: Change in HGF concentration (pg/ml) over time course of the infusion for the control and fish oil group.

The mean values at each time are displayed graphically in the next plot.
As with a number of the other outcomes, the HGF measurements were found to have a highly positively skewed distribution. Therefore, again the GEE analyses were performed with this outcome on the log scale.

The results of the analysis are summarised in the next table. Due to log transformation, the effect of time upon the outcome is given in the form of a ratio.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>Control</td>
<td>1.03 (0.98, 1.08)</td>
<td>0.29</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.96 (0.91, 1.01)</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.19: Statistical analysis of the change in HGF concentration over time for the control and FO group, and the interaction between the two groups.

The results suggested that, as a general trend, there was no significant change in HGF values over the course of the study for the placebo group. For the fish oil group there was very weak evidence of a reduction in values over time,
although this result was not quite statistically significant (p=0.09). In the fish oil group, on average, values reduced by 4% during each 10-hour period.

There was also some evidence of a significant interaction between time and group, although this result was of borderline statistical significance (p=0.05). This suggests some evidence that the change in HGF values over time varied significantly between the placebo and fish oil groups.

3.4.6 Angiopoitin-2

Angiopoitins (ANG) are proteins involved in the regulation of angiogenesis. ANGs act through the Tie2 tyrosine kinase receptor. The exact role of ANGs are poorly understood as is their exact function. It was initially thought that ANG-1 and ANG-2 were pro- and anti- angiogenic respectively. The characterization of ANG-1 and ANG-2 as pro- and anti- angiogenic was based on the ability of ANG-2 to bind to the Tie2 receptor and block pro-angiogenic effect of ANG-1 [Lobov 2002].

However, as understanding of ANG-2 develops it appears that ANG-2 has a dual pro- and anti-angiogenic capacity dependent on the surrounding environment and co-factors. ANG-2 expression is tightly controlled and almost entirely restricted to endothelial cells [Stratmann 1998, Fiedler 2006] and in 'normal' states is expressed at low levels. Examination of normal colonic mucosa and colon cancer specimens has revealed that there is a larger expression of ANG-2 in cancer tissue compared to ANG-1 [Ellis 2002]. This finding would contradict the initial hypothesis of ANG-2 being purely anti-angiogenic. It is thought that this imbalance of ANG-1 to ANG-2 makes the vasculature more plastic and amenable to sprouting [Wicki 2007].
In microvascular endothelial cells cultured in a three-dimensional collagen gel, ANG-2 can also induce Tie2 activation and promote formation of capillary-like structures [Mochizuki 2002]. At the same time, in vivo, ANG-2 is expressed during development at sites where blood vessel remodelling is occurring [Maisonpierre 1997], as well as in highly vascularised tumours [Eggert 2000, Etoh 2001].

In vivo it has been demonstrated that ANG-2 alone promotes endothelial cell death and vessel regression, but in the presence of VEGF-A it promotes rapid increase in capillary diameter, remodelling of basal lamina, proliferation and migration of endothelial cells. Thus it appears that VEGF can convert the consequence of ANG-2 stimulation from anti- to pro-angiogenic [Lobov 2002]. ANG-2 mRNA expression is also strongly induced by tissue hypoxia [Mandriota 1998, Oh 1999].

Vascular endothelial growth factor up regulation coincident with ANG-2 expression induces angiogenesis at the margins of the tumour, allowing the tumour to thrive [Benjamin 1999]. ANG-2 has been found to be elevated in numerous tumours including angiosarcoma [Amo 2004] and colorectal cancer [Gu 2006, Goede 2008]. Furthermore, during treatment with chemotherapy and bevacizumab ANG-2 levels fall in responders and rise in non-responders [Goede 2008].

Little data exists about the effect of n-3 PUFA on ANG-2 despite the large volume of evidence of the action of n-3 PUFA on angiogenesis. One study has looked at the effect of n-3 PUFA on HUVECs [Szymczak 2008] but there is no data on n-3 PUFA and ANG-2 in humans.
The study in HUVECs showed that n-6 PUFAs stimulate and n-3 PUFAs inhibit major proangiogenic processes, including the induction of ANG-2 and MMP-9, endothelial invasion and tube formation, that are all usually activated by AA. The COX-mediated conversion of PUFAs to prostanoid derivatives participated in modulation of the expression of ANG-2. Thus, the n-6 PUFA-derived PGE2 augmented ANG-2 induction, whereas the n-3 PUFA-derived PGE3 suppressed the induction of ANG-2 [Szymczak 2008].

3.4.6.1 Angiopoietin-2 Results

A summary of the results of the outcome for the two groups are shown in the next table. The figures are the mean and standard deviation values at each time point measure in pg/ml.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG-2</td>
<td>Baseline</td>
<td>975 (823)</td>
<td>723 (281)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>926 (625)</td>
<td>654 (366)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>958 (739)</td>
<td>612 (280)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>941 (695)</td>
<td>625 (369)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>1012 (703)</td>
<td>682 (296)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>1168 (747)</td>
<td>798 (429)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>1254 (0.11)</td>
<td>738 (422)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>1428 (1007)</td>
<td>864 (511)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>900 (650)</td>
<td>776 (374)</td>
</tr>
</tbody>
</table>

*Table 3.20: Change in ANG-2 concentration (pg/ml) over time course of the infusion for the control and fish oil group.*

The change in results is also shown graphically, for each of the two groups, in the next graph.
GEEs were used to examine if there was a trend over time for any of the pathways in each group, and also whether these trends varied between the two groups. The LR time point was omitted from these analyses. A summary of the analysis results is given in the next table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Coefficient (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG-2</td>
<td>Control</td>
<td>57.1 (16.8, 97.4)</td>
<td>0.006</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>20.2 (-24.4, 64.8)</td>
<td>0.38</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.21: Statistical analysis of the change in ANG-2 concentration over time for the control and FO group, and the interaction between the two groups.

The analysis results suggested that for the placebo group there was a significant increase in values over the course of the study. The values significantly increased over time, with a mean increase of 57 pg/ml over a 10 hour period. There was no statistically significant increase in the fish oil group.
Despite a significant increase in one group, but not the other, there was no evidence of a significant interaction between time and group.

3.4.7 Transforming Growth Factor-β

Transforming Growth Factor-β is a member of a family of dimeric polypeptide growth factors that includes bone morphogenic proteins and activins. All of these growth factors share a cluster of conserved cysteine residues that form a common cysteine knot structure held together by intramolecular disulfide bonds [Massague 1998]. Virtually every cell in the body, including epithelial, endothelial, hematopoietic, neuronal, and connective-tissue cells, produces TGF-β and has receptors for it. TGF-β, acting through its signalling pathway acts as a tumour suppressor gene, arresting the cell cycle in G1 to stop proliferation, induce differentiation, or promote apoptosis [Blobe 2000].

In the initial stages of tumourgenesis, a cell loses its TGF-β–mediated growth inhibition as a result of mutation or loss of expression of the genes for one or more components of the TGF-β signalling pathway. TGF-β mutation or loss of expression occurs in many malignancies. For example, 100 percent of pancreatic cancers and 83 percent of colon cancers have a mutation affecting at least one component of the TGF-β pathway [Villanueva 1998, Grady 1999]

TGF-β directly stimulates angiogenesis in vivo [Pepper 1997]. In mice, targeted deletion of either TGF-β1 or type II TGF-β receptors results in decreased vasculogenesis [Dickinson 1995, Oshima 1996]. In addition, expression of the TGF- β and its TGF-β receptor endoglin is greatly increased during angiogenesis. Stimulation of angiogenesis is one of many mechanisms by which TGF-β stimulates the growth of late-stage tumours [Blobe 2000].
3.4.7.1 Transforming Growth Factor-β Results

A summary of the results of the values for the two groups are shown in the next table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Baseline</td>
<td>230562 (94167)</td>
<td>192307 (91710)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>220511 (59872)</td>
<td>173150 (59512)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>229351 (89258)</td>
<td>173417 (47462)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>238086 (144197)</td>
<td>192086 (86186)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>25354 (84502)</td>
<td>184957 (65794)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>206276 (67226)</td>
<td>183362 (67922)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>217368 (77922)</td>
<td>136775 (38746)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>215051 (84208)</td>
<td>178400 (36390)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>238554 (80824)</td>
<td>217482 (59705)</td>
</tr>
</tbody>
</table>

Table 3.22: Change in TGF-β concentration (pg/ml) over time course of the infusion for the control and fish oil group.

The change in results can also be shown graphically, for each of the two groups, in the next graphs. The first graph considered TGF-β values on their original scale of measurement, whilst the second graph used the log-transformed values for the graph.
GEEs were used to examine if there was a trend over time in each group, and also whether these trends varied between the two groups. A summary of the analysis results is given in the following table.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Control</td>
<td>0.99 (0.96, 1.03)</td>
<td>0.56</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.98 (0.95, 1.02)</td>
<td>0.43</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.23: Statistical analysis of the change in TGF-β concentration over time for the control and FO group, and the interaction between the two groups.

The analysis results suggested that there was no significant change in values over the course of the study for either of the two study groups. Additionally there was no significant interaction between the groups, suggesting that the change in values over time did not vary between placebo and fish oil groups.

### 3.4.8 Platelet derived growth factor

Platelet-derived growth factor was discovered 3 decades ago in a search for serum factors that stimulate the proliferation of arterial smooth muscle cells [Ross 1974].

The PDGFs are a family of dimeric disulfide-bound growth factors that exert their biologic effects by activating 2 structurally related tyrosine kinase receptors, the PDGF-α and PDGF-β receptors [Heidin 1999]. Traditionally, PDGF-AA, PDGF-BB, and PDGF-AB were the only known PDGF ligands, however two new members of the PDGF family have been identified: PDGF-CC and PDGF-DD [Li 2000, LaRochelle 2001, Bergsten 2001]. The polypeptide chains of PDGF consist of 8 cysteine residues that are perfectly conserved between the 2 chains, a characteristic similar to that of the VEGF family [Joukov 1997]

Platelet derived growth factors play a significant role in tumourgenesis. They have a significant role in the control of cell growth, division and in particular
angiogenesis by stimulating fibroblast and vascular smooth muscle cell motility and acting as a chemo attractant [Ross 1974, Heldin 1999]. It is also known that PDGFs up regulate VEGF expression [Guo 2003].

The majority of studies looking at the effects of n-3 PUFA on PDGF have been related to PDGFs function in atherosclerosis. However these observations are also pertinent to PDGFs role in tumour angiogenesis. Fox and DiCorleto showed that in vitro production of PDGF was inhibited by n-3 PUFAs [Fox 1988]. Terano and colleagues demonstrated that EPA inhibited PDGF binding to its receptor, reducing receptor signal transduction. These effects lead to inhibition of smooth muscle proliferation a pre-requisite for angiogenesis [Terano 1996]. n-3 PUFAs have also been shown to significantly inhibit PDGF-induced cell migration of human aortic smooth muscle cell in vitro [Mizutani 1997]. In unstimulated and adherence-activated monocytes, Baumann and colleagues concluded that human gene expression for PDGF-A and PDGF-B can be reduced by dietary n-3 FAs [Baumann 1999].

3.4.8.1 Platelet Derived Growth Factor-AA Results

A summary of the results for the two groups during the course of the study is summarised in the next table.

Scatter plots showing the graphical relationship between groups over the course of the study are shown below. The first graph treats PDGFaa values on the original scale of measurement, whilst the log transformed scale is used in the second graph.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AA</td>
<td>Baseline</td>
<td>30054 (55769)</td>
<td>12463 (8237)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>19452 (20310)</td>
<td>14153 (9272)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>22183 (33236)</td>
<td>12243 (6606)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>12877 (7377)</td>
<td>12789 (7695)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>13040 (7276)</td>
<td>12897 (6171)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>12005 (6789)</td>
<td>12228 (7232)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>13270 (10058)</td>
<td>11069 (6671)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>13140 (7210)</td>
<td>13504 (10185)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>18436 (14271)</td>
<td>16519 (10952)</td>
</tr>
</tbody>
</table>

Table 3.24: Change in PDGF-AA concentration (pg/ml) over time course of the infusion for the control and fish oil group.

Figure 3.29: Mean change of PDGF-AA concentration in the control and FO group over time.
GEEs were used to examine how the values changed over time within and between the two groups.

![Figure 3.30: Log-transformed mean change of PDGF-AA concentration in the control and FO group over time.](image)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AA</td>
<td>Control</td>
<td>0.98 (0.93, 1.03)</td>
<td>0.37</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.99 (0.94, 1.05)</td>
<td>0.71</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.25: Statistical analysis of the change in PDGF-AA concentration over time for the control and FO group, and the interaction between the two groups.

The analyses suggested that there was no statistically significant change in PDGF-AA values over time for either of the two groups. Similarly there was no significant difference between these groups in terms of the values over time.

3.4.8.2 Platelet Derived Growth Factor-AB Results

Similar analyses were performed for the PDGFab outcome. A summary of the results is given in the next table.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AB</td>
<td>Baseline</td>
<td>36544 (15725)</td>
<td>34393 (10359)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>35588 (15744)</td>
<td>34632 (7887)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>39094 (15190)</td>
<td>31887 (7373)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>36343 (13611)</td>
<td>30346 (7875)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>35006 (14955)</td>
<td>28842 (10105)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>34000 (12754)</td>
<td>28739 (8145)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>32705 (12965)</td>
<td>27141 (6070)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>33979 (12582)</td>
<td>31161 (7980)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>39071 (18210)</td>
<td>32748 (17527)</td>
</tr>
</tbody>
</table>

Table 3.26: Change in PDGF-AB concentration (pg/ml) over time course of the infusion for the control and fish oil group.

The results are illustrated graphically in the next two scatter plots, the first on the original scale, and the second on the log scale.

![Mean change of PDGF-AB concentration in the control and FO group over time.](image)

Figure 3.31: Mean change of PDGF-AB concentration in the control and FO group over time.
Formal analyses were performed to compare the outcome values over time, and examine how these changes varied between groups. The results are summarised in the subsequent table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-AB</td>
<td>Control</td>
<td>0.99 (0.96, 1.02)</td>
<td>0.39</td>
<td>0.92</td>
</tr>
<tr>
<td>PDGF-AB</td>
<td>Fish Oil</td>
<td>0.99 (0.95, 1.02)</td>
<td>0.40</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.27: Statistical analysis of the change in PDGF-AB concentration over time for the control and FO group, and the interaction between the two groups.

The GEE analysis suggested that PDGF-AB values did not significantly change over time for either the placebo or fish oil group. Additionally there was no interaction between time and group, suggesting the pattern of values over time did not significantly vary over time.

3.4.8.3 Platelet Derived Growth Factor-BB Results

A summary of the results at each time point is given in the next table.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-BB</td>
<td>Baseline</td>
<td>1799 (748)</td>
<td>1345 (605)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>1869 (1472)</td>
<td>1181 (270)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>1643 (727)</td>
<td>1273 (398)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>1978 (2471)</td>
<td>1343 (429)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>2085 (1316)</td>
<td>1232 (303)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>1909 (873)</td>
<td>1258 (708)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>1669 (589)</td>
<td>1160 (380)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>1846 (763)</td>
<td>1236 (362)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>2502 (1680)</td>
<td>1544 (431)</td>
</tr>
</tbody>
</table>

Table 3.28: Change in PDGF-BB concentration (pg/ml) over time course of the infusion for the control and fish oil group.

The mean values at each time point for both placebo and fish oil groups are displayed graphically in the next plots. The first plot has PDGF-BB on the original measurement scale, whilst the log scale is used in the second scatter plot.

![Figure 3.33: Mean change of PDGF-BB concentration in the control and FO group over time.](image)
Figure 3.34: Log-transformed mean change of PDGF-BB concentration in the control and FO group over time.

