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Oesophageal Cancer: Association with Visceral Obesity, Inflammation and Optimisation of Postoperative Outcomes

A dissertation submitted to the University of Dublin

for the degree of Doctor of Philosophy



by

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April 2013

Department of Surgery



Thesis 10321

DECLARATION

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SUMMARY

The incidence of oesophageal cancer is rising in the developed world and has paralleled the rising prevalence of obesity. This is an aggressive malignancy with poor overall survival rates. Thus there is an impetus to identify mechanisms driving this increase in oesophageal cancer incidence and strategies to improve outcomes in patients receiving treatment. The aim of this thesis was to investigate further the relationship between visceral obesity and oesophageal cancer, in terms of both tumour biology and postoperative outcomes. In addition, the potential role of immuno-nutrition with eicosapentaenoic acid in improving postoperative outcomes in oesophagectomy patients was assessed.

Obesity associated cancer research is now focussing on the detrimental effects of specifically visceral adipose tissue excess with its associated pro-inflammatory insulin resistant state, collectively referred to as the metabolic syndrome. However, this field is hampered by the lack of an anthropometric threshold to classify visceral obesity in males and females. Utilising CT derived visceral fat area and metabolic syndrome status as a marker of whole body dysmetabolism, a pathologically relevant threshold for the classification of visceral obesity was determined. A visceral fat area of $\geq 160\text{cm}^2$ for men (82.7% sensitivity and 62.1% specificity) and $\geq 80\text{cm}^2$ for women (95.8% sensitivity and 71.4% specificity) are proposed as cut-off values for use in obesity-associated cancer research.

The insulin like growth factor (IGF) axis is altered in obesity and oesophageal cancer. Using *in vitro* models it was determined that IGF-1 induces significant proliferation of oesophageal adenocarcinoma cells. Utilising serum samples from a cohort of oesophageal cancer patients and non-cancer controls it was observed, for the first time, that circulating levels of IGF-1 are increased in visceral obesity and in oesophageal adenocarcinoma. The expression of the IGF-1 receptor was assessed in resected oesophageal tumour specimens and it was found that expression was increased at the tumour invasive edge. These novel findings suggest an important role for the IGF axis in oesophageal tumour growth and a plausible mechanism by which obesity increases oesophageal cancer risk.

The curative treatment for oesophageal cancer is resection and in this thesis the impact of visceral obesity on postoperative outcomes is assessed for the first time. Visceral obesity and the metabolic syndrome were associated with a heightened acute phase response postoperatively and an exacerbated inflammatory response to a complication, characterised by increased postoperative CRP and CRP:albumin levels. In addition, alterations were observed in the postoperative production of pro- and anti-inflammatory cytokines including IL-6, IL-12, TNF- α and TGF- β in obese and dysmetabolic patients. These findings provide insight into how visceral obesity impacts immune function postoperatively.

A double blind randomised controlled clinical trial was conducted to assess if peri-operative supplementation with eicosapentaenoic acid could attenuate the inflammatory stress response and catabolism in oesophagectomy patients. Supplementation with eicosapentaenoic acid significantly reduced the incidence of postoperative complications in patients. Furthermore it was associated with preservation of fat free mass postoperatively, suggesting anti-catabolic effects. EPA supplementation significantly altered the gene expression of the inflammatory mediators CCL4 and IL-10 in immune cells postoperatively and significantly increased the production of the circulating eicosanoids prostaglandin E₂ and prostacyclin I₂. This study presents novel findings regarding the pro- and anti-inflammatory effects of peri-operative EPA supplementation and its potential use in oesophagectomy patients.

The findings of this thesis add to the knowledge base regarding oesophageal cancer, which will hopefully result in improved treatment strategies and outcomes in patients suffering from this malignancy.

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PUBLICATIONS

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Donohoe CL, **Doyle SL**, Reynolds JV. Short Bowel Syndrome *in* Roshan Lall Gupta's Recent Advances in Surgery-13, January 2013; ISBN: 978-93-5090-382-7.

Doyle SL, Donohoe CL, Howard JM *et al.* IGF-1 and its receptor in esophageal cancer: association with adenocarcinoma and visceral obesity. *American Journal of Gastroenterology* 2012 107(2):196-204.

Donohoe CL, **Doyle SL**, McGarrigle SA *et al.* The insulin-like growth factor-1 axis in oesophageal adenocarcinoma: association with visceral adiposity. *British Journal of Surgery*. 2012 99(3):387-96.

Doyle SL, Donohoe CL, Lysaght J, *et al.* Visceral obesity, metabolic syndrome, insulin resistance and cancer. *Proceedings of the Nutrition Society* 2012 71(1):181-9.

Donohoe CL, **Doyle SL**, Reynolds JV. Visceral adiposity, insulin resistance and cancer risk. *Diabetology & Metabolic Syndrome* 2011 3: 12.

Reynolds JV, Donohoe CL, **Doyle SL**. Diet, obesity and cancer. *Irish Journal of Medical Science* 2011 180(2): 521-527.

Doyle SL, Lysaght J, Reynolds JV. Obesity and post-operative complications in patients undergoing non-bariatric surgery. *Obesity Reviews* 2010 11 (12) 875-886.

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"Visceral obesity alters expression of inflammatory mediators in patients undergoing gastrointestinal resection". July 2012, Nutrition Society Annual Meeting, Belfast. *Proceedings of the Nutrition Society*, **71**, E58.

"Visceral obesity alters expression of inflammatory mediators in patients undergoing gastrointestinal resection". January 2012, **Patey Prize plenary session**, Society of Academic and Research Surgery, Nottingham. *British Journal of Surgery* **99**, 1-5.

"Excess visceral adiposity negatively influences post-operative inflammatory response and risk of complications in patients undergoing gastrointestinal resection." January 2011, Society of Academic and Research Surgery, Dublin. *British Journal of Surgery* **98**, 6-39.

"Insulin-like growth factor-1 receptor is upregulated in leading edge of oesophageal adenocarcinoma and is associated with reduced survival". January 2010, **Patey Prize plenary session**, Society of Academic and Research Surgery, London. *British Journal of Surgery* **97**, 1-6.

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November 2011, Institute of Molecular Medicine Annual Meeting, Dublin.

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Chapter 1 Introduction	1
1.1 OESOPHAGEAL CANCER	1
1.1.1 Epidemiology	1
1.1.2 Pathogenesis	1
1.2 OBESITY AND OESOPHAGEAL CANCER.....	2
1.2.1 Obesity and Cancer - Epidemiology	2
1.2.2 Adipose Tissue, the Metabolic Syndrome and Cancer.....	3
1.2.3 Mechanisms Underlying Visceral Obesity and Tumourigenesis	4
1.2.4 Oesophageal Adenocarcinoma – A Model of Visceral Obesity, Metabolic Syndrome and Cancer	11
1.3 OBESITY AND POSTOPERATIVE RECOVERY	13
1.3.1 Obesity and Postoperative Morbidity.....	13
1.3.2 Visceral Adiposity and Risk of Postoperative Complications	16
1.3.3 The Metabolic Syndrome and Risk of Postoperative Complications	18
1.4 THE NUTRITIONAL MANAGEMENT OF POST-OPERATIVE OUTCOMES IN OESOPHAGECTOMY PATIENTS – A ROLE FOR IMMUNONUTRITION?	19
1.4.1 The Anti-Inflammatory Properties of Eicosapentaenoic Acid.....	21
1.4.2 Postoperative Inflammation Response and Recovery Following Oesophagectomy – A Role For EPA?	25
1.5 HYPOTHESIS AND AIMS	27
Chapter 2 Materials and Methods	29
2.1 REAGENTS.....	30
2.1.2 Cell Culture Medium	30
2.1.3 Hank’s Balanced Salt Solution	30
2.1.4 Dextran Solution.....	30
2.1.5 Monocyte Isolation Buffer	31
2.1.6 Hypotonic Buffer	31

2.1.7	Phosphate Citrate Buffer	31
2.1.8	ELISA Developing Solution	31
2.1.9	ELISA Stopping Solution	31
2.1.10	ELISA Washing Buffer.....	31
2.1.11	Fluorescence-Activated Cell Sorting (FACS) Buffer.....	32
2.1.12	FACS Blocking Buffer.....	32
2.1.13	RIPA Lysis Buffer	32
2.1.14	Separating Gels	32
2.1.15	5% (w/v) Stacking Gel	33
2.1.16	Laemmli Buffer.....	33
2.1.17	Electrode Buffer	33
2.1.18	Cold Transfer Buffer.....	33
2.1.19	Tris-Buffered Saline (TBS)	33
2.1.20	Tris-Buffered Saline Tween (TBST).....	34
2.1.21	Tris-Acetate-EDTA (TAE) Buffer	34
2.2	CELL CULTURE	34
2.2.1	Cell Subculture	34
2.2.2	Cell Counting.....	34
2.3	BRDU CELL PROLIFERATION ASSAY.....	35
2.4	WESTERN BLOTTING	36
2.4.1	Protein Extraction	36
2.4.2	Protein Quantification	36
2.4.3	Denaturing Polyacrylamide Gel (SDS-PAGE).....	36
2.4.4	Protein Electrophoresis and Transfer	36
2.4.5	Antibody Probing of Membranes	37
2.4.6	Densitometry	38

2.5	INVESTIGATION OF RNA EXPRESSION	38
2.5.1	RNA Isolation from Tumour Cell Lines using TriReagent Method	38
2.5.2	RNA Quantification and Purity Analysis	38
2.5.3	cDNA Synthesis.....	39
2.5.4	Reverse Transcription Polymerase Chain Reaction (RT-PCR)	39
2.5.5	Agarose Gel Electrophoresis	40
2.6	IMMUNOHISTOCHEMISTRY	41
2.6.1	Immunohistochemical Staining.....	41
2.6.2	Immunohistochemical Staining Quantification.....	42
2.7	ADIPOSE TISSUE BIOBANK.....	43
2.7.1	Anthropometry.....	43
2.7.2	Clinical Measurements.....	44
2.7.3	Blood Sampling and Biochemical Measurements.....	44
2.7.4	Definition of the Metabolic Syndrome	44
2.8	PATIENT RECRUITMENT FOR STUDY OF OBESITY AND POSTOPERATIVE INFLAMMATION	45
2.9.1	Endpoints.....	45
2.9.2	Inclusion and Exclusion Criteria	46
2.9.3	Randomisation	46
2.9.4	Test and Comparator Product.....	46
2.9.5	Treatment Administration.....	49
2.9.6	Method of Blinding.....	50
2.9.7	Anthropometric Measures	50
2.9.8	Nutritional Assessment	50
2.9.9	Routine laboratory measures of biochemical & immune function	50
2.10	BLOOD SAMPLE PROCESSING	53
2.10.1	Serum and Plasma Isolation	53

2.10.2	Peripheral Blood Mononuclear Cell Isolation	53
2.10.3	Neutrophil Isolation	53
2.10.4	Monocyte Isolation	54
2.10.5	Flow Cytometry.....	54
2.10.6	Preparation of Nuclear Extracts.....	55
2.11	RNA ISOLATION FROM IMMUNE CELLS USING QIAGEN RNEASY KIT	55
2.12	INFLAMMATORY GENE ARRAYS.....	56
2.12.1	RT ² First Strand cDNA Synthesis	56
2.12.2	Quantitative Polymerase Chain Reaction for Inflammatory Arrays	56
2.13	QUANTITATIVE PCR (qPCR).....	57
2.14	PBMC GENE EXPRESSION.....	57
2.15	NEUTROPHIL ACTIVATION	58
2.16	MONOCYTE ACTIVATION	58
2.15	MEASUREMENT OF NUCLEAR FACTOR- $\kappa\beta$ (NF- $\kappa\beta$) p65 SUBUNIT	59
2.16	ELISA.....	59
2.17	MEASUREMENT OF EICOSANOID METABOLITES.....	63
2.17.1	6-keto-Prostaglandin F _{1α} and Leukotriene B ₄	63
2.17.2	Prostaglandin E ₂	64
2.18	STATISTICAL ANALYSIS	64
Chapter 3 Establishing CT-Defined Visceral Fat Area Thresholds for Use in Obesity Related Cancer Research		66
3.1	INTRODUCTION.....	67
3.1.1	Specific Aims	69
3.2	RESULTS	70
3.2.1	Subject Characteristics.....	70
3.2.2	Determination of Abdominal Adiposity Thresholds Using Individual Markers of Dysmetabolism.....	72

3.2.3	Determination of Abdominal Adiposity Thresholds Using the MetSyn	74
3.2.4	Correlating Optimal Thresholds for Abdominal Adiposity with Waist Circumference	80
3.3	DISCUSSION	82
Chapter 4 IGF-1 and its Receptor in Oesophageal Cancer: Association with Adenocarcinoma and Visceral Adiposity		86
4.1	INTRODUCTION	87
4.1.1	Specific Aims.....	88
4.2	RESULTS.....	88
4.2.1	<i>In Vitro</i> mRNA and Protein Expression of IGF-1 and its Receptor	88
4.2.2	Changes in Cell Proliferation in Response to Exogenous IGF-1	91
4.2.3	Patient Characteristics	93
4.2.4	Serum IGF-1 levels.....	94
4.2.5	Tumour Expression of IGF-1R and CD68 ⁺ cells.....	96
4.3	DISCUSSION	104
Chapter 5 Investigating the Influence of Visceral Obesity on the Postoperative Inflammatory Response		108
5.1	INTRODUCTION	109
5.1.1	Specific Aims.....	111
5.2	RESULTS.....	111
5.2.1	Patient Characteristics of Retrospective Cohort.....	111
5.2.2	Incidence of postoperative complications in retrospective cohort	112
5.2.3	Postoperative expression of CRP and CRP:albumin in patients with no complications	113
5.2.4	Postoperative expression of CRP and CRP:albumin in patients who experienced a postoperative complication.....	116
5.2.5	Prospective investigation into postoperative inflammatory response following upper gastrointestinal resection	118

5.2.6	Alterations in inflammatory gene expression in peripheral blood mononuclear cells (PBMC) in obese and non-obese patients.....	119
5.2.7	Validation of significantly altered genes of interest by qPCR.....	121
5.2.8	Investigation of TNFSF-14, LT β and LT β R gene expression in obese and non-obese patients by qPCR.....	123
5.2.9	Expression of neutrophil activation markers in obese and non-obese patients pre and postoperatively	125
5.2.10	NF- κ B p65 in non-obese and obese patients in the postoperative period...	128
5.2.11	Circulating levels of inflammatory mediators in obese and non-obese patients postoperatively.....	129
5.2.12	Circulating levels of inflammatory mediators in MetSyn positive and MetSyn negative patients postoperatively.....	133
5.2.13	Influence of hyperglycaemia on circulating levels of inflammatory mediators postoperatively.....	137
5.2.14	Postoperative changes in clinical parameters.	139
5.2.15	Incidence of postoperative complications in prospectively recruited cohort.....	141
5.3	DISCUSSION.....	142
Chapter 6 The impact of an eicosapentaenoic acid enriched enteral feed on the postoperative inflammatory response and recovery in oesophagectomy patients.....		149
6.1	INTRODUCTION.....	150
6.1.1	Specific Aims	152
6.2	RESULTS	153
6.2.1	Recruitment and discontinuation of patients in the clinical trial	153
6.2.2	Characteristics of the study cohort.....	155
6.2.4	Alterations in inflammatory gene expression in peripheral blood mononuclear cells (PBMC) following oesophagectomy.....	157

6.2.5	Impact of EPA supplementation on inflammatory gene expression in PBMC postoperatively.....	158
6.2.6	Impact of EPA supplementation on the expression of monocyte phenotype indicators postoperatively	160
6.2.7	Impact of EPA supplementation of the expression of neutrophil activation markers postoperatively	163
6.2.8	Impact of EPA supplementation on C-reactive protein, albumin and white cell count postoperatively	165
6.2.9	Impact of EPA supplementation on circulating levels of a panel of inflammatory mediators postoperatively	167
6.2.10	Impact of EPA supplementation on circulating levels of eicosanoids postoperatively	171
6.2.11	Impact of EPA supplementation on muscle and fat mass postoperatively.	173
6.3	DISCUSSION	175
Chapter 7	General Discussion	181
7.1	FUTURE DIRECTIONS	191
REFERENCES		193
Appendix I		219

LIST OF FIGURES

Figure 1.1 The insulin-like growth factor (IGF) axis..... 8

Figure 1.2 Adipose tissue inflammation 11

Figure 1.3 The anatomical changes following oesophagectomy 20

Figure 1.4 Metabolism of arachidonic acid and eicosapentaenoic acid 22

Figure 1.5 Role of resolvins in inflammation resolution 24

Figure 1.6 The biphasic inflammatory response 26

Figure 2.1 Design of randomised controlled trial and collection of anthropometric
measures and blood samples 52