GEEs were used to analyse the data, and the results are summarised in the next tables.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-BB</td>
<td>Control</td>
<td>1.02 (0.97, 1.08)</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.99 (0.92, 1.05)</td>
<td>0.69</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.29: Statistical analysis of the change in PDGF-BB concentration over time for the control and FO group, and the interaction between the two groups.*

The analysis of results suggested that there was no significant change in PDGF-BB values over the course of the study for either of the two groups. Additionally, the change over time did not significantly vary between placebo and fish oil groups.
3.4.9 Matrix Metalloproteinase's and Tissue Inhibitors of Metalloproteinase's

The ability of cancer cells to invade other tissues and spread to distant organs is an often-fatal characteristic of malignant tumours. Proteolytic enzymes play a fundamental role in cancer progression providing access for tumour cells to the vascular and lymphatic systems, which support tumour growth and dissemination [Chambers 2002, Mareel 2003]. Among all the proteolytic enzymes potentially associated with tumour invasion, the members of the MMP family have reached an outstanding importance due to their ability to cleave virtually any component of the extracellular membrane and basement membranes, thereby allowing cancer cells to penetrate and infiltrate the subjacent stromal matrix [Brinckerhoff 2002].

Matrix metalloproteinase's are zinc dependent endopeptidases, distinguished from other endopeptidases by their dependence on metal ions as cofactors. MMPs play a major role in tumourgenesis and cell proliferation, inducing cell migration and adhesion, matrix degradation, differentiation, angiogenesis and apoptosis. This leads to them being an integral part of tumour growth and specifically invasion and metastases [Folgueras 2004]. However recent evidence suggests that some of the MMP sub family may play a paradoxical protective role in tumour progression [Folgueras 2004].

There are over 20 different subtypes of MMPs categorized and subdivided by their function – collagenases gelatinases, stromelysins, matrilysins and membrane type MMPs. Collagenases gelatinases, matrilysins and stromelysins are all secreted and present within the circulation allowing measurement within plasma [Vihinen 2002, Visse 2003, Folgueras 2004].

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The gelatinases, MMP-2 and MMP-9 are the key MMP effectors in tumourgenesis. Amongst the other mechanisms discussed above, MMP-2 and 9 play a critical role in proteolysis of the basement membrane – a key phase in sprouting angiogenesis [Spencer 2009]. They are also involved in capillary sprouting [Milkiewicz 2006]. Several pro-angiogenic factors such as VEGF, basic fibroblast growth factor and TGF-β are induced or activated by these enzymes, triggering the angiogenic switch [Bergers 2000, Yu 2000, Mohan 2000, Sounni 2002, Belotti 2003]. An additional connection between angiogenic factors and MMPs derives from the finding that MMP-9 is induced in tumour macrophages and endothelial cells promoting lung metastasis [Hiratsuka 2002]. Furthermore, host-derived MMP9 contributes to the malignant behaviour of ovarian carcinomas by promoting neovascularisation [Huang 2002].

Matrix metalloproteinase’s were heralded as a promising target for cancer therapy on the basis of their massive up-regulation in tumour tissue and the unique ability to degrade all components of the extracellular matrix. However, clinical trials using synthetic inhibitors of MMPs as anti-neoplastic agents have been disappointing. The inhibitors used have been broad-spectrum MMP inhibitors rather than ones focusing on MMP-2 and -9, for which there is maximal evidence of an a anti-tumour role [Folgueras 2004]. They have also performed poorly in trials due to adverse toxicity and side effects [Coussens 2002]. A naturally occurring inhibitor of MMPs may have a role to play in oncological treatment.

The activity of MMPs is controlled by a series of endogenous inhibitors. Some of them are general protease inhibitors such as α2-macroglobulin, which mainly blocks MMP activity in plasma and tissue fluids, whereas other inhibitors such as tissue inhibitors of metalloproteinase’s (TIMPs) are more specific
TIMPs reversibly inhibit MMPs in a stoichiometric manner [Gomis-Ruth 1997]. The inhibitory activities of TIMPs suggest that the net balance between MMPs and TIMPs is a major determinant of the proteolytic potential of tumours. This has been supported by several studies showing that overproduction of TIMPs reduces experimental metastasis [DeClerck 1994], whereas low levels of these inhibitors correlate with tumourgenesis [Khokha 1989]. Moreover, TIMP-2 inhibits endothelial cell proliferation in vitro and angiogenesis in vivo through a MMP-independent mechanism [Seo 2003]. TIMP-2 can also block the binding of VEGF to VEGF receptor-2, thereby inhibiting down-stream signalling and angiogenesis [Qi 2003]. However, several studies have shown that TIMP levels also increase during tumour progression. It is not known whether these levels are compensatory increases related to the associated MMP rise or whether they may exhibit growth-promoting activities. The role of TIMPs in cancer progression is much more complex than that derived purely from MMP inhibitory function [Baker 2002, Jiang 2002].

There is early data to suggest that n-3 PUFAs have an effect on MMP levels. EPA reduces MMP-2 and -9 mRNA production in models of VEGF induced angiogenesis [Tsuzuki 2007]. EPA has also been shown to decrease MMP-2 and -9 levels human breast cancer cells both in vitro and in vivo [Liu 1995]. n-3 PUFAs also inhibit major proangiogenic processes in human endothelial cells, including the induction of ANG-2 and MMP-9 in a COX dependent pathway [Szymczak 2008].

Recently in a human study, long-term dietary supplementation of n-3 PUFAs decreased levels of both MMP-2 and -9 when compared to baseline and placebo. Paradoxically the levels of TIMP-1 and -2 were also decreased [Derosa
2009]. However a study in rats showed decreased levels of TIMP-1 with n-3 PUFA supplementation [Vardar-Sengul 2008].

3.4.9.2 Matrix Metalloproteinase-2 Results

An examination of the MMP-2 values suggested that they were more normally distributed. As a result, a log transformation was not required for this outcome, and thus the values were analysed on their original scale of measurement. A summary of the mean and standard deviation value in each group at each time is shown below.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Baseline</td>
<td>226181 (115024)</td>
<td>163198 (73275)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>190300 (79050)</td>
<td>156420 (66590)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>183812 (63488)</td>
<td>143668 (50958)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>172757 (62147)</td>
<td>169923 (81884)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>206857 (81441)</td>
<td>166080 (70547)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>207942 (75639)</td>
<td>161548 (80060)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>199279 (70384)</td>
<td>147571 (95904)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>210003 (76679)</td>
<td>165867 (98450)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>212903 (69435)</td>
<td>172270 (115480)</td>
</tr>
</tbody>
</table>

Table 3.30: Change in MMP-2 concentration (pg/ml) over time course of the infusion for the control and fish oil group.

A graphical illustration of the mean MMP-2 value at each time point is shown in the next graph.
Figure 3.35: Mean change of MMP-2 concentration in the control and FO group over time.

GEEs were used to examine how the MMP-2 values varied over time. As no log transformation was required for this outcome, the regression coefficients are reported along with their corresponding confidence intervals. These represent the change in MMP-2 values for a 10-hour increase in time for each treatment group.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Coefficient (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Control</td>
<td>982 (-5782, 7746)</td>
<td>0.78</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>-1062 (-8540, 6415)</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.31: Statistical analysis of the change in MMP-2 concentration over time for the control and FO group, and the interaction between the two groups.

The analysis results suggested that there was no significant change in MMP-2 values over time for either the placebo or fish oil group. The analysis also indicated that the change in values over time was not significantly different between the two groups.
### 3.4.9.2 Matrix Metalloproteinase-9 Results

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>Baseline</td>
<td>584107 (685831)</td>
<td>501388 (771419)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>751558 (1030825)</td>
<td>487547 (687060)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>707110 (1262483)</td>
<td>428682 (516165)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>698120 (1128512)</td>
<td>353706 (423993)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>508928 (493479)</td>
<td>506745 (443190)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>875738 (829088)</td>
<td>904259 (1007234)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>742961 (707741)</td>
<td>553812 (426750)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>897822 (1060462)</td>
<td>375479 (349832)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>402135 (483960)</td>
<td>450254 (533063)</td>
</tr>
</tbody>
</table>

*Table 3.32: Change in MMP-9 concentration (pg/ml) over time course of the infusion for the control and fish oil group.*

Along with the majority of outcomes, this outcome was also positively skewed in its distribution. As a result the main analysis was performed on the log-transformed scale.

A graphical illustration of the results over time on both the original and log scales are shown in the next graphs.

GEEs were used to examine if there was a trend over time in each group, and also whether these trends varied between the two groups. As with the analysis of all outcomes, the LR time point was omitted from these analyses.
A summary of the analysis results is given in the next table. For this outcome, a linear relationship did not sufficiently indicate the shape of the relationship between time and MMP-9, so instead a curve relationship was used. As a result, there are two terms to capture this relationship for each group.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Term</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>Control</td>
<td>Linear</td>
<td>1.55 (1.20, 1.97)</td>
<td>0.003</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squared</td>
<td>0.95 (0.92, 0.98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>Linear</td>
<td>1.55 (1.20, 2.00)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squared</td>
<td>0.95 (0.92, 0.98)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.33: Statistical analysis of the change in MMP-9 concentration over time for the control and FO group, and the interaction between the two groups.

The results suggested that in both of the two groups, there was a significant change in the MMP-9 values over time. As the relationship between time and MMP-9 was non-linear, this is more difficult to picture the relationship from the regression coefficients alone, so the fitted relationship for each group is shown in the next graph.

![Fitted relationship for change in MMP-9 over time for the control and FO groups.](image)

Figure 3.38: Fitted relationship for change in MMP-9 over time for the control and FO groups.

The graph suggests an increase in MMP-9 values up to around 40 hours, after which these decreased again.
Although the values in the two groups both changed over time, there was no significant interaction between time and group ($p=0.95$). This suggests that the change in values over time did not vary between the two groups.

### 3.4.9.3 Tissue Inhibitor of Matrix Metalloproteinase-1 Results

A summary of the results of the outcome for the two groups are shown in the next table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1</td>
<td>Baseline</td>
<td>355223 (456446)</td>
<td>369544 (351913)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>300941 (363033)</td>
<td>268836 (247934)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>314989 (391999)</td>
<td>280201 (263419)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>244632 (289985)</td>
<td>273156 (250222)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>371608 (450439)</td>
<td>302381 (275617)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>373164 (389829)</td>
<td>374678 (341153)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>327381 (386239)</td>
<td>306468 (308488)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>306883 (326662)</td>
<td>315187 (343004)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>416973 (490515)</td>
<td>275011 (307257)</td>
</tr>
</tbody>
</table>

*Table 3.34: Change in TIMP-1 concentration (pg/ml) over time course of the infusion for the control and fish oil group.*

The variation in results is displayed graphically in the next plot, firstly on the original scale of measurement, and then on the log transformed scale.
GEEs were used to examine if there was a trend over time in each group, and also whether these trends varied between the two groups. A summary of the analysis results is given in the next table. The outcome was analysed on the log scale, the results are presented in the form of ratios.
Table 3.35: Statistical analysis of the change in TIMP-1 concentration over time for the control and FO group, and the interaction between the two groups.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Term</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1</td>
<td>Control</td>
<td>Linear</td>
<td>1.24 (1.03, 1.49)</td>
<td>0.05</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squared</td>
<td>0.98 (0.96, 1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish Oil</td>
<td>Linear</td>
<td></td>
<td>1.20 (1.00, 1.45)</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squared</td>
<td>0.98 (0.96, 1.00)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results suggested that there was some statistically significant effect of time upon the TIMP-1 values for both groups, although these results were only of borderline statistical significance (p=0.05). As the relationship between time and TIMP-1 was non-linear, it is more difficult to picture the relationship from the ratios alone, so the fitted relationship for each group is shown in the next graph.

Figure 3.41: Fitted relationship for change in TIMP-1 over time for the control and FO groups.

The graphs suggest that the TIMP-1 values increased for the first 40 hours, after which there was a reduction in values. This pattern was replicated for both groups. Additionally, there was no significant interaction between group and
time, suggesting that the change over time was not significantly different for the two groups.

3.4.9.4 Tissue Inhibitor of Matrix Metalloproteinase-2 Results

The next outcome considered was TIMP-2. A summary of the results in the two groups is given in the next table.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Time</th>
<th>Control Mean (SD)</th>
<th>Fish Oil Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-2</td>
<td>Baseline</td>
<td>91928 (29897)</td>
<td>91410 (35111)</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>90252 (35810)</td>
<td>85162 (32464)</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>87594 (24272)</td>
<td>81761 (27130)</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>85293 (24428)</td>
<td>76710 (222313)</td>
</tr>
<tr>
<td></td>
<td>20 hours</td>
<td>89708 (25718)</td>
<td>73597 (19483)</td>
</tr>
<tr>
<td></td>
<td>44 hours</td>
<td>100166 (44711)</td>
<td>72907 (29351)</td>
</tr>
<tr>
<td></td>
<td>68 hours</td>
<td>81972 (32652)</td>
<td>64249 (16001)</td>
</tr>
<tr>
<td></td>
<td>74 hours</td>
<td>84514 (28478)</td>
<td>74914 (28492)</td>
</tr>
<tr>
<td></td>
<td>LR</td>
<td>89989 (19686)</td>
<td>92013 (32934)</td>
</tr>
</tbody>
</table>

Table 3.36: Change in TIMP-2 concentration (pg/ml) over time course of the infusion for the control and fish oil group.

Graphical illustrations of the mean TIMP-2 values in the two groups at each time point is shown in the next graphs. The first graph used TIMP-2 on the original scale of measurement, whilst the second graph has the log-transformed values.
GEEs were used to examine how the results varied over time for the two groups, and the results are summarised in the next table. The effects of time upon the outcome in each group are given in the form of ratios, these indicating the relative change in TIMP-2 values for a 10-hour increase in time.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Group</th>
<th>Ratio (95% CI)</th>
<th>P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-2</td>
<td>Control</td>
<td>0.99 (0.96, 1.02)</td>
<td>0.42</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>Fish Oil</td>
<td>0.98 (0.94, 1.01)</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.37: Statistical analysis of the change in TIMP-2 concentration over time for the control and FO group, and the interaction between the two groups.

The results suggested that there was no significant change in TIMP-2 values over time in either the fish oil or placebo groups. Additionally there was no evidence of a significant interaction between time and group. In other words no significant evidence that the change in TIMP-2 values over time varied between the two groups.

3.4.10 Discussion

It was anticipated that a 72 hours infusion of EPA plus DHA would have a beneficial effect – anti-angiogenic - on the measured circulating cytokine concentrations. However in general this was not observed in a statistically significant way using the chosen method of analysis.

We utilized a statistical model based around GEEs to perform a single analysis of all time points combined, as our principle interest was how the two groups varied over time, and whether the profile of values varied between groups. Utilising this statistical method the results were overwhelmingly non-significant. The main reason behind this appears to be two-fold, firstly the cohort size is small and large differences would be required for a significant difference to be observed. This is further confounded by the heterogeneity of the cytokine baseline values between the patients. Unlike biochemical measurement used in everyday medicine such as haemoglobin, which have standardized ranges, cytokine levels are generally utilized in the experimental arena and I found the
levels differed greatly within the groups leading to a large standard deviation of measurements. However statistically significant changes were observed over time in EGF (both groups increased), HGF (decrease in n-3 group), ANG2 (increase in control group), and MMP-9 (both groups increased). The increases seen in EGF, ANG2 and MMP-9 are possibly related to TPN infusion.

On closer inspection however, there were some differences observed between the groups, for example the concentration of EGF and MMP-9 increased far more in the control group in comparison to the n-3 group. This stimulated my curiosity and further simplified analysis was carried out. As already discussed with such data, there are a number of different analyses that can be performed, such as comparisons of specific time points, or producing a single summary of all data points for analysis. If changes in cytokine concentration were looked for between baseline and specific time points within the group and differences between the n-3 and controls groups at specific time points we start to see statistical significance. Analysis was carried out looking at single time point differences using a Wilcoxon signed rank test.

VEGF A was significantly reduced compared to controls one hour after the start of the infusion (p=0.001). The statistically significant decrease persisted at all timepoints during the infusion and even when the infusion had been stopped at 74 hours. VEGF C was also significantly reduced compared to control (p=0.01) after 6 hours of infusion. The decrease remained throughout the infusion and levels returned towards baseline after the infusion had finished. There was a reduction in VEGFR2 but due to the sample size this was not statistically significant (p=0.056). EGF levels in the n-3FA group were significantly less than the control group at all time points with a maximal difference at 66 hours following the start of the infusion (p=0.008). ANG2 produced a similar trend
(p=0.018). Levels of both EGF and ANG2 recovered towards baseline 2 hours after cessation of the infusion in both the n-3FA and control groups.