Figure 3.1 Abdominal fat areas associated with the presence of the MetSyn in males
and females 75

Figure 3.2 Abdominal fat areas associated with presence of the MetSyn in males and
females using Harmonized Metabolic Syndrome Criteria..... 79

Figure 3.3 Correlation of optimal adiposity thresholds to waist circumference in
males and females. 81

Figure 4.1 Expression of IGF-1, IGF-2 and IGF-1R in oesophageal cancer cell lines... 90

Figure 4.2 IGF-1 stimulates oesophageal adenocarcinoma cell line growth in a dose-
dependent fashion..... 92

Figure 4.3 Serum IGF-1 levels are increased in oesophageal adenocarcinoma,
visceral obesity and the metabolic syndrome..... 95

Figure 4.4 Representative images of IGF-1R staining intensity..... 97

Figure 4.5 IGF-1R tumour expression is not associated with obesity or the metabolic
syndrome 98

Figure 4.6 IGF-1R expression is not associated with T-stage, nodal status and survival..... 99

Figure 4.7 Pattern of IGF-1R expression in oesophageal adenocarcinoma and
squamous cell carcinoma tumour sections 100

Figure 4.8 Representative image of staining for CD68⁺ cells in resected oesophageal
tumour. 101

Figure 4.9 Presence of CD68⁺ cells in oesophageal tumour is not associated with
obesity or the metabolic syndrome 102

Figure 4.10 Increased CD68⁺ cell numbers are present in the tumour associated
stroma compared to the tumour island 103

Figure 5.1	Changes in CRP and CRP:albumin ratio postoperatively in patients with no postoperative complications	115
Figure 5.2	Changes in CRP and CRP:albumin ratio postoperatively in patients with postoperative complications	117
Figure 5.3	Validation of gene expression of CCL3L1, INHBA and MGLL in PBMC from obese and non-obese patients pre and postoperatively.....	122
Figure 5.4	Gene expression of TNFSF14, LT β and LT β R in PBMC from obese and non-obese patients pre and postoperatively	124
Figure 5.5	Assessment of neutrophil purity by flow cytometry following isolation.	125
Figure 5.6	Gene expression of CD62L, CD11b and MPO by neutrophils from obese and non-obese patients pre and postoperatively	127
Figure 5.7	NF- κ B p65 in non-obese and obese patients pre and postoperatively...	128
Figure 5.8	Altered innate inflammatory response in obese patients postoperatively..	130
Figure 5.9	Altered adaptive inflammatory response in obese and non-obese patients postoperatively	131
Figure 5.10	Alterations in anti-inflammatory response in obese and non-obese patients postoperatively	132
Figure 5.11	Alterations in innate inflammatory response postoperatively in MetSyn positive and MetSyn negative patients	134
Figure 5.12	Alterations in adaptive inflammatory response postoperatively in MetSyn positive and MetSyn negative patients	135
Figure 5.13	Alterations in anti-inflammatory response postoperatively in MetSyn positive and MetSyn negative patients	136
Figure 5.14	Alterations in postoperative inflammatory response in hyperglycaemic and normoglycaemic patients.....	138
Figure 5.15	Changes in clinical parameters postoperatively in obese and non-obese patients	140
Figure 6.1	Flow chart of patient recruitment and discontinuation to clinical trial ..	154
Figure 6.2	Changes in gene expression of CCL4 and IL-10 in PMBC postoperatively in patients receiving EPA enriched versus standard enteral feeding.	159
Figure 6.3	Assessment of monocyte purity by flow cytometry following isolation .	161

Figure 6.4	Monocyte expression of arginase-1, IL-10, IL-12 and TNF- α postoperatively in patients receiving standard versus EPA enriched enteral feed	162
Figure 6.5	Expression of neutrophil activation and adhesion markers postoperatively in patients receiving standard versus EPA enriched enteral feed.....	164
Figure 6.6	The acute inflammatory response postoperatively in patients receiving standard enteral feed versus those receiving EPA enriched feed.	166
Figure 6.7	Alterations in circulating levels of pro-inflammatory in patients receiving standard versus enteral feed.....	168
Figure 6.8	Alterations in postoperative circulating levels of adaptive cytokines in patients receiving standard enteral feed versus EPA enriched enteral feed.....	169
Figure 6.9	Alterations in postoperative circulating levels of anti-inflammatory mediators in patients receiving standard enteral feed versus EPA enteral feed.....	170
Figure 6.10	Changes in circulating levels of eicosanoids postoperatively in patients receiving standard enteral feed versus EPA enriched enteral feed.	172
Figure 6.11	Changes in body composition postoperatively in patients receiving standard versus EPA enriched enteral feeding.	174

LIST OF TABLES

Table 2.1	Antibodies used in Western blotting	38
Table 2.2	Reagents and volumes used in cDNA synthesis	39
Table 2.3	Reagents and volumes used in RTPCR	40
Table 2.4	Primer sequences in RT-PCR.....	40
Table 2.5	Criteria for Metabolic Syndrome defined by IDF	44
Table 2.6	Composition of Test Product (Prosure) and Comparator (Ensure Plus)	48
Table 2.7	Routine Laboratory Tests Carried out by CPL in St. James's Hospital	51
Table 2.8	Antibodies Used for FACS Analysis.....	55
Table 2.9	qPCR Primer Probes Used to Detect Gene Expression in Immune Cells.....	58
Table 2.10	Antibody Concentrations and Reagents Used in ELISA	62
Table 2.11	EIA to detect 6-keto-Prostaglandin F ₁ α , Leukotriene B ₄ and Thromboxane B ₂	63
Table 3.1	Patient demographics according to gender and metabolic syndrome status	71
Table 3.2	Abdominal fat thresholds associated with increased risk of dysmetabolism.....	73
Table 3.3	Abdominal fat thresholds associated with increased risk of the MetSyn in males and females.....	74
Table 3.4	Abdominal fat thresholds associated with increased risk of the MetSyn in males with oesophageal and colorectal adenocarcinoma	76
Table 3.5	Abdominal fat thresholds associated with increased risk of the MetSyn in males and females (Harmonized Metabolic Syndrome Criteria)	78
Table 4.1	Oesophageal cancer patient demographics.....	93
Table 5.1	Patient characteristics of cohort for retrospective analysis of postoperative inflammation	112
Table 5.2	Incidence of postoperative complications in retrospective cohort, separated by obesity and metabolic syndrome status.	113
Table 5.3	Patient characteristics of prospectively recruited cohort.....	118
Table 5.4	Significantly altered inflammatory genes in obese compared to non-obese patients preoperatively and on day 1 and day 7 postoperatively	120
Table 5.5	Incidence of postoperative complications in the prospectively recruited upper GI cancer cohort (2010-2011).....	141
Table 6.1	Characteristics of the study cohort.	155

Table 6.2 Incidence of postoperative complications in patients receiving standard
feed versus EPA enriched feed 156

Table 6.3 Alterations in expression of inflammatory genes in PBMCpostoperatively ... 157

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ACTH	Adrenocorticotrophic Hormone
AKT-1	Anti-Apoptotic Serine Threonine Kinase
ANOVA	Analysis of Variance
AUC	Area under the Curve
AZU1	Azurocidin-1
BCA	Bicinchoninic Acid
BCP	1-Bromo-2 Chloropropane
BMI	Body Mass Index
Bo	Maximum Binding
BrdU	5-Bromo-2'-Deoxyuridine
BSA	Bovine Serum Albumin
BSE	Basal Layer of Normal Squamous Epithelium
CABG	Coronary Artery Bypass Grafting
CCL3L1	Chemokine Ligand 3-Like 1
cDNA	Complimentary Deoxyribonucleic Acid
CI	Confidence Interval
COX	Cyclooxygenase
CRC	Colorectal Adenocarcinoma
CRP	C-Reactive Protein
cRPMI	Complete RPMI
CRT	Chemoradiotherapy
CT	Computed Tomography
DAB	Diaminobenzidine
dH ₂ O	Deionised H ₂ O
DHA	Docosahexanoic Acid
dNTPs	Deoxyribonucleotide Triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay

EORTC	European Organisation for Research and Treatment of Cancer Questionnaires
EPA	Eicosapentaenoic Acid
EPA	Eicosapentaenoic Acid
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FN1	Fibronectin-1
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HBSS	Hank's Balanced Salt Solution
HDL-C	High Density Lipoprotein Cholesterol
HEPE	Hydroperoxyeicosapentaenoic Acid
HETE	Hydroxyeicosatetraenoic Acid
HPETE	Hydroperoxyeicosatetraenoic Acid
HRP	Horseradish Peroxidase
IDF	International Diabetes Federation
IE	Invasive Tumour Edge
IFN κ	Interferon- κ
IFN- γ	Interferon- γ
IGF	Insulin-Like Growth Factor
IGF-1	Insulin-Like Growth Factor-1
IGF-1R	Insulin-Like Growth Factor-1 Receptor
IGF-2	Insulin-Like Growth Factor-2
IGFBP	Insulin-Like Growth Factor Binding Protein
IL	Interleukin
INHBA	Inhibin β -A
iNKT	Invariant Natural Killer
LOX	Lipoxygenase
LTA ₄	Leukotriene A ₄
LTA ₅	Leukotriene A ₅
LTB ₄	Leukotriene B ₄
LTB ₅	Leukotriene B ₅
LT β	Lymphotoxin- β

LT β R	LT β Receptor
LXA ₄	Lipoxin A ₄
LXA ₅	Lipoxin A ₅
LXB ₄	Lipoxin B ₄
LXB ₅	Lipoxin B ₅
MAPK	Mitogen Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MetSyn	Metabolic Syndrome
MGLL	Monoacylglycerol Lipase
MIP-1 β	Macrophage Inflammatory Protein-1 β
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
MRI	Magnetic Resonance Imaging
NCEP-ATP	National Cholesterol Education Program-Adult Treatment Panel
NF- $\kappa\beta$	Nuclear Factor- $\kappa\beta$
NMR	Nuclear Magnetic Resonance
NSB	Non-Specific Binding
OAD	Oesophageal Adenocarcinoma
OLR1	Oxidized Low-Density Lipoprotein Receptor-1
OPD	o-Phenylenediamine Dihydrochloride
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PGE ₂	Prostaglandin E ₃
PGE ₃	Prostaglandin E ₃
PGF1 α	6-keto-Prostaglandin F1 α
PGI ₂	Prostacyclin I ₂
PGI ₃	Prostacyclin I ₂
PI3K	Phosphatidylinositide 3-Kinase
POAF	Postoperative Atrial Fibrillation
PUFA	Polyunsaturated Fatty Acid

PVDF	Polyvinylidene Fluoride
qPCR	Quantitative Polymerase Chain Reaction
ROC	Receiver Operating Characteristics
RPMI	Roswell Park Memorial Institute
RR	Relative Risk
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SFA	Subcutaneous Fat Area
SIRS	Systemic Inflammatory Response Syndrome
TA	Total Activity
TAM	Tumour-Associated Macrophage
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline Tween
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Total Fat Area
TGF- β	Transforming Growth Factor- β
Th	T Helper
TMB	3,3',5,5'-Tetramethylbenzidine
TNFSF14	Tumour Necrosis Factor Super Family-14
TNF- α	Tumour Necrosis Factor- α
TPN	Total Parenteral Nutrition
TS	Tumour Associated Stroma
TXA ₂	Thromboxane A ₂
TXA ₃	Thromboxane A ₃
TXB ₂	Thromboxane B ₂
VFA	Visceral Fat Area
WC	Waist Circumference
WHO	World Health Organization

Chapter 1 Introduction

1.1 OESOPHAGEAL CANCER

1.1.1 Epidemiology

Oesophageal cancer is the eighth most common cancer worldwide and sixth most common cause of death from cancer ^[1]. In Western industrialised countries oesophageal adenocarcinoma represents the dominant histological sub-type. Oesophageal adenocarcinoma has increased markedly in incidence over the past three decades, whilst the incidence of oesophageal squamous cell carcinoma continues to decrease ^[2-4]. This trend is mirrored in Ireland, where the incidence of oesophageal adenocarcinoma has doubled in the last two decades ^[5].

1.1.2 Pathogenesis

The pathogenesis of oesophageal cancer depends on the histological subtype. In oesophageal squamous cell carcinoma, smoking and alcohol consumption are the predominant risk factors ^[6]. Oesophageal adenocarcinoma is commonly associated with symptomatic gastro-oesophageal reflux disease, with Lagergren *et al.* reporting that the risk of oesophageal adenocarcinoma was almost eight times higher among persons in whom heartburn, regurgitation, or both occurred at least once a week ^[7]. The only known precursor to oesophageal adenocarcinoma is Barrett's oesophagus, a condition where the normal squamous lining of the oesophagus is replaced with specialized intestinal columnar epithelium, which then progresses through to dysplasia and ultimately adenocarcinoma ^[8].

The rising prevalence of oesophageal adenocarcinoma in Ireland coincides with the sharp increase in the prevalence of obesity ^[9]. A similar pattern is observed across Europe and this has stimulated much research into the associations between obesity and oesophageal adenocarcinoma. Epidemiological studies highlight a marked association between oesophageal adenocarcinoma and obesity, and a meta-analysis reported that a body mass index (BMI) $\geq 30 \text{ kg/m}^2$ is associated with a relative risk of 3.0 for developing oesophageal adenocarcinoma, a higher association than for any other cancer ^[10]. Furthermore, a study carried out by this centre in an Irish cohort reported that the risk of adenocarcinoma of the lower oesophagus was 11.3 times higher for individuals with a BMI $>30 \text{ kg/m}^2$ versus individuals with a BMI $<22 \text{ kg/m}^2$.

^[11]. Several other studies have also reported a strong association between obesity and oesophageal adenocarcinoma ^[12, 13].

Originally, the link between oesophageal adenocarcinoma and obesity was initially believed to relate solely to the physical/mechanical implications of excess abdominal fat leading to increased reflux and ultimately Barrett's oesophagus ^[14]. However, the observation that obesity is a risk factor for oesophageal adenocarcinoma independent of reflux ^[11, 15] suggests other mechanisms are at play. Thus oesophageal adenocarcinoma may be a paradigm model of obesity and cancer, and current molecular research is focused on mechanisms that underpin this association.

1.2 OBESITY AND OESOPHAGEAL CANCER

1.2.1 Obesity and Cancer - Epidemiology

Geographic and socioeconomic differences in cancer trends and incidences indicate that environmental factors affect the development of cancer ^[1]. Changes in cancer epidemiology over the last number of decades cannot be accounted for solely by changing population demographics, such as life expectancy, or improved screening programmes. Since not enough time has elapsed in order for significant genetic changes to account for the increased cancer incidence, it has to be attributed to environmental factors ^[16]. There has been an evolution in society structure from hunter-gatherer to peasant-agricultural and then to urban-industrialised. Alongside this, there have been a number of closely inter-related changes to body composition, physical activity, energy balance and diet and it is these environmental changes which are thought to be the cause of the alteration in cancer epidemiology ^[17, 18].

In Ireland, the prevalence of obesity amongst adults has increased significantly since 1990, rising from 8% to 26% in men, and from 13% to 21% in women ^[19]. This worrying pattern shows no signs of abating as obesity rates are increasing among children and adolescents ^[20] and overweight children tend to become overweight adults ^[21]. In countries where sedentary lifestyles and high-energy foods are abundant it is easy to see how energy intake exceeds that expended. It has been estimated that ingestion of 5% more energy than expended may result in an accumulation of 5 kg adipose tissue in a single year ^[22].

The effect that this passive overconsumption of energy and accumulation of adipose tissue has on the human body is calamitous. Epidemiological studies have demonstrated a robust link between obesity and cancer development at numerous sites, in particular the oesophagus, pancreas, colorectum, breast (postmenopausal), endometrium and kidney ^[12]. The World Cancer Research Fund estimates up to 28% of gallbladder cancers, 35% of pancreatic, 16% of colorectal, 17% of breast, 49% of endometrial, 28% of kidney and 35% of oesophageal cancers are attributable to obesity ^[23]. This association carries relative risk (RR) estimates of 1.1–1.6 per 5 kg/m² incremental increase in BMI ^[12]. Obesity also increases cancer-related mortality with studies reporting that obesity could account for 14% of all deaths from cancer in men and 20% in women ^[24]. The causative link between obesity and cancer is further strengthened by research in animal models which demonstrates that restricted caloric intake decreases spontaneous tumour occurrence ^[24, 25]. Furthermore, emerging clinical research suggests weight loss following bariatric surgery leads to a reduction in cancer incidence ^[26]. Thus, the potential mechanisms by which obesity increases both the incidence of certain malignancies, as well as death from malignancy, have become the focus of considerable research. In addition, the quantity and location of adipose, as opposed to overall body mass, has emerged as an essential factor in the study of obesity-associated pathologies.

1.2.2 Adipose Tissue, the Metabolic Syndrome and Cancer

The World Health Organisation defines obesity as an abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired ^[27]. Fat is principally deposited in two compartments – subcutaneously and viscerally. Attention was first drawn to the different patterns of fat distribution over 60 years ago by Jean Vague. He described 2 distinct types of fat deposition; upper-body, male-type fat (visceral) and lower-body, female-type fat (subcutaneous) ^[28]. He went on to demonstrate that upper-body, or visceral, fat deposition was associated with increased risk of type 2 diabetes, atherosclerosis and gout ^[29]. Visceral adipose tissue is largely comprised of omental adipose tissue and is more metabolically active than subcutaneous adipose tissue ^[30]. Further research has now confirmed that visceral adipose tissue has multiple endocrine, metabolic and immunological functions and has been shown to be central to the pathogenesis of the metabolic syndrome, a pro-

inflammatory, pro-coagulant state associated with insulin resistance ^[31]. The multiple risk factors that commonly appear together as the metabolic syndrome include abdominal obesity, atherogenic dyslipidemia (raised triglycerides and reduced high-density lipoprotein cholesterol), elevated fasting plasma glucose and hypertension ^[32]. The importance of adipose tissue location in terms of dysmetabolism risk is evident as an increased ratio of visceral fat area to subcutaneous fat area has been shown to be strongly related with disorders of glucose and lipid metabolism in obese subjects ^[33]. Furthermore, visceral obesity is more strongly associated with increased risk of insulin resistance, the metabolic syndrome and cardiovascular diseases than BMI alone ^[34].

Visceral fat has been identified as an independent risk factor for breast cancer ^[35], oesophageal adenocarcinoma ^[36], colorectal cancer ^[37] and colorectal adenomas ^[38, 39]. The other elements of the metabolic syndrome, i.e. dyslipidemia, hypertension and insulin resistance have also been independently linked with increased cancer risk ^[40-43]. The synergistic impact of these factors on cancer risk is the focus of the Me-Can Study, a prospective international population-based study of 580,000 people ^[44]. Initial findings suggest that a combination of components of the metabolic syndrome is associated with risk of colorectal cancer development (Men: RR: 1.25 (95% CI; 1.18-1.32); Women: RR 1.14 (95% CI; 1.02-1.18)^[45], endometrial cancer (RR 1.37, 1.28-1.46)^[46], bladder cancer in men (RR: 1.1, 1.01-1.18) ^[47] and pancreatic cancer in women (RR,1.58; 1.34-1.87) ^[48] Similarly, in studies of pancreatic and colorectal cancer a high prevalence of metabolic syndrome, central obesity and systemic inflammation has been identified ^[49-51] and has been shown to be associated with advanced tumour stage and reduced survival ^[52-55].

1.2.3 Mechanisms Underlying Visceral Obesity and Tumourigenesis

The mechanisms by which excess visceral adiposity and the metabolic syndrome influence tumour progression are manifold. Adipose tissue may promote tumour development in a paracrine manner. Studies in breast and ovarian cancer have demonstrated that co-culture with adipocytes promotes tumour migration and invasion, with omental adipocytes more efficient in promoting invasion of ovarian cancer cells than subcutaneous adipocytes ^[56, 57]. In studies using a murine mammary

carcinoma cell line, it was found that tumour growth was enhanced following injection into the mesenteric and ovarian fat pads and the mammary gland, but not in the subcutis or peritoneal cavity ^[58, 59]. In addition, a proteomic study of mammary fat revealed the production of a wide variety of proteins involved in diverse processes such as cell communication, growth, immune response, apoptosis and numerous signalling molecules including hormones, cytokines and growth factors ^[60]. The paracrine influence of adipose tissue on tumourigenesis and the role of “cancer-associated adipocytes” is less fully investigated. Much research to date has focused on the systemic effects of visceral adiposity that are putatively involved in cancer biology. These include alterations in adipokine production, insulin and insulin-like growth factor pathways and adipose tissue immune cell infiltration which are discussed in further detail below.

1.2.3.1 *Altered adipokine production*

The existence of adipose tissue derived factors that may influence metabolism has been suggested since the 1980s ^[61, 62], however it was the discovery of the adipokines leptin ^[63] and adiponectin ^[64] that led to the concept of adipose tissue as an endocrine organ. The altered secretion of these and other adipokines in obesity has been implicated in the pathogenesis of obesity-associated pathologies, including cancer ^[65]. Excess visceral obesity results in a state of chronic systemic low-grade inflammation, attributed to the production and release of these inflammatory cytokines by both adipocytes and infiltrating immune cells which in turn creates an inflammatory pro-tumourigenic environment ^[66].

Adiponectin is the most abundant adipokine and is secreted mainly from visceral fat adipocytes, however circulating levels are inversely correlated with obesity ^[67]. It has been shown to be both anti-angiogenic and anti-inflammatory and to inhibit tumour growth in animal models. In addition, circulating levels are lower in cancer patients ^[68]. Leptin may be considered the antithesis of adiponectin. It is an adipokine that acts centrally to regulate appetite and bodyweight ^[69] and circulating levels are positively associated with adiposity ^[70]. *In vitro* studies confirm promotion of cell proliferation, angiogenesis and metalloproteinase expression in oesophageal and colonic cancer cell

lines ^[71, 72]. Furthermore serum levels of leptin are positively correlated with cancer risk ^[73]. Increased production of leptin and decreased production of adiponectin, correlating with amounts of visceral adiposity and the metabolic syndrome, have been associated with cancer promoting pathways such as cellular proliferation, angiogenesis and metalloproteinase expression across a wide variety of cancer subtypes ^[67, 72, 74].

1.2.3.2 *Hyperinsulinaemia, insulin resistance and the insulin-like growth factor-1 axis*

A core component of the metabolic syndrome is insulin resistance and associated hyperglycaemia. Nutritionally induced insulin resistance develops as a metabolic adaptation to increased circulating levels of non-esterified fatty acids, which are constantly released from adipose tissue, thus is related to adipose tissue mass. Increased non-esterified fatty acid levels force liver, muscle and other tissues to shift towards increased storage and oxidation of fats for their energy production. Consequently, insulin secretion rises to compensate for the decreased capacity to handle glucose ^[75-77]. Despite this, there is a decreased expression of insulin-receptor levels and reduced intracellular insulin signalling in response to insulin receptor binding ^[78] resulting in hyperinsulinaemia and insulin resistance.

The ratio of visceral to subcutaneous fat is central to insulin resistance. The rate of lipolysis is higher in visceral adipose tissue than subcutaneous adipose tissue, leading to increased circulation of non-esterified fatty acids ^[79]. Therefore, the risk of developing insulin resistance and hyperinsulinaemia increases with increasing visceral adipose tissue accumulation. Adipokines are believed to be central to the pathogenesis of insulin resistance ^[80], with high concentrations of tumour necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β , and low concentrations of adiponectin, having deleterious effects on glucose homeostasis leading to chronic hyperinsulinaemia and insulin resistance ^[81]. Several epidemiological studies have shown that insulin resistance status, characterised by hyperinsulinaemia, is associated with an increased risk for a number of malignancies, including carcinomas of the breast, prostate and colon ^[41, 68, 82, 83]. Data from the Me-Can study reported that every 1 mmol/l increment in glucose increased risk of incident cancer in men (RR 1.05, 1.01-1.1) and women (1.11, 1.05-

1.16) and a further increased RR for mortality (Men: 1.15, 1.07-1.22; Women: 1.21, 1.11-1.33)^[82]. Interestingly, studies of type 2 diabetics have found that the risk of colorectal cancer is higher in those treated with insulin^[84].