These results are by no means proof and show how statistics can be manipulated to give significance, however they do suggest that there maybe mileage in further investigation of the effect of n-3 PUFA on circulating cytokine level in humans, especially with the profound changes in-vivo and in-vitro reported in the literature. This may suggest that n-3 PUFA infusion may have an effect on circulating cytokines but the limitations within this study precluded meaningful statistically significant detection.
3.5.0 The use of Magnetic Resonance Imaging as a surrogate marker of angiogenesis to detect changes in tumour microenvironment.

3.5.1 Introduction

Unlike conventional cytotoxic chemotherapies, which are often titrated to maximal tolerated dose, angiogenesis inhibitors may achieve therapeutic levels before toxicities arise [Kummar 2007]. Thus, it is necessary to identify biomarkers or surrogate clinical end points that reflect the effect of a drug on its target to predict response to treatment at different drug exposures [Citrin 2006, Jubb 2006, Korn 2001].

Biomarkers of angiogenesis inhibition can be divided into three categories: invasive measures, minimally invasive measures, and non-invasive measures [Brown 2008]. Each provides different information regarding the effect of the agent on tumour vasculature. However, it is not yet clear whether a lone marker could be a ‘gold-standard’ or whether a panel of markers may be required for an optimal assessment of angiogenesis.

Invasive measures require target tissue sampling to characterize tumours and utilise techniques such as microarray, immunohistochemistry and proteomic analysis. Minimally invasive methods include blood or other bodily fluid sampling and medical imaging techniques. Blood tests include sampling of circulating markers of angiogenesis. This includes measurement of growth factors and cytokines as performed in this study using ELISA and newer markers such as endothelial cell surface molecules and circulating endothelial and endothelial precursor cells [Brown 2008].
Medical imaging methods are based upon the utilization of existing and novel imaging techniques which allow measurement of a wide range of parameters relating to molecular, physiologic or metabolic factors such as tumour perfusion, oxygenation and glucose metabolism [Harry 2010]. They have a key potential advantage over other techniques in that they allow serial measurements ‘in vivo’ allowing spatial mapping of factors within the whole tumour and all tumours in the body. This is important as tumour heterogeneity and phenotype may be important to treatment and response decisions [Jackson 2007, Morgan 2011, Marusyk 2012].

Molecular and functional imaging utilises tracers or biomarkers to reveal specific physiological activities within an anatomical location. Biomarkers interact chemically with their surroundings and subsequently alter the image according to molecular changes occurring within the area of interest. Molecular or functional imaging modalities that have been used to assess tumour response to therapy include ultrasound scanning (USS) [De 2005, Drevs 2006, Schirner 2004, Cosgrove 2003, Forsberg 2004, Abdollahi 2003, Fury 2007], functional computed tomography (CT) [Cuenod 2006, Lee 2003, Miles 2003, Taeishi 2001 & 2002, Yi 2004] and positron emission tomography (PET) [Drevs 2006, Willwt 2007, Herbst 2002, Jennens 172, Mullamitha 173, Turner 2007]. These techniques are an integral part of the clinical assessment of tumours; however in the quantification of angiogenic response these techniques are limited. The use of USS is restricted by the requirement for experienced operators to reduce inter- and intra-observer variability [Mross 2005]. CT is limited by concerns about recurrent ionising radiation exposure [Brown 2008] and results are similar to those seen with MRI [Kim 2004]. $^{18}$FDG-PET is currently limited by cost, poor anatomical resolution, the requirement for a radioactive isotope and lack of understanding of

The current ‘gold-standard’ imaging modality for assessment of early tumour changes in response to anti-angiogenic agents is probably Dynamic Contrast Enhanced MRI (DCE-MRI) [Hylton 2006]. DCE-MRI can be performed on most standard clinical MRI systems available in the majority of centres (albeit with increased complexity and potential out-sourcing of image analysis), does not involve ionising radiation, is non-invasive, does not use toxic contrast agents and provides relatively good spatial resolution.

3.5.1.1 Dynamic Contrast Enhanced Magnetic Resonance Imaging

Dynamic contrast enhanced magnetic resonance imaging can provide data to measure parameters that relate to the microvascular structure and function of tumours utilising an injected contrast agent (gadolinium (Gd) chelate). This is an extra-vascular, extra-cellular contrast agent as defined by it rapidly leaking from intra-tumoural vessels (capillaries and venules) into the tumour extravascular space. This is passive transfer rather than by active pump mechanisms - see figure 3.44 [Su 1998].

Clinically available MRI contrast agents do not leak into the intracellular space (V_i) in normal physiological conditions, although it may occur following cell death. Contrast agent leakage is governed by the concentration difference between the plasma and the extracellular extravascular space and by the permeability and surface area of the capillary endothelia (P).
Dynamic contrast enhanced magnetic resonance imaging will acquire multiple images before and dynamically after a contrast injection - normally by bolus (Leech 2012). The majority of approaches manually select a region of interest (ROI) - either single or divided into multiple voxels - to measure contrast signal changes within the tumour and relate this to contrast concentration in a supplying artery (the arterial input function or AIF). This allows for changes in physiological variations in cardiac output, vascular tone, renal function and injection timing to be eliminated [Dowlati 2002, Liu 2005]. Some centres use a 'modelled' AIF based on injected contrast concentrations as imprecision due to cardiac output may be outweighed by imprecision in measuring the AIF accurately [Morgan 2006, Morgan 2003, Thomas 2005]. When planning scan sequences for acquisition of DCE-MRI images concessions between special resolution, time resolution and spatial coverage must occur and to increase spatial resolution image capture times are prolonged. However compromise is required because image acquisition needs to be rapid to reduce the risk of patient movement - and associated artefact - and fully capture the dynamic
contrast uptake in sequentially captured images over a set period of time [Morgan 2008].

To achieve high temporal resolution and avoid the need for breath holding scan parameters using single slices and individual image acquisition times of less than 500 ms can be used, effectively freezing motion owing to respiration. When imaging liver tumours, respiratory motion in the abdomen and lower part of the lung is largely in the cranio–caudal direction so a coronal or sagittal-oblique plane can be used to keep the tumour within the imaged slice during the dynamic run [Morgan 2006].

Within the tumour ROI - a defined region used for measurement that encompasses all or part of the tumour - signal enhancement from Gd on T₁-weighted images is dependent upon alterations in plasma volume ($V_p$), vascular permeability and the available extra-vascular, extra-cellular space (EES or $V_e$) [Tofts 1999]. Measuring these parameters requires creating physiological models for the pharmaco-kinetics of the agent and estimating the parameters from the observed concentration-time curves for the feeding artery (arterial input function, AIF) and the tumour [O'Connor 2007] (figure 3.50). To allow more robust analysis, conversion of signal intensity to contrast concentration is ideal, as the relationship between signal intensity and contrast agent concentration is non-linear and varies with differing MRI pulse sequences [Morgan 2008, Tofts 1999, Parker 2005]. The concentration-time curve can be analyzed qualitatively, semi-quantitatively or quantitatively using physiological models of varying complexity. The possible methods of measuring the MRI contrast parameters are discussed below.
Qualitative analysis may look at the shape of the curve for benign or malignant patterns and are now used in clinical breast cancer imaging [Warren 2005]. Curves are evaluated against defined standards to determine whether a lesion is benign or malignant.

Semi-quantitative methods include peak enhancement or the peak slope of the enhancement curve. These are simple descriptions of contrast agent distribution and show considerable variation between acquisition method and individual examinations making comparison between patients and trials difficult [O’Connor 2007]. A more robust semi-quantitative approach is measuring the area under the contrast agent concentration curve to a fixed time after the contrast injection – initial area under the enhancement curve (IAUC) [Morgan 2007, O’Connor 2007]. IAUC measurement gives no direct physiological meaning but is robust, repeatable and depends on tumour vascularity [Morgan 2006]. IAUC has a complicated and incompletely defined relationship with underlying tumour physiology and represents a composite of physiological processes, which will vary depending on whether the IAUC is measured to 60 or 180 seconds [Tofts 1999, Evelhoch 2005].
Pharmacokinetic models can be applied to enable estimates of physiological characteristics, such as flow and capillary endothelial permeability. Modelled parameters are in theory more physiologically meaningful than simple descriptors, such as IAUC [O’Connor 2007, Leach 2005]. Theoretically they should be independent of acquisition protocol and solely reflect tissue characteristics but it is highly unlikely that intra patient reproducibility would be acceptable using different MRI platforms or sequences in reality [Leach 2012].

Modelled parameters require us to make assumptions that the imaged tumour or ROI is composed of compartments (see figure 3.49) and that there is no active membrane transport mechanism between these compartments, thus leak/flux of contrast is solely dependent on concentration gradients between the plasma and the EES [Tofts 1999]. Clinically available MRI contrast agents do not leak into the intracellular space in normal conditions thus giving us a 2-compartment model consisting of the $V_p$ and the EES. Consensus opinion suggests that in clinical trials, assessment of anti-angiogenic and vascular disrupting agents is based on the 2-compartment model, assessing the volume transfer coefficient of contrast between blood plasma and the EES or leak rate constant ($K_{\text{trans}}$) and the size of the EES or $V_e$ [Leach 2005 and Leech 2012].

The two compartmental modelled bi-directional transfer constant $K_{\text{trans}}$ is expressed as [Tofts 1999]:

$$\frac{dC_t}{dt} = K_{\text{trans}} \left( C_p(t) - \frac{C_t(t)}{V_e} \right)$$

Where $C_p$ is the concentration of contrast agent in the blood plasma (AIF); $C_t$ is the contrast agent in the tissue, average over the whole tissue; $K_{\text{trans}}$ is the
transfer constant; and $V_e$ is the ESS volume fraction. Recently, Sourbron and Buckley have suggested that the original model from Tofts is only accurate if the tissue is weakly vascularised with a small blood volume and that for highly perfused areas a more complex extended Tofts model is required [Sourbron 2011]. However increasing the complexity of the model may not be appropriate if the data are too noisy.

The process of determining $K^{\text{trans}}$ and $V_e$ from a tissue and AIF (either measured or modelled) concentration-time curve is by fitting the model to the observed data and finding a solution for the unknown parameters in an iterative fashion. The parameters that fit the curve as closely as possible are given as the result [Horsfield 2004]. The quality of the curve fit can also be given as a result but it should be recognized that introducing an extra variable might improve curve fits even if the variable has no physiological relevance. Scans are performed pre and post treatment and parameters of interest can then be compared following therapy. Two baseline scans are recommended in order to study intra patient reproducibility and act as a quality control for the study [Galbraith 2003, Morgan 2006, Leach 2005, Leech 2012].

3.5.1.2 Clinical trials using Dynamic Contrast Enhanced-Magnetic Resonance Imaging

Numerous clinical trials utilising T1-weighted DCE-MRI have been reported [O’Connor 2007, 2012a, 2012b]. It is apparent that day-to-day variation in measured DCE-MRI parameters occurs due to random error, biological variation and systemic inaccuracies and as a consequence many investigators consider a rate of change in $K^{\text{trans}}$ of $> 40\%$ correlates with disease stability/response [Roberts 2006]. The majority of trials also utilise two baseline scans to reduce intra-patient variability and measure reproducibility for each trial.
dataset, which allows the investigator to have confidence that any significant parameter change is due to drug effect [O’Connor 2007, Galbraith 2003, Jayson 2005, Padhani 2006].

There is a growing recognition that biomarkers - including those derived from functional imaging - will play an increasingly important role in the drug development process [Padhani 2010]. DCE-MRI has been incorporated into several phase I and II trials of anti-angiogenic drugs with definitive evidence of drug efficacy [De Langen 2008, O’Connor 2007].

Several studies of various VEGFR-2 tyrosine kinase inhibitors (TKI) have demonstrated reductions in IAUC and $K^{\text{trans}}$ that are consistent with reductions in VEGF-dependent tumour perfusion and vascular permeability in patients with advanced cancer. However the clinical meaning of this response is still unclear. AG-013736 [Liu 2005] and AZD2171 [Drevs et al, 2005] have all shown dose-dependent reductions in $K^{\text{trans}}$ and IAUC without demonstrating clinical response [O’Connor 2007]. Conversely a recent study using vandetanib – an oral inhibitor of VEGFR, EGFR and RET signalling pathways did not show any modulation of IAUC or $K^{\text{trans}}$, despite previously demonstrating anti-tumour activity [Mross 2009].

In a phase II study, patients with advanced breast cancer were treated with bevacizumab and significant reductions in $K^{\text{trans}}$ and $v_o$ were seen, which persisted when cytotoxic chemotherapy was added. These were correlated with increased apoptosis and a reduction in VEGFR expression. However changes in $K^{\text{trans}}$ did not predict response rate [Wedam 2006].
Few trials have demonstrated a relationship between a reduction in DCE-MRI measurement parameters and clinical outcome measure. Correlation of $K^{\text{trans}}$ reduction with response rate and progression-free survival has been shown with BAY 43-9006 [O’Dwyer 2005]. A 40% cut off in reductions of $K^{\text{trans}}$ have predicted outcome in glioblastoma multiforme and colorectal liver metastases in a trial of PTK787/ZK222584 (PTK/ZK) a VEGFR TKI [Morgan 2003] [Conrad 2004]. A recent study of DCE-MRI in patients with progressive multiple myeloma treated with thalidomide found amplitude A, which is proportional to $K^{\text{trans}}$, to be a good prognostic indicator of progression-free survival [Hillehngas 2007].

Treatment with PTK/ZK in patients with colorectal carcinoma liver metastases demonstrated a mean reduction in $K^{\text{trans}}$ of 43% at day 2. This was greatest in higher-dose groups where $K^{\text{trans}}$ was reduced by 58% on day 2 and 53% at the end of cycle 1. The reduction in $K^{\text{trans}}$ from baseline at day 2 and end of cycle 1 was positively correlated with reduction in tumour size and predicted clinical response [Morgan 2003]. Statistically significant dose-dependent changes in $K^{\text{trans}}$ from baseline at day 2 and day 28 were identified in two subsequent studies of PTK/ZK in patients with mixed solid tumours [Thomas 2005]. These studies, as in trials of other TKI’s, AZD2171 [Drevs 2007] and BMS-582664 [Rosen 2006] have helped define the biologically effective dose to take into phase II studies. However in subsequent randomised phase III trials benefit from treatment with PTK/ZK compared with placebo in combination with chemotherapy did not reach statistical significance [Hecht 2005, Koehne 2006].

3.5.1.3 Other MRI methods used to assess tumour micro-environment

Recently, interest in the use of diffusion-weighted magnetic resonance imaging (DW-MRI) to measure pharmacological response of tumours has increased significantly [Padhani 2009]. DW-MRI depends on the microscopic-
mobility of water (Brownian mobility), which is due to thermal agitation and is highly influenced by the cellular environment of water and the structural integrity of tissues. Thus changes demonstrated on DW-MRI could be an early harbinger of biologic abnormality [Padhani 2009]. Presently the most established clinical indication for DW-MRI is the assessment of cerebral ischemia (stroke) where DW-MRI findings precede all other MR techniques [Yoshikawa 2008].

Within tumours, diffusion is classically restricted by high cellularity, tissue disorganization and extracellular tortuosity. A high apparent diffusion coefficient is suggestive of necrosis and has been linked to lesion aggressiveness as well as being a predictive marker of poor response to chemotherapy [Dzik-Jurasz 2004]. It is this quantification of necrosis that makes DW-MRI attractive as an indicator of tumour response to therapy [Thoeny 2005]. Furthermore parameters derived from DW-MRI are also appealing as imaging biomarkers because the acquisition does not require any exogenous contrast agents unlike DCE-MRI and can be obtained relatively rapidly and easily incorporated into routine patient evaluations. DW-MRI has already been shown to be useful in monitoring therapy in a number of different tumours and its use remains an area of burgeoning investigation [Padhani 2009].

Blood oxygen level dependent (BOLD) contrast MRI is an indirect measure of assessing vasculature maturation and may predict response to anti-vascular therapy [Eberhard 2000, Taylor 2001]. BOLD uses heavily T2*-weighted imaging that can depict change in blood oxygenation. The T2* signal is affected by vasodilatation, thus assessment can be made of blood vessel maturity as a marker of angiogenesis, because immature vessels do not respond to vasodilatation due to lack of smooth muscle activity. However the most useful applications of this technique require inhalation of carbogen (oxygen and high
carbon dioxide concentration) to produce hyperoxia and hypercapnia which is unpleasant [Taylor 2001].

However, in its basic form the T2* relaxation rate harnessed in BOLD-MRI is linearly related to the level of oxygen saturation. An increase in the blood oxygen fraction reduces the paramagnetic effect of deoxyhaemoglobin causing a signal increase on T2* due to transverse relaxation. Tumour hypoxia is an important marker of angiogenesis and has been shown to correlate with tumour invasiveness, response to treatment and survival in several types of cancers [Brizel 1996].

Another MR technique to assess tumours is MR spectroscopy. The subtle shift in resonance frequency of protons in different chemical environments, known as ‘chemical shift’, allows estimation of the concentration of some common metabolites in the body. The technique initially had poor sensitivity, however with the increased availability and use of high magnetic field MR scanners (≥ 3 Tesla) the sensitivity has improved. Elevated choline levels detected by MR spectroscopy correlate with malignancy and cell proliferation, and can be used successfully to monitor treatment of patients with brain tumours [Murphy 2004] and for neoadjuvant breast cancer therapy [Meisamy 2004]. However quantifying MR spectroscopy data is difficult. Results are highly dependent on where the area for measurement (‘region of interest’) is set, which can change after treatment [Morgan 2011].

I quantified the effect of a 72 hours intravenous infusion of n-3 PUFA, derived from FO, on MRI parameters (DCE-MRI, DWI-MRI and T2*) of hepatic colorectal liver metastases versus controls. My aim was to measure the reproducibility of the MRI measurements in this patient group and answer the
following question: ‘does FO cause a physiologically meaningful change in MRI parameters in the study cohort or individual receiving FO compared with control?’

3.5.2 Methods

Each patient underwent a total of three MRI scans – two ‘baseline’ prior to treatment 4 to 14 days apart and one within 2-4 hours post cessation of the FO infusion. See appendix 1 for the timing of scans and trial protocol, and appendix 2 for formal scan guidelines.

All MR images were acquired using the same Siemens Symphony 1.5 Tesla whole body scanner with phased array body surface coils. Initially the T2* study was performed in the coronal plane. Three separate sequences were carried out with a constant time to echo (TE) of 4.76 msec and a second TE of 19 msec, 33 msec and 47.6 msec respectively.

Subsequently the DWI sequence was captured in the axial plane with TR of 4500 ms and a TE of 77 ms. Pulsed field gradients of b0 and b650t were used.

For the DCE-MRI images a single slice saturation recovery FLASH (fast low-angle shot) spoiled gradient recalled echo sequence was performed. To achieve a high temporal resolution and avoid the need for breath hold, an individual image acquisition time of less than 500 msec was used with TR 3.3 msec, effectively avoiding motion artefact due to respiration. Since respiratory motion around the liver is largely cranio-caudal all image acquisition was in the coronal plane. This kept the tumour within the imaged slice during the dynamic run. A representative disease site in the liver was selected. Suitable lesions were all solid or non-necrotic lesions with a minimum size of 3 cm. This became the
imaged tumour for all subsequent scans. A single axial slice was acquired to include liver, tumour and the aorta for determination of AIF. T1-weighting was generated with an effective TI (time between saturation pulse and centre of k-space) of 655 msec and a flip angle of 16°. The echo time (TE) was 1.4 msec. The dynamic run consisted of 150 measurements taken 3 seconds apart (TR0 = 3000msec). The field of view was selected depending on subject size and remained constant for subsequent scans. Low molecular weight Gadolinium-chelate 0.2 ml/kg (Magnevist®, Bayer Schering Pharma) up to 20 ml max was injected as a rapid bolus through an arm vein in less than 6 seconds followed by a saline flush. After initial calculation for weight, injection volume was kept the same for all 3 visits. Injection commenced after the first 6 measurements to allow magnetisation to reach a steady state and provide non-contrast enhanced images. Formal scan guidelines are shown in the appendix.

After acquisition, data was anonymised to a trial code and transferred to an in-house image analysis package (Jim, Version 4.0, Xinapse). Prior to analysis all the DICOM header was checked on all scans to ensure the correct sequences had been applied. Further the time-intensity curves from the ROI over the feeding blood vessel or aorta were assessed qualitatively to check the quality of the injection. Major differences in the scale and shape of the curve in comparison to other scans of the same patient were excluded as injection was assumed to be inadequate. Images were analysed blind to patient identity and time point. Defined regions of interest were manually drawn around the tumour of interest and aorta.
Figure 3.46: Image set showing a T1 (A) and T2 (B)-weighted image, demonstrating the tumour and the planned dynamic slice position (white line). Image (C) is the post contrast S0 image. The dynamic contrast enhancement series is represented by a precontrast (D), and early and late contrast enhanced images (E and F). The inset image in (D) shows how the ROI is drawn for analysis.

The ROIs were transposed to all slices and then manually adjusted on a slice-by-slice basis to ensure accurate spatial registration. The mean image intensity data for the ROI was then used to create a concentration time curve to generate IAUC, $K_{\text{trans}}$ and $V_e$ for statistical analysis.

3.5.2.1 Statistical analysis

Dynamic contrast-enhanced MR imaging is applied to assess changes caused by treatment, with patients acting as their own controls. Previous experience shows a wide inter-patient variability in pre treatment $K_{\text{trans}}$ and IAUC (coefficient of variation (CoV) of 61%). Changes due to treatment and variability are related to the magnitude of the baseline value and therefore the percentage rather than absolute changes of enhancement parameters are more useful for analysis [Morgan 2006, Morgan 2003].
Initially the reproducibility was evaluated of the observed parameters - IAUC, $k_{\text{trans}}$ and $V_e$ - between the two baseline scans (B1 and B2) after data transformation to percentage change as % error was not proportional to magnitude [Bland 1996a]. The within-subject coefficient of variation (wsCOV) can be used to predict the lowest mean percentage change that could be detected above random variation in any cohort. The repeatability shows the range within which 95% of measurements will fall for an individual and is therefore helpful in assessing the significance of individual patient results. This was calculated for all the scans performed with no distinction made for treatment group.

Statistical analysis of treatment effect was performed on the percentage changes of the observed parameter between the 2nd baseline scan and post treatment scan (D3). The mean changes were calculated and expressed as percentages [Bland and Altman, 1996a]. These percentages were then used for statistical comparison for within cohort change between B2 and D3 within the cohorts and subsequently for percentage change to D3 between the cohorts using students ‘t’ test.

3.5.3 Results

Twenty patients were randomised to receive either active substance - fish oil group, Lipidem® 20%, B Braun, UK) or control - Lipofundin® MCT 20%, B Braun, UK. Nineteen patients underwent the sequence of three MRI scans - B1, B2 and D3; nine in the fish oil group and 10 in the control group. One patient (from the control group) underwent B1 and B2 scanning but unfortunately prior to D3 scanning the MRI scanner developed a fault and we were unable to proceed with the scan. In total 59 MRI scans were performed. All scans were performed within the set time criteria and the time between B1 and B2 was < 14 days and
between B2 and D3 74 – 80 hours. The mean time between B1 and B2 scans was 9 days; 9 in the fish oil group and 8 in the control group. All D3 scans were performed between 2 and 4 hours post treatment cessation.

On review of the DCE-MRI images 15 scans had to be excluded from analysis due to protocol errors. Seven had problems with the contrast injection, as shown by quality control using the AIF concentration-time curve, 4 had incorrect field of view or scan plane, 2 had incorrect flip angles and two had incorrect image processing. A further 2 images were not included in analysis as 2 scans had already been excluded from that patient leaving no scans available for comparison and statistical analysis. As previously discussed the patients were well matched for sex, age and weight, and all were Caucasian.

3.5.3.1 DCE-MRI results - \( \kappa_{\text{trans}} \) results

Initially repeatability was calculated between B1 and B2 for the whole cohort. After exclusions there were 10 matched pairs of B1 and B2 scans used for analysis.

The wsCOV of B1 to B2 scans was 16.7%, which suggests the test can detect cohort differences of this magnitude. The repeatability was 46.3% (95% of \( \kappa_{\text{trans}} \) measurements would be expected to fall within +/-46.3%) assuming no treatment effect and therefore an individual change outside this could be considered significant.
<table>
<thead>
<tr>
<th>Percent difference of baseline 1 to 2</th>
<th>Coefficient of variation (B2 to B1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-9.14%</td>
<td>0.42%</td>
</tr>
<tr>
<td>10.10%</td>
<td>0.51%</td>
</tr>
<tr>
<td>10.33%</td>
<td>0.53%</td>
</tr>
<tr>
<td>2.68%</td>
<td>0.04%</td>
</tr>
<tr>
<td>-9.85%</td>
<td>0.49%</td>
</tr>
<tr>
<td>-7.49%</td>
<td>0.28%</td>
</tr>
<tr>
<td>31.45%</td>
<td>4.95%</td>
</tr>
<tr>
<td>61.01%</td>
<td>18.61%</td>
</tr>
<tr>
<td>15.58%</td>
<td>1.21%</td>
</tr>
<tr>
<td>12.91%</td>
<td>0.83%</td>
</tr>
<tr>
<td>wsCOV</td>
<td>16.7%</td>
</tr>
<tr>
<td>Repeatability</td>
<td>46.3%</td>
</tr>
</tbody>
</table>

Table 3.39: Percentage change in $K_{\text{trans}}$ between baseline 1 and 2 scans, with calculated wsCOV and repeatability.

<table>
<thead>
<tr>
<th></th>
<th>Percentage change from Baseline to D3</th>
<th></th>
<th>Percentage change from Baseline to D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>Fish oil group</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>4</td>
<td>69.3%</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6</td>
<td>6.4%</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>7</td>
<td>-47.0%</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>9</td>
<td>-32.4%</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>16</td>
<td>20.9%</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>17</td>
<td>-9.9%</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>18</td>
<td>70.7%</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>18</td>
<td>-10.2%</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>18</td>
<td>-1.6%</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>-3.7%</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td></td>
<td>14.9%</td>
</tr>
</tbody>
</table>

Table 3.40: Percentage change of $K_{\text{trans}}$ from baseline to D3 post treatment of control group and fish oil group with cohort mean and standard error of the mean.

The mean percentage change of $K_{\text{trans}}$ from baseline to D3 in the fish oil group was + 3.4% (SEM +/-14.5%) versus - 3.7% (SEM +/-14.9%) in the control group. There was no statistical significance within the fish oil group, the control group or between the groups ($p = 0.8728$, 0.9866 and 0.7373 respectively).
3.5.3.2 DCE-MRI results - $V_e$ results

Initially repeatability was calculated between B1 and B2 for the whole cohort. After exclusions there were 10 matched pairs of B1 and B2 scans used for analysis.

<table>
<thead>
<tr>
<th>Percent difference of baseline 1 to 2</th>
<th>Coefficient of variation (B2 to B1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.82%</td>
<td>0.70%</td>
</tr>
<tr>
<td>-48.92%</td>
<td>11.97%</td>
</tr>
<tr>
<td>-22.56%</td>
<td>2.54%</td>
</tr>
<tr>
<td>6.93%</td>
<td>0.24%</td>
</tr>
<tr>
<td>0.75%</td>
<td>0.00%</td>
</tr>
<tr>
<td>-22.26%</td>
<td>2.48%</td>
</tr>
<tr>
<td>17.46%</td>
<td>1.52%</td>
</tr>
<tr>
<td>28.46%</td>
<td>4.05%</td>
</tr>
<tr>
<td>1.32%</td>
<td>0.01%</td>
</tr>
<tr>
<td>5.35%</td>
<td>0.14%</td>
</tr>
<tr>
<td>wsCOV</td>
<td>15.4%</td>
</tr>
<tr>
<td>Repeatability</td>
<td>42.6%</td>
</tr>
</tbody>
</table>

*Table 3.41: Percentage change in $V_e$ between baseline 1 and 2 scans, with calculated wsCOV and repeatability.*

The wsCOV of B1 to B2 scans was 15.4% and the repeatability was 42.6%. This suggests that 95% of $V_e$ measurements will fall within +/- 42.6% of the baseline scan, assuming no treatment effect. Thus a D3 measurement outside this region maybe considered important for further analysis.

The mean percentage change of $V_e$ from baseline to D3 in the fish oil group was - 9.3% (SEM +/- 5.4%) versus - 10.20% (SEM +/- 8.1%) in the control group. There was no statistical significance within the fish oil group, the control group or between the groups.
<table>
<thead>
<tr>
<th>Control</th>
<th>Percentage change from Baseline to D3</th>
<th>Fish oil group</th>
<th>Percentage change from Baseline to D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.6%</td>
<td>4</td>
<td>-22.2%</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>7</td>
<td>-18.3%</td>
</tr>
<tr>
<td>3</td>
<td>-29.2%</td>
<td>9</td>
<td>-14.8%</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>16</td>
<td>-17.6%</td>
</tr>
<tr>
<td>13</td>
<td>-29.0%</td>
<td>16</td>
<td>16.9%</td>
</tr>
<tr>
<td>14</td>
<td>-23.7%</td>
<td>17</td>
<td>4.4%</td>
</tr>
<tr>
<td>15</td>
<td>21.9%</td>
<td>18</td>
<td>-13.4%</td>
</tr>
<tr>
<td>19</td>
<td>-16.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>-10.2%</td>
<td>Mean</td>
<td>-9.3%</td>
</tr>
<tr>
<td>SEM</td>
<td>8.1%</td>
<td>SEM</td>
<td>5.4%</td>
</tr>
</tbody>
</table>

Table 3.42: Percentage change of $V_e$ from baseline to D3 post treatment of control group and fish oil group with cohort mean and standard error of the mean.

3.5.3.3 Diffusion Weighted Imaging results

On review of the DW-MRI images 30 scans had to be excluded from analysis. Repeatability was calculated between B1 and B2 for the whole cohort. After exclusions there were 10 matched pairs of B1 and B2 scans used for analysis.

<table>
<thead>
<tr>
<th>Percent difference of baseline 1 to 2</th>
<th>Coefficient of variation (B2 to B1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.87%</td>
<td>0.00%</td>
</tr>
<tr>
<td>2.86%</td>
<td>0.04%</td>
</tr>
<tr>
<td>2.22%</td>
<td>0.02%</td>
</tr>
<tr>
<td>6.82%</td>
<td>0.23%</td>
</tr>
<tr>
<td>-8.23%</td>
<td>0.34%</td>
</tr>
<tr>
<td>-1.36%</td>
<td>0.01%</td>
</tr>
<tr>
<td>-9.27%</td>
<td>0.43%</td>
</tr>
<tr>
<td>-2.14%</td>
<td>0.02%</td>
</tr>
<tr>
<td>-14.42%</td>
<td>1.04%</td>
</tr>
<tr>
<td>0.49%</td>
<td>0.00%</td>
</tr>
<tr>
<td>wsCOV</td>
<td>4.6%</td>
</tr>
<tr>
<td>Repeatability</td>
<td>12.9%</td>
</tr>
</tbody>
</table>

Table 3.43: Percentage change in diffusion between baseline 1 and 2 scans, with calculated wsCOV and repeatability.
<table>
<thead>
<tr>
<th>Control</th>
<th>Percentage change from Baseline to D3</th>
<th>Fish oil group</th>
<th>Percentage change from Baseline to D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.6%</td>
<td>4</td>
<td>0.31%</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6</td>
<td>-3.8%</td>
</tr>
<tr>
<td>3</td>
<td>-6.1%</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>9</td>
<td>-1.9%</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-9.1%</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>16</td>
<td>-1.6%</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>17</td>
<td>-2.0%</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>6.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-2.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>-1.2%</td>
<td>Mean</td>
<td>-1.8%</td>
</tr>
<tr>
<td>SEM</td>
<td>0.55%</td>
<td>SEM</td>
<td>0.82%</td>
</tr>
</tbody>
</table>

Table 3.44: Percentage change of diffusion from baseline to D3 post treatment of control group and fish oil group with cohort mean and standard error of the mean.