The tumourigenic effects of insulin could be directly mediated by insulin receptors in the pre-neoplastic target cells^[37, 85]. *In vitro* studies demonstrate that insulin increases the neoplastic proliferation of cell lines at both physiological and pharmacological doses and the insulin receptor is commonly expressed in human neoplasms^[86]. It has also been proposed that the insulin-like growth factor (IGF) system mediates the effects of hyperinsulinaemia and may be more relevant than insulin. In the IGF axis, binding of IGF-1 or IGF-2 to the IGF-1 receptor leads to cell proliferation, differentiation and protection from apoptosis and a dysregulation in this pathway can give rise to malignancy (Figure 1.1)^[87, 88]. Levels of IGF are influenced by circulating insulin levels, with increasing insulin leading to decreased levels of IGF-binding proteins (IGFBP) -1 and -2, thus increasing the bioavailability of IGF^[89]. The IGF-1 axis has been implicated in the progression of breast, pancreatic, colorectal and oesophageal cancer^[90-93]. Obesity increases levels of bioactive free IGF-1^[94, 95], high levels of which are associated with increased risk of malignancy, thus the IGF axis represents a plausible mechanism by which obesity increases cancer risk. Murine models of IGF-1 deficiency have yielded interesting results regarding the association of IGF-1 and cancer in obesity. Diet-induced obesity increased both local tumour growth and metastases in wild type mice compared to lean controls, but this was not observed in IGF-1 gene deficient mice. In addition, the expression of inflammatory cytokines and cell adhesion molecules was upregulated in obese compared to lean mice, but chronic IGF-1 deficiency was associated with a reduction in these indicators^[96]. Similar anti-tumour effects of IGF-1 deficiency were observed in other murine studies of colon and pancreatic cancer^[97, 98]. Hence it is possible that in obesity IGF-1 can affect tumour development both directly, by stimulating tumour growth and indirectly, by creating a microenvironment that is permissive for tumour growth.

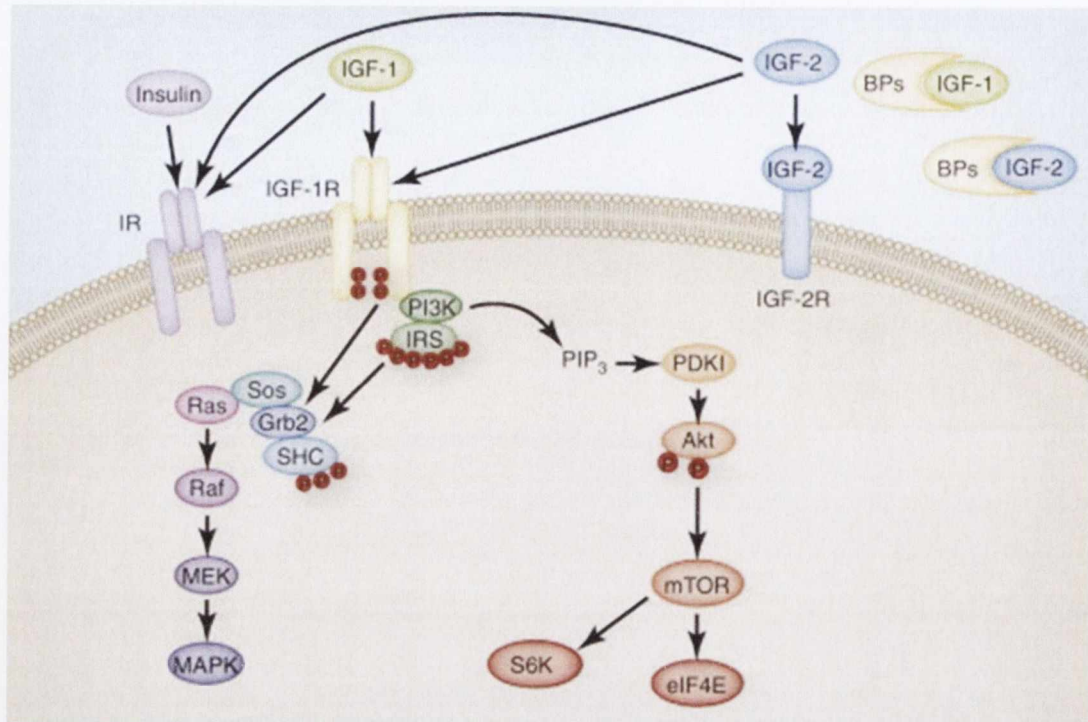


Figure 1.1 The insulin-like growth factor (IGF) axis.

The insulin-like growth factor (IGF) axis comprises of three ligands: insulin, IGF-1 and IGF-2. IGF-1 and IGF-2 levels in circulation are regulated by IGF binding proteins (BPs). The IGF-1 receptor (IGF-1R), is a heterotetrameric tyrosine kinase receptor consisting of two α and two β subunits. Binding of the ligands IGF-1 and IGF-2 to IGF-1R results in receptor autophosphorylation and activation of multiple signalling pathways, including the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/anti-apoptotic serine threonine kinase (Akt-1) pathways, leading to cell proliferation and protection from apoptosis. Figure adapted from Alberini *et al.*^[99].

1.3.2.3 Visceral obesity, inflammation and cancer

In 1863, Virchow first hypothesized that the origin of cancer was at sites of chronic inflammation. Today we know that there is an increased risk of malignancy associated with chronic inflammation arising from carcinogenic agents (tobacco and lung cancer), autoimmune and inflammatory reactions of uncertain aetiology (ulcerative colitis and colon cancer) and infectious agents (*Helicobacter pylori* and gastrointestinal cancer, hepatitis C and liver cancer)^[100]. In chronic inflammation repeated tissue damage and regeneration in the presence of highly reactive nitrogen and oxygen species released from inflammatory cells, leads to permanent genomic alterations in proliferating

epithelium ^[101]. Key features of cancer related inflammation include the infiltration of immune cells, the presence of cytokines and chemokines, such as TNF- α , IL-1 β , IL-6, monocyte chemoattractant protein-1 (MCP-1) and IL-8 ^[102]. Thus, cancer related inflammation is a key component of the tumourigenic process and is increasingly referred to as the seventh hallmark of cancer.

Viscerally obese individuals have been shown to have higher circulating levels of pro-inflammatory mediators such as C-reactive protein (CRP), TNF- α , IL-6 and MCP-1 ^[103-108]. The level of adipocytokine release from adipose tissue is strongly influenced by immune cell populations present in adipose tissue ^[109-112]. Adipose tissue in obese individuals is infiltrated with macrophages and the number of macrophages correlates with the degree of adiposity ^[113]. Peripheral monocytes are recruited into adipose tissue by MCP-1 and TNF- α , and can differentiate into activated macrophages ^[114]. The products of these activated macrophages can then impact on adipocyte function and are postulated to be involved in altering adipose tissue glucose handling and thus contribute to insulin resistance ^[115, 116]. Research has shown that co-culture of adipocytes with macrophage-conditioned media causes increased adipokine and inflammatory cytokine production by adipocytes ^[117], further supporting this hypothesis. Furthermore, in obesity it has been demonstrated that visceral adipose tissue resident macrophages undergo a phenotypical change from the anti-inflammatory M2 to a pro-inflammatory M1 state ^[118], further potentiating metabolic dysfunction, insulin resistance and inflammation in obese individuals.

Whilst the majority of studies to date evaluating the immune cell properties of visceral adipose tissue have predominantly focused on macrophages, emerging research has suggested a role for T cells as key regulators of adipose tissue inflammation. Nishimura *et al.* reported in a murine model of diet induced obesity that there was a significant increase in CD8⁺ T cell infiltration in expanding visceral adipose tissue and this preceded macrophage infiltration. Furthermore, depletion of CD8⁺ T cells lead to a reduction in macrophage infiltration (M1 phenotype) and a reduction in inflammatory mediators in visceral adipose tissue ^[119]. Research within this group recently demonstrated that visceral adipose tissue is a rich source of CD8⁺ T cells. In addition, a

higher percentage of CD4⁺ and CD8⁺ omental T cells were activated with an inflammatory T helper (Th) -1 phenotype in oesophageal adenocarcinoma patients ^[120]. These findings suggest that visceral adipose tissue is a reservoir of activated T cells in cancer patients, promoting inflammation and possibly influencing cancer progression.

Recently the role of invariant natural killer (iNKT) cells in obesity has been investigated by Lynch *et al.* in both human and murine studies ^[121, 122]. Upon activation, iNKT cells rapidly release Th1 and Th2 type cytokines and can demonstrate cytotoxic activity ^[123]. Research in human visceral adipose tissue samples demonstrated that it is a rich source of iNKT cells but levels inversely correlated with BMI. Interestingly, following weight loss, circulating levels of iNKT rise ^[122]. In a further study, iNKT depleted mice demonstrated enhanced weight gain and dysmetabolism, with increased adipose tissue infiltration of M1 type macrophages. To further confirm the association between iNKT cells and visceral obesity, adoptive transfer of iNKT cells into obese mice was performed and this resulted in weight loss and improved metabolic function. Furthermore, injection of the iNKT agonist alpha-galactocylceramide into wild type obese mice resulted in weight loss, decreased fasting glucose and decreased circulating levels of pro-inflammatory cytokines ^[121].