The wsCOV of B1 to B2 scans was 4.6% and the repeatability was 12.9%. This suggests that 95% of DWI measurements will fall within +/-12.9% of the baseline scan, assuming no treatment effect. Thus a D3 measurement outside this region may be considered individually significant.

The mean percentage change of diffusion from baseline to D3 in the fish oil group was -1.8% (SEM +/-0.82%) versus -1.2% (SEM +/-0.55%) in the control group. There was no statistical significance within the fish oil group, the control group or between the groups.

3.5.3.4 $T2^*$ results

On review of the $T2^*$ images 24 scans had to be excluded from analysis. Initially repeatability was calculated between B1 and B2 for the whole cohort. After exclusions there were 13 matched pairs of B1 and B2 scans used for analysis.
The wsCOV of B1 to B2 scans was 11.5% and the repeatability was 31.7%. This suggests that 95% of DWI measurements will fall within +/-31.7% of the baseline scan, assuming no treatment effect. Thus a D3 measurement outside this region may be considered individually significant.

The mean percentage change of T2* from baseline to D3 in the fish oil group was -15.2% (SEM +/-7.1%) versus +9.7% (SEM +/-3.7%) in the control group. There was no statistical significance within the fish oil group (p=0.056), the control group or between the groups.
<table>
<thead>
<tr>
<th></th>
<th>Percentage change from Baseline to D3</th>
<th>Fish oil group</th>
<th>Percentage change from Baseline to D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.5%</td>
<td>4</td>
<td>-30.8%</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6</td>
<td>-30.6%</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.1%</td>
<td>9</td>
<td>-24.7%</td>
</tr>
<tr>
<td>8</td>
<td>32.9%</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-3.3%</td>
<td>12</td>
<td>-5.9%</td>
</tr>
<tr>
<td>14</td>
<td>34.9%</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>18</td>
<td>16.1%</td>
</tr>
<tr>
<td>19</td>
<td>-1.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-3.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>-15.2%</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td></td>
<td>7.1%</td>
</tr>
</tbody>
</table>

Table 3.56: Percentage change of T2* from baseline to D3 post treatment of control group and fish oil group with cohort mean and standard error of the mean.

3.5.4 Discussion

This study used $K^{\text{trans}}$ and $V_e$ DCE-MRI, DWI and T2* parameters as biomarkers of angiogenesis to investigate the effect of intravenous n-3 PUFAs in 20 patients with advanced colorectal cancer and liver metastases. The DCE-MRI variables of $V_e$ and $K^{\text{trans}}$ along with DWI and T2* did not show any statistically significant changes from baseline for either the n-3 PUFA group or the control group. There was also no significant difference between the two groups. Although the T2* results almost reach $p=0.05$, this is one of 4 parameters assessed and therefore using Bonferroni corrections the suitable target would be $p=0.0125$. Therefore, the study did not support the hypothesis that EPA and DHA have effects on tumour vasculature and angiogenesis, as defined by changes in gadolinium uptake measured by $V_e$ and $K^{\text{trans}}$ or tumour structure as defined by alterations in the apparent diffusion coefficient. There was no evidence to suggest there was any change in tumour oxygenation or degree of hypoxia as defined by T2* properties.
When analysing the data further and utilising the wsCOV and repeatability figures there are 2 cases with DCE-MRI scans within both the control and treatment group have $K_{\text{trans}}$ percentage changes that lie outside the repeatability test of 46.27% (Fish oil group -56.40% and 70.67%, control group 69.32% and -47.02%). However there is one increase and one decrease in $K_{\text{trans}}$ within both groups, which would suggest that these outliers are non-specific. It is also noted that these findings are not replicated in the $V_e$ analysis and there is no relation to any changes within the n3-PUFA plasma and tissue levels or the circulating cytokines.

In the control group there were two T2* results marginally outside the repeatability test of 31.75% (32.88% and 34.87%) with increased T2* signal which would indicate an increase in the blood oxygen fraction secondary to a reduction in deoxyhaemoglobin. These changes are non-specific.

There are several possible explanations for the absence of detectable changes in gadolinium uptake between the control group and the fish oil group. Firstly that n-3 PUFA do not have a significant effect on tumour angiogenesis. This would seem to be plausible in light of the non-significant results previously discussed relating to circulating cytokine markers of angiogenesis, in particular VEGF-A and VEGFR-2.

However, the majority of trials showing significant changes in DCE-MRI parameters have used TKIs, which have instant and dramatic effects on tumour vasculature, with changes of 40% or more being common [Liu 2005, Mross 2005b, Drevs 2005 Mross 2005a, Thomas 2005, Rosen 2006]. It is likely that this study would have identified such changes despite the poor reproducibility.
demonstrated, but n-3 PUFA have not been shown to act as TKI in previous in-
vivo studies and it is likely that any effect would be smaller and not be
demonstrated using DCE-MRI, especially within such a short time period after
treatment. It is also likely that when large changes in MRI derived parameters are
seen in metastatic disease treated by anti-VEGF TKIs they actually represent
more of an anti-vascular effect relating to loss of immature vessels depending on
high circulating levels of VEGF [Jain 1998, Benjamin 1999, Morgan 2003].
Therefore n-3 PUFAs may not cause these large effects whilst still having anti-
angiogenic properties. If so more rigorous MRI protocols would be required to
detect these more subtle changes. Also n-3 PUFAs may have no effect as a
single agent and may be best used in combination with other biologically active
compounds.

However, this study has a major weakness. More scans than expected
were lost due to protocol error, and the reproducibility of the remaining cases
were not as good as expected, compared with a previous local study assessing
liver metastases [Morgan 2006]. The previous study had a wsCOV of 19.1% and
a reproducibility of 36.1% in tumours > 2 cm, but for tumours of > 3cm this
improved to 15.5% and 30.6% respectively, with one protocol violation in 22
scans, leading to the loss of one of 11 patients. Our study had a wsCOV of
16.7% and 15.4% for $K_{trans}$ and Ve respectively and a reproducibility of 46.3%
and 46.2% with 14 protocol violations and the loss of six patients from $K_{trans}$
and Ve analysis. Most studies accept a change in $K_{trans}$ of >40% as likely to represent
a true difference due to drug effect [Jackson 2007]

The reproducibility of the T2* (wsCOV 11.5%, reproducibility 31.8%) and
DWI (4.6%, 12.9%) imaging was superior to the DCE-MRI reproducibility,
however little comparative data exists on the reproducibility of DWI and T2*
assessment of liver tumours in the medical literature. Even though the reproducibility was improved with these imaging techniques, which do not rely on contrast, the high number of protocol violations again lead to a high number of scans being unsuitable for analysis.

Despite the data loss in this study being 23% - 14 of 60 scans - in comparison to 5% in the previous study, data loss when using DCE-MRI of up to 36% has been described [Chica 2005].

The poor reproducibility of the DCE-MRI and high number of protocol violations compared to our local standard was not expected, but the scans were performed at a separate hospital to the original study and with different staff. Staff at the previous hospital were trained in a similar manner but had more experience in using contrast enhanced research protocols for both cardiac and oncology scans, whereas for this study the staff had no previous research experience. Furthermore in the original study, staff were initially supervised by a consultant radiologist with greater MRI experience compared to a research fellow with very little MRI experience in this study. Interestingly in some cases where protocol errors occurred the radiographer admitted to being unsure of the process but instead of reviewing the study manual they improvised using previous clinical experience. In clinical practice these improvisations may have been satisfactory but not for a research protocol. This emphasizes the importance of training staff in research methodology and the importance of protocol compliance. It also emphasizes that all research scans should be supervised by somebody experienced in research methodology, which may be difficult in a busy NHS hospital.

In response to the high number of protocol violations and therefore data
loss separate to this thesis I carried out a qualitative thematic analysis of radiographer’s beliefs and perspectives related trial scanning as an aide to identify the source and reasons behind protocol violation prevent protocol violations in future studies.

Thematic analysis is the most common form of analysis in qualitative research [Guest 2012]. It is used to examine patterned response or meaning from data known as themes [Daly 1997]. Themes are generated by a process of coding a subject's response to questioning [Saldanna 2009], going beyond surface meanings to make sense of the answers and tell an accurate story of what the data means [Braun 2006]. This form of analysis may seem alien to clinicians, but it is used widely within the social sciences to appreciate a unified and coherent portrayal of human experience [Moustakas 1994, Strauss 1990].

This process involves grounded research theory described by Glaser and Strauss [Glaser 1967]. It is designed to unravel the elements of ‘experience’ enabling the researcher to understand the nature and meaning of an experience for a particular group of people in a particular setting. This involves careful analysis of transcribed interviews or questionnaire responses to allow reflective analysis and construct a theory of causality [Strauss 1987].

The aim was to perform this analysis by requesting written responses to a free text questionnaire exploring radiographers thoughts and mind-set related to trial scanning to allow interpretation of experiences & themes, to allow us to address any ingrained personal or grouped beliefs related to trial scans. By addressing these beliefs & problems, we can hopefully reduce the number of protocol violations in future studies.
In summary, the results showed that there is general despondence towards research scans and the radiographers performing the scans in this trial neither enjoy, nor see the benefit of performing the research scans; they are seen as a chore. There also seemed to be major concerns related to training and the complexity of MR protocols. This was in contrast to a group of radiographers at a different site where the original DCE-MRI trial was performed, who carry out high volume of trial scans. This group had a general positivity towards research scanning, and seem to understand why research scans are needed and what benefit they bring. It also seems to give them satisfaction. There was also general understanding of where to seek help if required and in stark contrast there was no problem with the apparent complexity of the MR protocols.

The negativity towards training was surprising as there was formal training prior to initiation of this trial. I and other research fellows were on hand to explain the background to the study and help when there were problems/questions related to the protocol, which we did on numerous occasions, and all the radiographers involved in the trial received feedback on the outcome, but this was done informally. However, all respondents echoed these sentiments so there must have been a problem with the training and support. I have subsequently discussed these comments with the radiographers involved and they seem to reflect their views towards all trial scanning performed at this site, not specifically this trial.

It was also unexpected that the radiographers did not seem to know the processes in places to get help with trial scans, I am unsure as to why this is. A clear message is that introducing the same protocols, training and back up in two separate units does not have the same result, due to the impact of existing knowledge, culture and infrastructure. Therefore translating any complex
process into a new environment requires an approach tailored to that environment, which may not be predictable.

The radiographers in this study rarely carry out trial scans and generally perform less than ten per year each, thus the training and support mechanisms are possibly forgotten and time taken to progress up the learning curve is prolonged. This small number will also potentially lead to these scans being thought of as an annoyance.

To address these issues will be difficult if volume remains low and the solution maybe that either trial scanning is abandoned at this unit, or that many more trial scans should be performed, so it becomes part of the routine days scanning workload. If more are carried out then better support and direct supervision are a must, with explicit pre-trial briefings – to include a scanning protocol review to highlight any misunderstandings.
Chapter 4

Discussion
4.1 Discussion

It was anticipated that a 72 hour infusion of EPA and DHA in this double-blind randomised controlled pilot clinical study, would elevate the concentrations of those FAs in circulating PPLs and liver tissue of patients with hepatic colorectal metastases leading to a measurable decrease in angiogenesis as assessed by measurement of circulating cytokines and tumour MRI characteristics, however this was not confirmed.

Positively, the study demonstrated a marked and rapid statistically significant increase in PPL EPA and DHA concentrations in the study group, with an associated decrease in the concentration of AA. We showed that our data mirrored that of other published studies, which show a significant rise in levels by 44 hours and steady state levels reached by 66 hours [Mayer 2003, Nau 1993, Morlion 1996, Wachtler P 1997, Dupont 1999] and that intravenous FO infusion leads to rapid PL enrichment compared to oral supplementation [Mayer 2003]. These results would have also supported the hypothesis that any significant changes in the subsequent analysis of biomakers of angiogenesis in this thesis are likely to be related to these increased EPA and DHA levels along with an associated decrease in AA levels.

However, when I quantified the n-3 FA levels within normal liver tissue and tissue from hepatic colorectal liver metastases there were no significant differences between the two groups. Nevertheless, I did observe a statistically significant trend for n-3 PUFA concentration to be higher in tumour tissue of the patients who received EPA plus DHA and underwent tissue sampling earlier. It is widely accepted that upon cessation of the supplementation membrane levels of n-3 PUFA return to pre-supplementation levels. This rate of return to baseline has been shown to mirror the original rate of incorporation thus a brisk
enrichment will be followed by an equally brisk return to pre-supplementation levels [Katan 1997, Calder 2009], thus we are likely to have missed any significant cohort rise due to the delayed time to tissue sampling post cessation of the FA infusion. When I extrapolated the tissue data back to the time the infusion was stopped it indicated that the FO infusion increased n-3 PUFA levels by 45% in normal liver tissue and 56% in tumour tissue. A way around this may have been to combine oral and intravenous supplementation with ‘dual’ enrichment. However the oral supplementation would have had to be started many weeks before resection and the third trial scan, and thus led to delays in patient care. Robust data is required to directly compare oral, intravenous and combinations of oral and intravenous supplementation and define the ‘ideal’ route to allow rapid sustained enrichment with EPA and DHA.

When quantifying the levels of cytokines involved in the angiogenic pathway in serum samples using ELISA it was anticipated that a 72 hours infusion of EPA plus DHA would have a anti-angiogenic on the measured circulating cytokine concentrations. This was not observed in a statistically significant way using the chosen method of analysis. GEE analysis was selected as a robust and sound method to perform a single analysis of all time points combined, as my principle interest was how the two groups varied over time, and whether the profile of values varied between groups. There were some significant changes demonstrated in EGF, HGF, ANG2 and MMP-9, though these were non-specific, but possibly related to the TPN infusion. As the initial data showed, which I presented at the European Society of Clinical Nutrition using a less robust method of analysis there was some evidence of statistically significant differences between the groups FO and non FO groups as discussed previously, suggesting there is mileage in further investigation of the effect of n-3 PUFA on circulating cytokine level in humans. The rate-limiting factor in the cytokine
measurement was the huge variation in the baseline levels for each patient leading to a large standard deviation/error of the mean within the cohorts, which could not be overcome statistically in such a small cohort subjects. I suspect to demonstrate any marked effect on cytokines an extremely large study cohort will be required to overcome this heterogenicity in baseline measurements.

The MRI results were disappointing and limited two-fold, firstly by the poor reproducibility of the scans leading to even less numbers for analysis. DCE-MRI is recognised as a complex procedure [Leech 2012] and a high degree of test re-test variability is expected [Chica 2005]. In many cases analysis is centralised to a core lab to avoid problems with analysis protocol. In this case all analysis was centralised, however the rate-limiting step was not with analysis but reproducibility. Previous research at our institution using similar scanning protocols had far better reproducibility; therefore the poor reproducibility of the DCE-MRI and high number of protocol violations in this study was not expected.

As discussed, on reflection the scans for the previous studies were performed at a different site, with different staff in comparison to this study. Training of both groups of staff was similar, however the staff in this study had less ‘hands-on’ experience of research scanning, particularly with contrast enhanced research protocols, of which they had no previous experience, this has been addressed and all trial scanning has been centralised to the original centr.

But, the fundamental flaw was presuming that EPA and DHA would potentially act in a way akin to TKI’s, which have dramatic effects on tumour blood flow that can be detected by DCE-MRI. If EPA and DHA had caused the type of effect TKI’s have on tumours these would have still been evident despite the poor MRI reproducibility. But there was no evidence to suggest there was any n-3 PUFA change in the tumour micro-environment as defined by changes in
DCE-MRI, apparent diffusion coefficient or T2* properties. The tumour microenvironment changes need to be dramatic to be detected by MRI, as such histological analysis – microscopy, immunohistochemistry, vessel counts etc. - of the tumour samples may have revealed more subtle changes as was seen in a recent study from the Leeds group [Cockbain]

The MRI techniques are still robust and offer a non-invasive safe biomaker, which requires no radiation. When the study was conceived the utilisation of these techniques in the clinical arena was limited and generally used to aide diagnosis e.g. DCE-MRI in breast tumour characterisation and, DCE-MRI and diffusion properties to define liver lesions [Khaled, Kele]. However, there now is a vogue to use these properties – DCE-MRI and particularly DWI – day to day to define tumour phenotype and aggressiveness, and assess response to treatment [Chen, Jung, Bozgeyik]. Other adjuncts are being developed to enhance the already vast worth of MRI, using markers to type the tumour at a functional level such as PET-MRI and MRI spectroscopy [Gallagher], along with molecular techniques such as Dynamic Nuclear Polarisation, which allows carbon metabolism to be imaged non-invasively; this technique has been used to image tumour pH, enzyme activity and cellular necrosis [Brindle]. These techniques will hopefully allow us to detect cancer earlier as well as assessing early response of tumours to treatment.

As is evident from what I have stated above and within the discussion sections of each results chapter there are two major flaws underpinning the outcome of this research study, one is the small cohort numbers and two, the misunderstanding of the way in which EPA and DHA would act on tumourgenesis. As this trial was a pilot/mechanistic study, in reflection we should have performed a phase II exploratory study with patients acting as there own
control (baseline measurements, which was done for all the biomarkers – PPL n-3 levels, MRI and cytokines) with all patients receiving FO emulsion and not a randomised control study. This would have increased the numbers, even within our financial constraints and subsequently enhanced any statistical analysis.

What we have shown is that rapid enrichment of the lipid raft is possible with IV FO infusion, but sustained or repeated infusions eliminating washout are likely to be required to demonstrate any tangible responses caused by FO. As a research group we have used this knowledge on a series of further studies demonstrating

Omega-3 fish oil are not the panacea for cancer as I once naively hoped when I embarked on this thesis, but the recent results published from within the Leicester group are exciting and show there maybe a role for EPA and DHA supplementation in the treatment of cancer and other disease processes. Sustained and significant decreases in PDGF and FGF secondary to weekly FO infusion lead to an increase in overall survival and progression free survival respectively in patients treated with Gemectabine for advanced pancreatic cancer [Arshad 2012]. The group has also recently presented data showing significant and beneficial alterations in inflammatory cytokines in response to EPA and DHA infusion in a trial of patients with acute severe pancreatitis and a second trial enrolling intensive care patients with mulit-organ dysfunction. Preliminary data from both these studies has demonstrated improved outcomes in the patients receiving FO infusion versus control [Al-Leswas 2013, Hall 2013].
### Appendices

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Appendix One: Trial Protocol

Aims and hypothesis
The aim was to assess the effect of parenteral n-3 PUFA upon markers of angiogenesis in patients with hepatic colorectal metastases using TPN containing either n-3 PUFA containing lipid emulsion (Lipidem®, B Braun, Melsungen) or control substance without n-3 PUFA (Lipofundin® MCT 20%, B Braun, Melsungen).

The null hypothesis for this investigational study is that the administration of parenteral nutrition containing n-3 PUFA for 72 hours will not alter the outcome measures detailed below in study subjects compared to controls without n-3 PUFA infusion.

Trail design
The study was designed as a Phase II, single centre, 2-arm placebo controlled randomised trial with 10 patients in each arm (n=20).

Ethics
Regional ethical approval was granted (REC number: 06/Q2501/16) along with approval from the Medicines and Healthcare Products Regulatory Agency (EudraCT number: 2006-000044-71) prior to study commencement.

The trial ‘Randomised Controlled Trial on the Effect of Fish Oils on Human Hepatic Colorectal Metastases’ was registered on the U.S. Institutes of Health clinical trails registry (ClinicalTrials.gov identifier NCT00942292).

Patient identification
Patients were identified from the University Hospitals of Leicester Hepatobiliary Multi-Disciplinary team database. They were then approached and I gave a verbal explanation of the study and were given a patient information leaflet. They were then left to consider taking part in the study for 72 hours as per GCP guidelines. At which point the patient was invited to take part in the study and asked to provide written informed consent.

**Inclusion criteria**

All patients aged 18-80 whom were able to give written informed consent referred to the University Hospitals of Leicester Hepatobiliary Multi-Disciplinary team meeting with hepatic colorectal metastases deemed amenable to curative surgical resection after routine staging investigations were considered for trial inclusion. The target lesion needed to be >3 cm in size to allow adequate visualisation and analysis on MRI scanning.

**Exclusion criteria**

Patients were not eligible for the study if they had:

- Hypersensitivity to fish-, egg-, or soy protein, or to any of the active substances or constituents in the lipid emulsion.
- Hyperlipidaemia.
- Severe blood coagulation disorders.
- Severe renal insufficiency (Creatinine >200).
- Any general contra-indications to infusion therapy – pulmonary oedema, hyperhydration, decompensated cardiac insufficiency.
- Any unstable medical conditions – uncontrolled diabetes mellitus, acute myocardial infarction, stroke, embolic disease, metabolic acidosis, sepsis, pancreatitis.
• Received conventional neo-adjuvant chemotherapy within 3 months of study enrolment.

If a patient met any of the following criteria, they could not undergo MRI scanning and were excluded from the trial:

• Cardiac pacemaker.
• Other ferromagnetic metal implants not authorised for use in MRI such as some types of cerebral aneurysm clips.
• Claustrophobia.
• Body weight or circumference beyond the MRI scanner’s capacity.

Randomisation
Patients were randomised using random number tables at a secondary institution (Clinical Trials Pharmacy Department, Sheffield Teaching Hospitals NHS Trust) and all investigators remained blinded until after analysis of results.

Drug supplies
Standard Lipidem® (active substance) and Lipofundin® MCT 20% (control) were supplied along with Nutriflex basal TPN by B Braun UK directly to the clinical trials pharmacy department at Sheffield Teaching Hospitals NHS Trust.

Formulation and Packaging
Standard formulations of Nutriflex® basal, Lipidem® 200 mg/ml and Lipofundin® MCT/LCT 20% were used as per the Summary of Product Characteristics for each product (appendix 4).

TPN was compounded at Sheffield Teaching Hospital – during the trial Sheffield lost their license to produce medicinal products for clinical trial investigation so compounding was transferred to Royal Free Hospitals NHS Trust Clinical Trial.
Unit - to include 2000 ml of Nutriflex® basal with 500 ml of Lipidem® (n-3 containing investigational product) or Lipofundin® (control substance) in a single chamber TPN bag as per randomisation prior to study commencement for each patient. The product was labeled on a named patient basis and delivered one day prior to the date required in temperature-controlled storage to Leicester General Hospital Pharmacy Department.

Administration

TPN containing Nutriflex® basal with either Lipidem 200mg/ml OR Lipofundin® MCT 20% in a ratio of 4:1 (2000 ml Nutriflex® basal to 500 ml Lipidem® 200mg/ml OR Lipofundin® MCT 20%) was infused via a dedicated peripherally inserted central catheter for 72 hours (3 days infusion of fish oil has been shown to result in a significantly higher total ω-3 FA and lower total ω-6 FA in plasma phospholipids [Roulet 1997], and to achieve “steady state”).

Dosage/rate of administration

Total fluid intake intravenously should be approximately 1.5 ml/kg/hr and the suggested intravenous fat intake in TPN for adults is 1-2 g fat per kg body weight per 24 hours, corresponding to 5-10 ml of Lipidem 200 mg/ml or Lipofundin MCT/LCT 20% per kg body weight per day.

Dosage was calculated per patient body weight at 1.5 ml/kg/hr of TPN. This represented the following for an 80 kg patient:

80 kg x 1.5 ml = 120 ml of TPN per hour

or

120 ml x 24 hr = 2880 ml per 24 hours

which corresponds to
2880 \div 5 \text{ (ratio of basal compound to lipid 4:1) } = 576 \text{ ml of 200mg/ml or 20\% lipid}

576 \text{ ml } \times 0.2 \text{ g/ml} = 115.2 \text{ g fat per 24 hours}

or

115.2 \text{ g } \div 80 \text{ kg} = 1.44 \text{ g/kg/24hr of fat.}

Potential risks

The potential risks of this project are virtually identical to those of standard clinical management, as all that is being altered is the balance of the lipid constituents in TPN. The risks of lipid parenteral nutrition are outlined below.

Potential risks of parenteral fish-oil infusion are:

- Anaphylactic/hypersensitivity reactions (<1/10,000)
- Metabolic and nutritional disorders (<1/10,000)
  - Metabolic acidosis
  - Hyperglycaemia
  - Hyperlipidemia/ Fat overload syndrome (see below)
  - Ketoacidosis
- Hyper- and hypotension (<1/10,000)
- Dyspnoea (<1/10,000)
- Priapism (<1/10,000)
- General disorders/administration site conditions (<1/10,000)
  - Headache
  - Flushing
  - Nausea and vomiting
  - Lack of appetite
  - Erythema at infusion site

Fat Overload Syndrome
This is caused by impaired capacity to eliminate triglycerides, which may be caused by an overdose. There are various causes:

- Genetic predisposition
- Fat metabolism may be impaired by illness
- May be associated with sudden changes in the patients condition – e.g. renal impairment or sepsis

It is characterised by hyperlipidemia, fever, fat infiltration, hepatomegaly with or without jaundice, splenomegaly, anaemia, leukopenia, thrombocytopenia, coagulopathy, haemolysis and reticulocytosis, abnormal liver function tests and coma. The symptoms are reversible if the infusion of fat emulsion is discontinued. Serum triglycerides will be monitored and if they rise above 3 mmol/L during infusion of lipid emulsion the infusion will be stopped.

Potential risks associated with MRI scanning include hypersensitivity to the contrast agent and claustrophobia.

**Concomitant medication(s)/Interactions**

Standard management continued for all patients on this trial as per standard clinical practice and no medications were withheld.

Heparin induces a transient release of lipoprotein lipase into the bloodstream. This may initially lead to increased plasma lipolysis, followed by a transient decrease in triglyceride clearance and thus as per protocol triglyceride levels were monitored.
Soya-bean oil (found in both Lipidem 200 mg/ml and Lipofundin MCT 20%) has a natural content of vitamin K1. The content is however so low that it does not significantly influence coagulation in patients treated with coumarin derivatives. Nevertheless, the coagulation status was monitored as per standard protocol in patients treated concomitantly with anticoagulants.

There are no other known interactions.

**Trial pathway**

- Pre-trial serum lipids, full blood count, liver function tests, renal function, glucose and coagulation screen were performed on all patients and a thorough history and physical examination performed to screen for exclusion criteria. Baseline blood samples were taken for storage and subsequent analysis (secondary outcome measures).
- Patients fulfilling all inclusion criteria and who had no exclusion criteria giving written informed consent were recruited into the study.
- They were randomised to receive TPN with (Lipidem® 200mg/ml, B Braun, Melsungen) or without (Lipofundin® MCT 20%, B Braun, Melsungen) n-3 PUFA by sealed envelope system from random number tables.
- Patients underwent an initial MRI at this stage.
- Within the following 2 weeks, subjects were admitted to Leicester General Hospital.
- This is the normal clinical management of these patients, and staging laparoscopy and curative intent surgery was not delayed by the study for any subject.
• On the day of admission, the day before staging laparoscopy, a peripheral inserted central cannula (PICC line) was sited in the arm of subjects for administration of TPN.

• A second MRI was performed, comparison with the first scan permitted calculation of a rate of change of vascularity and vascular permeability prior to the intervention of the study.

• TPN will be commenced.

• The TPN bags were made up according to patient prescription at Sheffield Teaching Hospitals/Royal Free Hospital and blinded to the study and surgical teams caring for the patient, with regard to the presence or absence of n-3 PUFA. A sealed envelope with a code break informing the trial team of which product the study participant was receiving was kept in pharmacy in the event of a serious adverse reaction.

• Routine haematological and biochemical blood tests were monitored every 24 hours while the patient was receiving the TPN infusion.

• Patients were permitted only free clear fluids orally whilst on TPN to reduce the interference of diet on the PUFA uptake results.

• Participants underwent a staging laparoscopy and intra-operative ultrasound scan which is routinely used in staging of colorectal metastases.

• Patients remained inpatients for 48 hours after the laparoscopy, during this time they continued on the TPN regimen.

• During the admission, parallel to the trial, standard clinical pre-operative preparation and investigation proceeded as normal.

• Throughout the TPN infusion at 7 time-points, blood samples were taken for storage and subsequent analysis of secondary outcome measures (see below re timings and method of preparation).
• After 72 hours of TPN, a third and final MRI scan was performed, to allow comparison with the second scan which will provide a new rate of change in the vascular characteristics of the lesion of interest to assess the impact of the interventions.
• Post TPN trial blood samples were taken for storage and subsequent assay.
• The patients proceeded to surgical resection of metastases when this was not contra-indicated by laparoscopy findings. At the time of resection a further set of routine clinical blood tests and trial blood samples were taken. Tumour samples and normal liver tissue was taken from the excised histological specimen to allow analysis PUFA composition and histological examination of the vasculature.
• After the resection the patient exited the trial, and proceeded with standard clinical management of their disease.

**Monitoring**

Patients were carefully monitored for any signs or symptoms or anaphylactic reaction, if present the infusion would have been immediately interrupted. At the start of the infusion, the patient had hourly observations taken (temperature, pulse rate, blood pressure, respiratory rate), changing after the first 6 hours to 4 hourly observations for the duration of the treatment. The patients were also be carefully monitored for signs of fat overload syndrome (see above). All patients had daily bloods taken including full blood count, renal function, liver function tests, serum lipids, glucose, coagulation screen, bicarbonate and trace elements (calcium, phosphate, magnesium). Any persistent metabolic acidosis, deteriorating renal or liver function, coagulopathy, anaemia, leukopenia, or thrombocytopenia would have resulted in discontinuation of the infusion and premature trial exit.
Safety reporting

- All serious adverse events were immediately reported to the trial sponsor (University Hospitals of Leicester). This was followed by a detailed report. These reports were anonymised and patients referred to only by their trial code number.
- The sponsor would have ensure that all relevant information regarding serious unexpected adverse reactions that were fatal or life threatening was recorded and reported to the MHRA and ethics committee within a seven-day period following the incident –however there were no serious unexpected adverse reactions that were fatal or life threatening.
- All other serious unexpected adverse reactions were reported to the MHRA and ethics committee within fifteen days.
- All investigators were made aware of any adverse reactions.
- A detailed record was kept of all adverse events by the chief investigator and these will be available to the ethics committee and MHRA as necessary.
- The sponsor provided the MHRA and ethics committee with this report once a year throughout the duration of the trial.
- Following an adverse event, the investigators met to discuss any additional measures necessary.

Remaining participants will be informed of any serious adverse reactions and counselled as to whether they wish to continue with the trial.

Patient's withdrawal criteria

Withdrawal criteria:

- Patients decision to exit the trial
- Fat overload syndrome
- Disturbance of monitored biochemical and haematological parameters described above
- Anaphylaxis/hypersensitivity reaction to the products
- Inability to insert a PICC line
- Unable to tolerate MRI
Appendix 2: DCE-MRI Radiographer Guidelines

Patient Preparation

Subjects must refrain from food and liquid (except water) for 4 hours before each DCE-MRI procedure. Site imaging staff will need to liaise with the trial co-coordinator on these days, in order that the DCE-MRI session can be booked at the right time.

Whatever conditions imposed for the baseline scans they must also be in place for the post-treatment scans to ensure consistency. Please give full explanation and reassurance to the patient. Pre-scan please complete the MRI safety questionnaire, inform the patient of the contrast injection, the approximate length of the scanning session and the importance of keeping still. Ensure the patient visits the toilet before entering the scan room, remove all metallic objects and ask the patient to change into a hospital gown.

On the first visit, record the IV dose, archive disc number (if appropriate) and the field-of-view for both axial sequences and the dynamic scan.