The findings of these studies and others investigating immune cell activity in visceral obesity confirm the absolute association between excess visceral adiposity and inflammation. Chronic inflammation is a hallmark of cancer and it may be deduced that visceral obesity increases cancer risk by creating a state of systemic inflammation that is pro-tumourigenic (Figure 1.2).

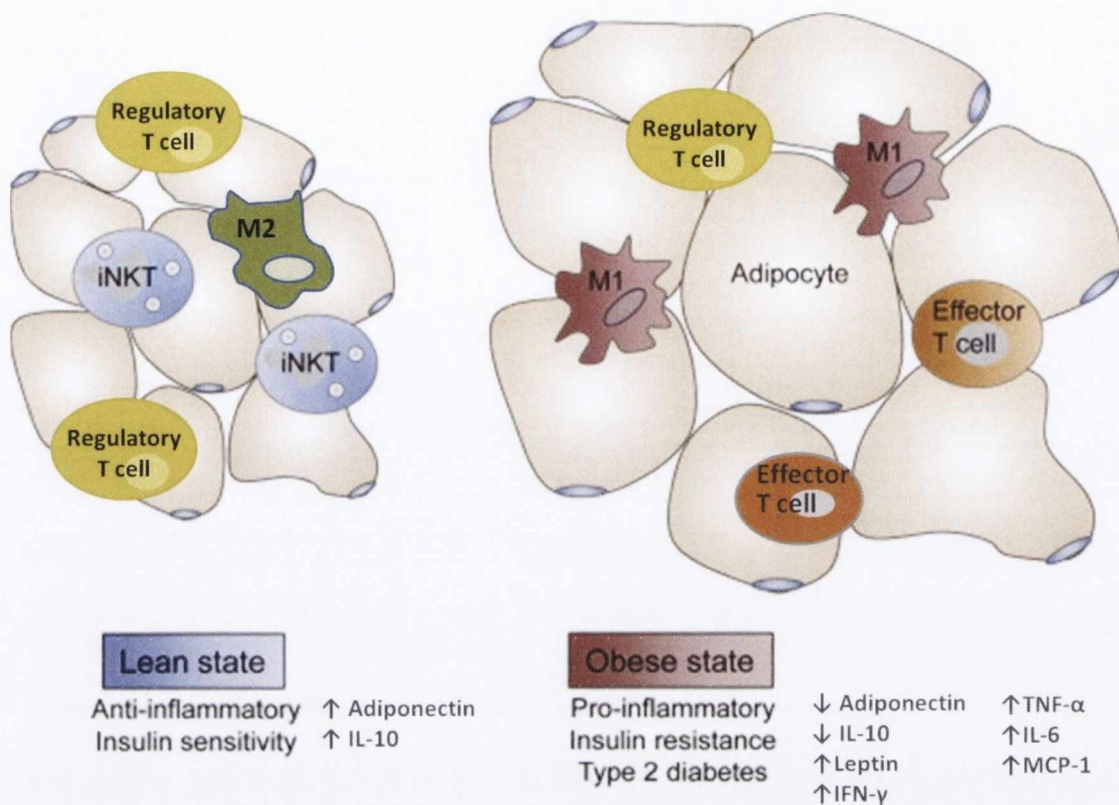


Figure 1.2 Adipose tissue inflammation

Immune cell infiltration is altered in visceral obesity. There is a shift in T cell infiltration, with a decrease in CD4⁺ regulatory T cells and an increase in CD4⁺ and CD8⁺ effector T cells, with an associated increase in Th1 type cytokine production. Increased production of monocyte chemotactic protein-1 (MCP-1) recruits macrophages into the visceral adipose tissue, which undergo a phenotypic shift to the more inflammatory M1 type macrophages. Emerging research has identified depleted iNKT cells in obesity. The combination of this altered inflammatory infiltrate in visceral obesity is the release of pro-inflammatory cytokines and the creation of systemic inflammation and dysmetabolism. Adapted from Lukens *et al.* ^[124].

1.2.4 Oesophageal Adenocarcinoma – A Model of Visceral Obesity, Metabolic Syndrome and Cancer

The robust epidemiological evidence linking obesity with oesophageal adenocarcinoma makes it is an exemplar model for investigating the molecular mechanisms

underpinning obesity-associated malignant progression. Research within this group not only established a link between oesophageal adenocarcinoma and obesity as measured by BMI, but also demonstrated a strong association with visceral obesity, as measured by visceral fat area (VFA) $\geq 130\text{cm}^2$ [36]. Obesity and the metabolic syndrome have also been shown to be associated with the progressive sequence of oesophageal adenocarcinoma, from Barrett's oesophagus through to malignancy. A study at this centre in 2008 reported that the prevalence of metabolic syndrome in Barrett's oesophagus patients far exceeded population norms, with metabolic syndrome present in 46% of patients and central obesity in 78%. The syndrome was also significantly associated with the length of Barrett's; 60% of patients with long-segment Barrett's oesophagus had metabolic syndrome and 92% were centrally obese compared with 23.8% and 62% respectively in short-segment Barrett's oesophagus. Long-segment Barrett's oesophagus was also associated with systemic inflammation, with significantly higher serum levels of IL-6 observed in patients [125].

The obesity, inflammation, malignancy association is further strengthened by explant studies of human visceral adipose tissue carried out within this research group. Co-culture of oesophageal adenocarcinoma cells with visceral adipose tissue samples has been shown to upregulate the expression and gelatinase activity of markers of invasion and metastasis, matrix metalloproteinase (MMP)-2 and MMP-9, and decrease the expression of the tumour suppressor, p53 [126]. In addition, visceral adipose condition media from obese patients has been found to drive the proliferation of oesophageal adenocarcinoma cells [127]. Research has also been carried out in resected tumour tissue and it has been found that expression of the epithelial mesenchymal transition genes, plasminogen activator inhibitor 1 and SNAI2, which also play an important role in tumour invasion and metastases, is elevated in tumours of obese oesophageal cancer patients [128]. These findings provide mechanistic evidence to confirm the epidemiological observation that visceral obesity increases oesophageal adenocarcinoma risk.

1.3 OBESITY AND POSTOPERATIVE RECOVERY

As discussed above, visceral obesity and the metabolic syndrome play an important role in the pathogenesis of many cancer types including gastrointestinal, breast, endometrium and prostate ^[12]. Other pathological conditions such as cardiovascular disease and osteoarthritis are also associated with obesity ^[129, 130]. Many of these obesity-related diseases require surgical intervention, e.g. in the form of a resection for malignancy, coronary artery bypass graft or joint arthroplasty. Studies have reported that 46-54% of hospital patients are overweight and 32% are obese ^[131-133] and as the prevalence of obesity and obesity-related co-morbidities continues to rise, it is inevitable that there will be an increase in the prevalence of obese surgical patients. Given that a pro-thrombotic, pro-inflammatory, disordered metabolic state is observed in obesity; it may be hypothesised that obesity impacts on recovery following major surgery. The following sections review the evidence relating to obesity and postoperative outcomes.

1.3.1 Obesity and Postoperative Morbidity

Obesity has historically been considered a risk factor for adverse postoperative outcomes due to its negative impact on cardiovascular and respiratory function. This association is not clear-cut, however, and there is a lack of consensus in the literature on the risk between obesity, as measured by BMI, and specific complications, in particular relating to infection, wound healing and respiratory complications.

1.3.1.1 *Respiratory complications*

In addition to the obesity related pulmonary disorders of sleep apnea and hypoventilation syndrome, obesity is also associated with decreased vital capacity, functional residual capacity, forced expiratory volume in 1 second and arterial oxygen tension ^[134-136]. Whilst some studies have identified overweight and obesity as a risk factor for postoperative pneumonia, atelectasis and pulmonary embolism ^[137-140], other studies have found no association between overweight and obesity and postoperative respiratory complications ^[141-143]. A study carried out at this institute involving patients undergoing oesophagectomy found that whilst obesity was

associated with an increased risk of minor respiratory complications postoperatively, there was no association with respiratory failure, pneumonia or acute respiratory distress syndrome ^[144].