Exclusions from MRI

If a patient meets any of the following criteria, he/she should not undergo MRI scanning:
- Cardiac pacemaker
- Other ferromagnetic metal implants not authorised for use in MRI such as some types of cerebral aneurysm clips
- Claustrophobia
- Body weight or circumference beyond the MRI scanner's capacity.
**Patient Positioning**

Inset an intravenous cannula into the basilic/brachial vein before entering the MRI scan room. Calculate the contrast does (single dose) on the first visit and maintain this doe for subsequent visits (Magnevist 0.2 ml/kg, max 20 ml).

Connect the pump injector to the patient’s intravenous cannula. Set a rate of 3 ml/sec followed by a 30 ml saline flush (use keep vein open button (KVO)).

The patient should be positioned supine with the upper part of the liver at the centre of the magnet (please ensure the patient is comfortable prior to entering the magnet).

**Summary List of Scan Sequences**

The following summarises the list of scans to be performed at each visit.

Every single step is vital.

Before starting, ensure the correct coils are plugged ion. Breath-held sequences are to be performed as the patient inspires then breath-holds. Maintain slice thickness at 8mm, and once the filed of view is decided for a patient, it should be kept the same for all visits of that patient.

1. Localiser 3-plane

**Standard sequences**

2. T1-weighted FLASH – breath held (t1-tra) Liver

3. T2-weighted TSE – breath-held (t2-tse) Liver

4. VIBE – breath-held (t1-vibe) Liver
**T2 star study**

All breath-held

**Target lesion selection**

This will be done by trial coordinator (>3 cm)

Use scan 2 or 3 to plan

5. T2 star sequence (TE 4.76 & 19.0) Coronal through tumour

6. T2 star sequence (TE 4.76 & 33.0) Coronal through tumour

7. T2 star sequence (TE 4.76 & 47.6) Coronal through tumour

**Diffusion sequence**

Non breath-hold

8. Diffusion sequence (run 4 times) Axial through tumour

**DCE-MRI saturation-recovery (dynamic)**

All non breath-hold

Drag down sequence 2 (ti-tra) from above and repeat during gentle breathing

9. T1 weighted FLASH Target area

Plan DCE using scan 9 Plan through tumour and aorta

Use ‘dynamic’ sequence

10. 1 measurement check positioning of slice
• Drag down scan 10, change Magnetisation Preparation to NONE
• Change flip angle to 5°

11. M0 // 1 1 measurement
• Drag down and change flip angle to 16°

12. MO // 2 1 measurement
• Drag down and reselect Magnetisation Preparation (Non Sel SR)
• Confirm flip angle is set at 16°
• Change to 150 measurements
• Insert 1 second pause after measurement ‘Pause after Meas’
  (i.e. this will acquire a slice every 2 seconds)
• Check pump injector is set for 3 ml per second followed by 30 ml flush
• Ask patient to stay as still as possible and breathe gently
• Arm pump

13. Dynamic run (150 measurements) IV Contrast injection on 6th measurement

14. Drag down and repeat sequence 11; M0 //1 1 measurement
  (5° flip angle)
15. Drag down and repeat sequence 11; M0 //1 1 measurement
  (16° flip angle)

Post contrast scan

16. Drag down and repeat sequence 4 (t1-VIBE) 1 measurement

Please make a ‘hard-copy’ image of the target lesion with positioning slice for future positioning (both T1 and T2 weighted images)

Clinical observations

Any concomitant medications administered during the DCE-MRI procedure
(including details of contrast agent used) are recorded in the subject’s medical notes. In addition details of any adverse events during the DCE-MRI procedure should also be recorded.

In the event of an adverse event the Principal Investigator should be informed immediately and a study-specific SAE reporting procedure followed.

**Data transmittal to central reading facility**

Please transfer all image files to PACS to allow trial coordinators to download the images for transfer to the image analysis system.
Appendix Three: Gas Chromatography Guidelines

Materials

Solvents

- Choloroform: Fisher (Loughborough, UK)
- Methanol: Fisher (Loughborough, UK)
- Hexane: Fisher (Loughborough, UK)
- Toluene: Fisher (Loughborough, UK)

Others

- SPE cartridges (Bond Elut™ Certify) Aminopropylsilica (100mg): Varian (California, US)

Methods

A) Preparation of a total lipid extract from plasma:

1. Plasma samples were thawed at room temperature, mixed by vortexing and spun in the centrifuge at 13,000 rpm for 5 minutes to remove denatured protein.

2. 0.5 ml of plasma transferred to a screw-cap glass tube, the volume adjusted with 0.9% NaCl to 0.8 ml.

3. 5.0 ml chloroform:methanol (2:1) containing BHT (50mg/l) anti oxidant was added to each sample.

4. 1.0 ml of 1M NaCl added mixed thoroughly by vortexing.

5. Samples were spun in the centrifuge at 2,000 rpm for 10 minutes (low brake, room temp).

6. The lower phase was collected by aspiration with a Pasteur pipette and transferred to a screw-cap glass tube.

7. The collected samples were dried under nitrogen at 40°C.
A cont) Preparation of a total lipid extract from tissues:

Replace steps A) 1 and 2 in the preparation of a total lipid extract from plasma protocol as follows for tissue, then continue as above:

1. One hundred mg of tissue was cut approximately with a scalpel before thawing started.

2. Ice cold 0.9% NaCl (0.8ml) added to the tissue and homogenised in a plastic bijou using an Untraturrax. Mixture was then transferred to a glass test tube and internal standards added to make up the volume to 1 ml and then tube put on vortex.

B) Separation of lipid classes by solid phase extraction (SPE):

1. The SPE tank was connected to a vacuum pump.

2. The aminopropylsilica SPE cartridge was placed on the SPE tank. A waste tube was placed under each cartridge.

3. 2ml of chloroform added using a dispenser to each cartridge and allowed to drip through under gravity. Vacuum was switched on when no drips fell to remove remaining fluid. The waste tube removed and contents discarded.

4. New glass tubes labelled PC put under cartridges.

5. 2.0 ml dry chloroform:methanol (60:40, v/v) added under vacuum until it was dry. The washes containing PC removed and dried under nitrogen at 40 °C.

6. PC fraction was dried under nitrogen at 40 °C.
C) Preparation of fatty acid methyl esters:

1. 0.5 ml dry toluene was added to the purified lipid classes and mixed by vortexing.

2. Five mls of methylation reagent (methanol containing 2% (v/v) H$_2$SO$_4$) was added for each batch followed by 1 ml of H$_2$SO$_4$ and then mixed.

3. Add 1.0 ml methanol containing 2% (v/v) H$_2$SO$_4$. Mix gently. Tubes were capped securely and heated at 50°C for 2 hours.

4. After 2 hours tubes were removed from heating block and allowed to cool.

5. 1.0 ml of neutralising solution was added to each tube (0.25M KHCO$_3$ (25.03g/l)), 0.5M K$_2$CO$_3$ (69.10g/l)) followed by 1.0 ml dry hexane. Each tube was then vortexed and spun in centrifuge at 1,000 rpm for 2 minutes at room temp (low brake).

6. The upper phase containing the FAMEs (Fatty Acids Methyl Esters) was collected and transferred to a new glass tube and dried under nitrogen at 40°C.

7. After dryness, 75µl of dry hexane was added, vortexed and transferred to the insert of a GC autosampler vial.

8. Step (7) was repeated

D) FAME analysis:

Samples were loaded into Gas Chromatography machine: Agilent, Hewlett Packard (California, USA). Each analysed sample produced a Graph Chromatogram. The peaks of the graph represented different fatty acids (depending on their retention time from known standards). Area under the curve was calculated for each peak corresponding to the fatty acids of interest using Chemstation software.
E) Calculation of fatty acid concentration:

Amount of each fatty acid was represented as a percentage of overall fatty acids within the particular tissue.
Appendix 4: Summary of Product Characteristics for Lipofundin

1. NAME OF THE MEDICINAL PRODUCT
Lipofundin MCT/LCT 20 %

2. QUALITATIVE AND QUANTITATIVE COMPOSITION
1000 ml of emulsion contain:
- Soya oil: 100.00g
- Medium-chain Triglycerides: 100.00g

For excipients, see 6.1

3. PHARMACEUTICAL FORM
Emulsion for intravenous infusion

4. CLINICAL PARTICULARS

4.1. Therapeutic indications
Lipofundin MCT/LCT is indicated as a source of calories and essential fatty acids for patients requiring parenteral nutrition.

4.2. Posology and method of administration

4.2.1. Recommended dosage schedule

1. Adults and school-age children
1-2 g fat per kg body weight per day, corresponding to 5 - 10 ml of Lipofundin MCT/LCT 20 % per kg body weight per day.

2. Neonates, infants and pre-school children

Neonates
2-3 g (up to 4 g) of fat per kg body weight per day, corresponding to 10 - 15 ml (up to 20 ml) of Lipofundin MCT/LCT 20 % per kg body weight per day.

Especially in preterm infants and low-birth-weight neonates, the ability to eliminate infused lipids is not yet fully developed. Therefore maximum fat doses should not be administered to these patients and serum triglyceride and fatty acid levels should be carefully monitored.

At the end of the daily fat-free interval, the fat must have been cleared from the serum.

Infants and pre-school children
1-3 g of fat per kg body weight per day, corresponding to 5 - 15 ml of Lipofundin MCT/LCT 20 % per kg body weight per day.

3. The elderly

There is no evidence to suggest that dosage should be different from that recommended for other adult patients. Nevertheless, metabolic rates and patterns can vary in the elderly, so careful monitoring of this particular group of patients is always prudent.

Infusion rates

In general, fat emulsions should be infused at as low rate as possible. During the first 15 minutes the infusion rate should not exceed 0.05 - 0.1 g of fat per kg body weight per hour, corresponding to 0.25 - 0.5 ml Lipofundin MCT/LCT 20 % per kg body weight per hour. If no adverse reactions are observed during this initial phase, the infusion rate may be increased to 0.15 - 0.2 g fat per kg body weight per hour, corresponding to 0.75 - 1.0 ml of Lipofundin MCT/LCT 20 % per kg body weight per hour. The daily fat infusions should be administered over not less than 15 hours, preferably as continuous infusion over 24 hours.

Duration of use

In total parenteral nutrition, Lipofundin MCT/LCT is normally administered over 1-2 weeks (up to 4 weeks max.). In elective cases, if fat infusions are further indicated and appropriate monitoring is instituted, the period of use of Lipofundin MCT/LCT may be extended beyond 4 weeks.

4.2.2. Method of administration

Lipofundin MCT/LCT should be administered as part of a total parenteral nutrition regimen via peripheral vein or central venous catheter. Lipofundin MCT/LCT can be infused into the same central or peripheral vein as the carbohydrate and amino acid solutions by means of a short Y-connector near the infusion site. This allows for mixing of the solutions immediately before entering the vein. Flow rates of each solution should be controlled separately by infusion pumps, if these are used.

For safe administration of intravenous fluids from non-collapsible containers a giving set with an integral airway is recommended.

Infusion sets with in-line filters are not to be used for administration of fat emulsions.

4.3. Contra-indications

The administration of Lipofundin MCT/LCT is contra-indicated in patients demonstrating disturbances in normal fat metabolism such as pathologic hyperlipaemia, lipoid nephrosis, or acute pancreatitis if accompanied by hyperlipaemia. It is further contra-indicated in patients with ketoacidosis or hypoxia, in thromboembolism and in acute shock states.
4.4. Special warnings and special precautions for use

4.4.1. Special warnings

Dependence, tolerance: not applicable

Other:

The too rapid infusion of fat emulsions can cause fluid and/or fat overloading resulting in dilution of serum electrolyte concentrations, overhydration, congested states, pulmonary oedema, impaired pulmonary diffusion capacity.

A too rapid infusion of Lipofundin MCT/LCT can also cause hyperketonaemia and/or metabolic acidosis, especially when carbohydrates are not administered simultaneously.

Vitamin E may have an influence on the effect of vitamin K in the synthesis of coagulation factors. Therefore, in patients receiving oral anticoagulants and suspected to have vitamin K deficiency, monitoring of the coagulation status is recommended.

4.4.2. Special precautions for use

Caution should be exercised in administering intravenous fat emulsions in patients with metabolic acidosis, severe liver damage, pulmonary disease, sepsis, diseases of the reticuloendothelial system, anaemia or blood coagulation disorders or when there is danger of fat embolism.

Administration of Lipofundin MCT/LCT should be accompanied by simultaneous carbohydrate infusions making up to 40 % (at least) of the total calorie intake. When Lipofundin MCT/LCT is administered, the patient's capacity to eliminate the infused fat from the circulation must be monitored. The lipaemia must clear between daily infusions. Especially where fat emulsions are administered for extended periods of time, the patient's haemogram, blood coagulation, liver function and platelet count should be closely monitored.

4.5. Interactions with other medicaments and other forms of interaction

None known; for Incompatibilities, however, see para. 6.2.

4.6. Use in pregnancy and lactation

The safety of Lipofundin MCT/LCT during pregnancy and lactation has not been assessed, but its use during these periods is not considered to constitute a hazard. Nevertheless, medicines should not be used in pregnancy, especially during the first trimester, unless the expected benefit is thought to outweigh any possible risk to the foetus.

4.7. Effects on ability to drive and use machines

Not applicable
4.8. **Undesirable effects**

Adverse reactions directly related to fat emulsions in general are of two types:

(a) Immediate (acute) reactions: dyspnea, cyanosis, allergic reactions, hyperlipaemia, hypercoagulability, nausea, vomiting, headache, flushing, hyperthermia, sweating, chills, sleepiness, chest and back pain.

(b) Delayed reactions: hepatomegaly, jaundice due to central lobular cholestasis, splenomegaly, thrombocytopenia, leucopenia, transient increases in liver function tests, and overloading syndrome. The deposition of a brown pigmentation in the reticuloendothelial system, the so-called "intravenous fat pigment", has also been reported. The cause and the significance of this phenomenon ar unknown.

4.9. **Overdose**

In the event of fat overload during therapy, stop the infusion of Lipofundin MCT/LCT, until visual inspection of the plasma, determination of triglyceride concentrations, or measurement of plasma light-scattering activity by nephelometry indicate the lipid has cleared. Re-evaluate the patient and institute appropriate corrective measures.

5. **PHARMACOLOGICAL PROPERTIES**

5.1. **Pharmacodynamic properties**

Lipofundin MCT/LCT 20 % provides a source of energy and essential (polyunsaturated) fatty acids for the patient requiring parenteral nutrition. Medium-chain triglycerides are cleared from the bloodstream at a faster rate and are oxidised more completely for energy production than long-chain triglycerides. For that reason they serve as preferential fuel for the body, especially in conditions where the oxidation of long-chain triglycerides is impaired, e. g. due to carnitine deficiency (EFAD) or diminished carnitine palmitoyltransferase activity, resp.

The polyunsaturated fatty acids, which are only provided by long-chain triglycerides, prevent the biochemical disorders of essential fatty acid deficiency (EFAD) and correct the clinical manifestations of the EFAD syndrome.

5.2. **Pharmacokinetic properties**

Because of the i. v. administration of Lipofundin MCT/LCT 20 %, no data on absorption are provided, for the same reason, the bioavailability is 100 per cent.

The maximum serum triglyceride concentrations during infusion of Lipofundin MCT/LCT 20 % mainly depend on the actual dose and infusion rate as well as on the patient's individual metabolic status.
and other patient-related factors, e.g. the fasting triglyceride level. In
general, however, serum triglyceride concentrations will not exceed 5 
µmol/l as long as recommended doses and all other directions for use
are observed.

The plasma half life time of triglycerides infused in the form of
Lipofundin MCT/LCT 20 % is approx. 9 minutes. Although the affinity
of long-chain fatty acids to albumin is somewhat greater than that of
medium-chain fatty acids, albumin binding of both types of fatty acids
is virtually complete, provided the recommended doses are not
exceeded. Therefore, medium- and long-chain fatty acids do not pass
over into the cerebrospinal fluid. No data are presently available as to
passage across the placental barrier and into breastmilk.

Triglycerides and free fatty acids are not excreted via the kidneys. In
view of the intended nutritive effect of Lipofundin MCT/LCT 20 %, such
excretion is not even desirable. Poisoning requiring rapid
elimination of the toxic agent is not to be expected with Lipofundin
MCT/LCT 20 % because this product only contains physiological
nutrient substances.

5.3. Preclinical safety data

The pharmacological and toxicological studies conducted with the
product did not reveal any effects indicating specific pharmacological
activity or toxicity of the product relevant to its use in man at the
recommended dose levels.

6. PHARMACEUTICAL PARTICULARS

6.1. List of excipients:

Glycerol, egg lecithin, all-rac-α-tocopherol, sodium oleate, water for
injections

6.2. Incompatibilities

As a general rule fat emulsions should not be mixed with electrolytes,
drugs or any other additives in the infusion bottle.

Lipofundin MCT/LCT 20 % may be used with nutrient mixing bag
systems only if such resultant mixtures are compatible and stable.

6.3. Shelf life

In unopened state the Lipofundin MCT/LCT 20 % has a shelf life of 2
years.

6.4. Special precautions for storage

Do not store above 25 °C.
Protect from freezing. If accidentally frozen, discard bottle.
6.5. **Nature and contents of container**

Lipofundin MCT/LCT 20 % is supplied in glass bottles sealed with rubber stoppers.

Contents: 100 ml, 250 ml, 500 ml

6.6. **Instructions for use / handling**

Lipofundin MCT/LCT 20 % is supplied in single dose containers. Unused contents must be discarded and should not be stored for later use.

Infusion sets with in-line filters should not be used for administration of fat emulsions.

Do not use any bottle in which there appears to be a separation (oiling out) of the emulsion.

7. **MARKETING AUTHORISATION HOLDER**

B. Braun Melsungen AG
Carl-Braun-Strasse 1
34212 Melsungen
GERMANY

8. **MARKETING AUTHORISATION NUMBER**

Lipofundin MCT/LCT 20 % : 3551/0016

9. **DATE OF FIRST AUTHORISATION / RENEWAL OF AUTHORISATION**

23 June 1995

10. **DATE OF (PARTIAL) REVISION OF THE TEXT**

April 2003
Appendix 5: Summary of Product Characteristics for Lipidem

1. NAME OF THE MEDICINAL PRODUCT

Lipidem 200 mg/ml emulsion for infusion

2. QUALITATIVE AND QUANTITATIVE COMPOSITION

1000 ml emulsion contains:
- Medium-chain triglycerides 100.0 g
- Soya-bean oil, refined 80.0 g
- Omega-3-acid triglycerides 20.0 g

Content of essential fatty acids per liter:
- Linoleic acid (omega-6) 48.0 – 58.0 g
- Alpha-linolenic acid (omega-3) 5.0 – 11.0 g
- Eicosapentaenoic acid and docosahexaenoic acid (omega-3) 8.6 – 17.2 g

20% correspond to total content of triglycerides.

- Total energy per liter 7900 kJ (1910 Kcal)
- Osmolality 410 mOsm/kg
- Titration (to pH 7.4) < 0.5 mmol/l NaOH or HCL
- pH 6.5 – 8.5

For excipients, see section 6.1

3. PHARMACEUTICAL FORM

Emulsion for infusion

White, homogeneous emulsion.

4. CLINICAL PARTICULARS

4.1 Therapeutic indications

Supply of lipids, including essential omega-6 fatty acids and omega-3 fatty acids, as part of a parenteral nutrition regimen for adults, when oral or enteral nutrition is impossible, insufficient or contra-indicated.

4.2 Posology and method of administration

Adults:
Dosage should be adjusted to the individual patient’s needs.
Recommended dosage:

1 - 2 g fat per kg body weight per day

equivalent to:
- 5 - 10 ml of Lipidem 200 mg/ml per kg body weight per day

Infusion rate:
The infusion should be administered at the lowest possible infusion rate. During the first 15 minutes the infusion rate should only be 50% of the maximum infusion rate to be used.

Maximum infusion rate:
Up to 0.15 g lipids per kg body weight per hour, equivalent to:
- up to 0.75 ml of Lipidem 200 mg/ml per kg body weight per hour

The infusion rate should be reduced in undernourished patients.

As clinical experience with long-term use of Lipidem 200 mg/ml is limited, it should normally not be administered for longer than one week. Only if clearly needed the emulsion may be administered longer, with careful metabolic monitoring.

Lipidem 200 mg/ml is suitable for both central and peripheral intravenous infusion.

Paediatric patients:
Safety and efficacy in children and adolescents have not been established.

4.3 Contraindications

Lipidem 200 mg/ml must not be used in any of the following conditions:
- Severe hyperlipidemia
- Severe blood coagulation disorders
- Intrahepatic cholestasis
- Severe liver failure
- Severe renal failure without access to haemofiltration or dialysis.
- Acute phase of myocardial infarction or stroke
- Acute thromboembolic disease, lipidembolism
- Hypersensitivity to egg, fish, or soya-bean protein or to any of the active substances or excipients.

The following conditions are general contraindications to infusion therapy:
- Unstable hemodynamic status with compromised vital functions (conditions of collapse and shock)
- unstable metabolic conditions (e.g. severe post-traumatic conditions, uncompensated diabetes mellitus, severe sepsis, acidosis)
- Acute pulmonary edema
- Hyperhydration
- Decompensated cardiac insufficiency
- Hypotonicdehydration
- Hypokalaemia
4.4 Special warnings and precautions for use

Serum triglycerides should be monitored during the infusion of Lipidem 200 mg/ml. In patients with suspected disorders of lipid metabolism, fasting lipaemia should be ruled out before the start of the infusion. Hypertriglyceridemia 12 hours after the administration of lipids is also indicative of abnormal lipid metabolism. Transient hypertriglyceridaemia or elevated blood glucose levels may arise, depending on the patient's metabolic status. If the plasma triglyceride concentration rises to more than 3 mmol/l during administration of the lipid emulsion, it is recommended to reduce the infusion rate. If the plasma triglyceride concentration remains higher than 3 mmol/l, the infusion should be stopped until the plasma triglyceride concentration is normalized.

Electrolytes, fluid balance or body weight, acid-base balance, blood glucose levels, and, during long-term administration, full blood counts, coagulation status, and liver function should be monitored.

Infusion of Lipidem 200 mg/ml should be discontinued in case of appearance of any sign of allergic reaction, e.g. fever, shivering, rash, dyspnoea.

An overdose may lead to fat overload syndrome, see section 4.8.

There is as yet no clinical experience of the use of Lipidem 200 mg/ml in children and adolescents, and there is only limited experience of its use in patients with diabetes mellitus or renal failure.

There is as yet only limited experience of the use of Lipidem 200 mg/ml for periods longer than seven days.

Caution should be exercised in patients with conditions associated with disturbed lipid metabolism, such as renal insufficiency, diabetes mellitus, pancreatitis, hepatic insufficiency, hypothyroidism (in the presence of hypertriglyceridaemia), pulmonary disease and sepsis.

Lipids may interfere with certain laboratory tests (such as bilirubin, lactate dehydrogenase, oxygen saturation, haemoglobin measurement) when the blood sample is taken before the lipids have been eliminated from the bloodstream. In most patients the lipids are eliminated within 5 to 6 hours after the end of the infusion.

Energy supply with lipid emulsions only could cause metabolic acidosis. This may be avoided by the concurrent administration of carbohydrates. It is therefore recommended to infuse an adequate quantity of intravenous carbohydrates or carbohydrate-containing amino acid solutions along with the fat emulsion.

Vitamin E can interfere with the effect of vitamin K in clotting factor synthesis. This should be considered in patients with blood coagulation disorders or suspected vitamin K deficiency.

Lipidem 200 mg/ml contains 2.6 mmol/l sodium. This should be taken into consideration by patients on a controlled sodium diet.

4.5 Interaction with other medicinal products and other forms of interaction
Heparin induces a transient release of lipoprotein lipase into the bloodstream. This may initially lead to increased plasma lipolysis, followed by a transient decrease in triglyceride clearance.

Soya-bean oil has a natural content of vitamin K1. The content is however so low in Lipidem 200 mg/ml that it is not expected to significantly influence the coagulation process in patients treated with coumarin derivatives. Nevertheless, the coagulation status should be monitored in patients treated concomitantly with anticoagulants.

4.6 Pregnancy and lactation

Pregnancy
There is no experience of the use of Lipidem 200 mg/ml in pregnant women. Parenteral nutrition may become necessary during pregnancy. Lipidem 200 mg/ml should only be given to pregnant women after careful consideration.

Lactation
There is no experience of the use of Lipidem 200 mg/ml in nursing mothers. It is as yet not known if Lipidem 200 mg/ml crosses the placental barrier or is excreted in breast milk. No respective data are available from animal experiments either. Breast-feeding is in general not recommended to mothers on parenteral nutrition.

4.7 Effects on ability to drive and use machines

Not relevant.

4.8 Undesirable effects

The following adverse reactions include a number of systemic reactions that are very rarely associated with the use of Lipidem 200 mg/ml:

Blood and lymphatic system disorders
Very rare (<1/10,000): Hypercoagulation

Immune system disorders
Very rare (<1/10,000): Allergic reactions

Metabolic and nutritional disorders
Very rare (<1/10,000): Hyperlipidemia, hyperglycemia, metabolic acidosis, keto-acidosis.

However, the frequency of the undesirable effects listed here is dose-dependent. They are likely to occur as symptoms of absolute or relative overdose. A.m. frequency applies to conditions of correct use, in terms of dosing monitoring, observation of safety restrictions and instructions.

Central and peripheral nervous system disorders
Very rare (<1/10,000): Drowsiness

Vascular disorders
Very rare (<1/10,000): Hypertension or hypotension

Respiratory, thoracic, and mediastinal disorders
Very rare (<1/10,000): Dyspnea, cyanosis
Gastrointestinal disorders
Very rare (<1/10,000): Nausea, vomiting

General disorders and/or administration site conditions
Very rare (<1/10,000): Headache, flushing / erythema, elevated body temperature, sweating, chills, chest and back pain, Fat overload syndrome (see below).

Should these adverse reactions occur or should the triglyceride level rise above 3 mmol/l during infusion, the infusion of Lipidem 200 mg/ml should be stopped or, if necessary, continued at a reduced dosage. If the infusion is restarted, the patient should be carefully monitored, especially at the beginning, and serum triglycerides should be determined at short intervals.

Triglycerides that contain omega-3 fatty acids may increase bleeding time and inhibit platelet aggregation. In patients with aspirin-induced asthma, pulmonary function may deteriorate as well.

Lipidem 200 mg/ml should always be a part of a complete parenteral nutritional treatment including amino acids and glucose. Nausea, vomiting, lack of appetite and hyperglycemia are symptoms related to conditions indicating parenteral nutrition and may sometimes be associated with parenteral nutrition.

Fat overload syndrome
Impaired capacity to eliminate triglycerides can lead to “Fat overload syndrome” which may be caused by overdose. Possible signs of metabolic overload must be observed. The cause may be genetic (individually different metabolism) or the fat metabolism may be affected by ongoing or previous illnesses. This syndrome may also appear during severe hypertriglyceridemia, even at the recommended infusion rate, and in association with a sudden change in the patient’s clinical condition, such as renal function impairment or infection. The fat overload syndrome is characterised by hyperlipemia, fever, fat infiltration, hepatomegaly with or without icterus, splenomegaly, anemia, leukopenia, thrombocytopenia, coagulation disorder, hemolysis and reticulocytosis, abnormal liver function tests and coma. The symptoms are usually reversible if the infusion of the fat emulsion is discontinued. Should signs of a fat overload syndrome occur, the infusion of Lipidem 200 mg/ml should be discontinued immediately.

4.9 Overdose

Overdose leading to fat overload syndrome may occur as a result of a too rapid infusion rate, or chronically at recommended rates of infusion in association with a change in the patients clinical conditions e.g. renal function impairment or infection. Overdosage may lead to undesirable effects (see section 4.8).

Substantial overdosage with a fat emulsion that contains medium-chain triglycerides may lead to metabolic acidosis, especially when no carbohydrates are given concomitantly.

Treatment: In case of an overdose, the infusion must be stopped immediately. Other therapeutic measures will depend on a patient's specific symptoms and their severity. If the infusion is restarted after symptoms have subsided, the infusion rate should be increased gradually with close monitoring.
5 PHARMACOLOGICAL PROPERTIES

5.1 Pharmacodynamic properties

Pharmacotherapeutic group: Solutions for parenteral nutrition, fat emulsions ATC code: B05BA02

Lipidem 200 mg/ml is intended for supply of energy and polyunsaturated ("essential") omega-6 and omega-3 fatty acids as part of parenteral nutrition regimens. Lipidem 200 mg/ml therefore contains medium-chain triglycerides, soya-bean oil (medium-chain triglycerides), and triglycerides containing omega-3 fatty acids (long-chain triglycerides).

Medium-chain triglycerides are hydrolyzed faster, eliminated faster from the bloodstream, and oxidized faster than long-chain triglycerides. Only the long-chain omega-6 and omega-3 triglycerides supply polyunsaturated fatty acids. They are primarily intended for the prevention and treatment of essential fatty acid deficiency, but also as a source of calories. Lipidem 200 mg/ml supplies essential omega-6 fatty acids, mainly in the form of linoleic acid, and omega-3 fatty acids in the form of alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid.

The ratio of omega-6/omega-3 fatty acids in Lipidem 200 mg/ml is approximately 3:1.

5.2 Pharmacokinetic properties

The dose, the infusion rate, the patient’s metabolic status and other individual factors (such as fasting levels) should be considered when the maximum serum triglyceride concentration is determined.

Medium-chain fatty acids have lower affinity for albumin than long-chain fatty acids. When administered in accordance with the dosing guidelines, however, plasma albumin binding of both types of fatty acid is almost 100%. When the dosing guidelines are complied with, neither medium-chain nor long-chain fatty acids therefore cross the blood-brain barrier or pass into the cerebrospinal fluid.

5.3 Preclinical safety data

Preclinical studies with a developmental version of Lipidem 200 mg/ml (containing twice the amount of omega-3 acid triglycerides present in the final product and a correspondingly smaller amount of long chain triglycerides) revealed no effects other than those expected following administration of high doses of lipids. In a rabbit reproductive toxicity study no evidence of embryotoxicity or teratogenicity was seen at a dose of 2g lipid/kg body weight per day for 12 days.

6 PHARMACEUTICAL PARTICULARS

6.1 List of excipients

Egg Lecithin,
Glycerol,
Sodium Oleate,
Ascorbyl Palmitate,
all-rac-α-T ocopherol, 
Sodium Hydroxide for pH adjustment, 
Water for Injections.

6.2 Incompatibilities

In the absence of compatibility studies, this medicinal product must not be mixed with other medicinal products.

6.3 Shelf life

Unopened: 2 years. After first opening the medicinal product should be used immediately.

6.4 Special precautions for storage

Do not store above 25°C. Store in the original package, in order to protect from light. Do not freeze.

6.5 Nature and contents of container

The emulsion is packed in a Type II glass bottle with a butyl rubber stopper or in a soft plastic container in a protective outer bag. An oxygen absorber is placed between the inner and outer bag. The primary container consists of a three-layer plastic laminate with a polyamide outer layer and polypropylene. The butyl rubber injection and additive ports are self-sealing.

6.6 Instructions for use and handling and disposal

Use only if the emulsion is homogeneous from intact containers. Inspect the emulsion visually for phase separation prior to administration. For single use only. Any unused emulsion should be discarded. Products that have been frozen should be discarded.

Before infusing a lipid emulsion together with other solutions via a Y connector or bypass set, the compatibility of these fluids should be checked, especially when co-administering carrier solutions to which drugs have been added. Particular caution should be exercised when co-infusing solutions that contain divalent electrolytes (such as calcium).

The emulsion should always be brought to room temperature prior to infusion.

If filters are used, these must be permeable to lipids.

7 MARKETING AUTHORIZATION HOLDER

B. Braun Melsungen AG Carl-Braun-Strasse 1 P.O. Box 1110 + 1120 34209 Melsungen, Germany

8 MARKETING AUTHORIZATION NUMBER

PL 03551/0099
9 DATE OF FIRST AUTHORIZATION

October 2004
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