1.3.1.2 Atrial arrhythmia

Atrial fibrillation is the most prevalent form of cardiac arrhythmia and is the most common complication after cardiac surgery ^[145]. The occurrence of atrial fibrillation is associated with increased postoperative morbidity and mortality ^[145]. Obesity is associated with changes in atrial and ventricular structure ^[146-148] and ventricular diastolic function ^[149] and has been identified as a risk factor for atrial fibrillation ^[150]. Several studies have reported obesity to be associated with atrial arrhythmia post coronary artery bypass grafting (CABG) ^[141, 145, 150-153]. The findings of studies by Kuduvali *et al.* ^[151] and Zacharias *et al.* ^[145] suggested that the risk of arrhythmia was proportional to the degree of obesity, with odds ratios (OR) in the region of 1.25 for BMI $\geq 30 \text{ kg/m}^2$, 1.5 for BMI $\geq 35 \text{ kg/m}^2$ and 2.39 for BMI $\geq 40 \text{ kg/m}^2$ ^[145, 151]. However, a meta-analysis by Wanahita *et al.*, ^[154] reported that whilst obesity increased the risk of developing atrial fibrillation in the general population, it did not increase the risk of atrial fibrillation post cardiac surgery ^[154].

1.3.1.3 Infectious complications

There is a large body of evidence to suggest that obesity increases the risk of postoperative infection. A study by Choban *et al.* ^[155] retrospectively compared the nosocomial infection rate in 849 obese and normal weight patients undergoing a range of surgical procedures and reported that the incidence of infections in the normal weight group was 0.5%, which was significantly less than the rates observed in the obese group (2.8%, $p < 0.05$) ^[155]. In a similar study of 395 surgical patients by Canturk *et al.* ^[156], 24.3% acquired one or more nosocomial infections (surgical site infections, urinary infections, and pulmonary infections), with significantly more infections occurring in obese patients compared with normal-weight patients ($p < 0.05$) ^[156]. Wound infection is a major cause of morbidity following various types of surgery ^[157] and is associated with increased mortality ^[158], wound dehiscence ^[159], and increased hospital stay ^[160]. Karunakar *et al.* ^[161] retrospectively examined the impact of obesity

on postoperative complications in 169 patients undergoing open reduction of acetabular fracture. The study reported that increasing BMI was associated with increased risk of wound infection ($p = 0.002$), with patients with a BMI $\geq 40 \text{ kg/m}^2$ five times more likely to have a wound infection^[161]. Similarly Namba *et al.*^[162] reported that in patients with a BMI $\geq 35 \text{ kg/m}^2$, the risk of postoperative infection was 6.7 times higher when undergoing total knee arthroplasty and 4.2 times higher when undergoing total hip arthroplasty when compared to patients with a BMI $\leq 35 \text{ kg/m}^2$ ^[162]. In a study carried out Merkow *et al.*^[138] the impact of BMI on short-term outcomes after colectomy was assessed in 3,202 patients using prospectively collected data from the American College of Surgeons National Surgical Quality Improvement Project database. The results demonstrated that morbid obesity (BMI $\geq 35 \text{ kg/m}^2$) was a strong risk factor for wound complications. When compared to patients with a normal BMI, morbidly obese patients were 2.6 times more likely to incur a surgical site infection, superficial or deep, and 3.5 times more likely to experience wound dehiscence^[138]. Sternal wound infections and mediastinitis are serious potential complications following CABG procedures that are associated with increased mortality^[163, 164]. In the UK Lu *et al.*^[165] prospectively collected data over 4 years on 4,228 patients undergoing CABG procedures and, after multivariate analysis, identified BMI $\geq 30 \text{ kg/m}^2$ as an independent risk factor for sternal wound infection (OR 2.0 (95% CI 1.3–2.9); $p < 0.001$)^[165]. Several similar studies have also identified a BMI $\geq 30 \text{ kg/m}^2$ as a significant risk factor for deep and superficial sternal wound infections post CABG^[151, 160, 166-168]. In addition, Kuduvalli *et al.*^[151] reported that morbidly obese patients were 4.17 ($p < 0.001$) times more likely to develop harvest site infections compared with normal weight patients^[151].

A number of different explanations for why obesity is a risk factor for wound infection have been postulated. Adipose tissue is a relatively avascular mass, leading to tissue hypoperfusion and decreased oxygen tension. Oxidative killing mediates the bactericidal activity of neutrophils and is a critical defence against surgical pathogens^[169]. Thus, the reduced oxygen tension in the tissues at the site of surgical incision in the obese patient may result in suboptimal neutrophil oxidative killing and increased risk of infection^[170]. It has also been suggested that a failure to adjust dosing of antibiotics for the increased volume of distribution, giving rise to inadequate tissue

drug levels, may predispose obese patients to surgical site infections ^[171]. Another factor to consider is that obesity is associated with insulin resistance ^[172] and deranged glucose metabolism is associated with impaired macrophage function, angiogenic response, collagen accumulation and growth factor production leading to an increased risk of wound infection and impaired wound healing ^[173].

1.3.2 Visceral Adiposity and Risk of Postoperative Complications

Whilst there is evidence that obesity, as measured by BMI, increases the risk of certain complications following surgery; there is still no consensus in the literature as to whether obesity is a risk factor for overall morbidity and mortality. A possible explanation for this is the reliance on BMI as a measure of obesity. BMI is a convenient tool, but it is also an imperfect tool as it does not distinguish lean tissue mass from adipose tissue mass and does not reflect adipose tissue distribution ^[174, 175]. Thus when attempting to evaluate the adverse effects obesity may have on postoperative outcomes, it is more relevant to look specifically at visceral adiposity, a central factor in the development of dysmetabolism and comorbidities in obesity. Computed tomography (CT) and magnetic resonance imaging (MRI) are considered the gold standards for determining visceral adiposity ^[176], with waist circumference representing the best surrogate measure ^[175]. In recent years there has been an increase in research examining the effect of specifically visceral obesity on postoperative outcomes.

A study by House *et al.* ^[177] examined the impact of obesity on risk of complications post pancreaticoduodenectomy using both BMI and body fat distribution as indicators of obesity. Abdominal wall thickness, hip girdle fat thickness and retrorenal visceral fat were calculated from CT and/or MRI of the abdomen. It was found that whilst BMI ≥ 30 kg/m² was only associated with a higher risk of wound infection, visceral fat greater than or equal to 2.5 cm was associated with nearly 3 times greater risk of postoperative complications and nearly 2.5 times greater risk of developing a pancreatic fistula ^[177]. This finding has been supported in other studies of pancreatic surgery, which reported that high visceral fat area ($>100\text{cm}^2$), but not BMI, was an independent predictor of postoperative fistula formation ^[178]. Conversely, a study by

Balentine *et al.* reported no association between intra-abdominal fat or BMI and postoperative complications following pancreaticoduodenectomy^[179].

Tanaka *et al.*^[180], investigated the impact of body composition on postoperative complications in 191 patients undergoing gastrectomy. The results showed that BMI $\geq 25 \text{ kg/m}^2$ was associated only with increased blood loss, but high visceral fat area ($\geq 100 \text{ cm}^2$), estimated from cross sectional CT scans taken at the level of the umbilicus, was associated with a 6.5 fold increased risk of pancreatic fistula ($p=0.0006$)^[180]. Other studies in gastrectomy patients report that visceral fat area, not BMI, is associated with greater risk of postoperative infection and anastomotic leak^[181, 182]. Similar studies in patients undergoing colorectal resections revealed that when patients were classed as obese using BMI $\geq 25 \text{ kg/m}^2$ and visceral fat area, patients with elevated visceral fat area were at increased risk of wound infections, overall complications, operative time and length of hospital stay^[183, 184]. In another study by Tsukada *et al.*^[185] intra-abdominal and subcutaneous fat levels were assessed from CT scans in 139 gastric and colorectal cancer patients. High levels of fat were defined as intra-abdominal fat $\geq 160 \text{ cm}^2$ in males and $\geq 120 \text{ cm}^2$ in females; and subcutaneous abdominal fat $\geq 180 \text{ cm}^2$ in males and $\geq 250 \text{ cm}^2$ in females, as described by Kobayashi *et al.*^[186]. Interestingly, high intra-abdominal fat was associated with a 9.6 fold increased risk of pneumonitis and atrial fibrillation, whereas increased subcutaneous fat was associated with a 10 fold increased risk of surgery related complications including anastomotic leaks, abdominal collection and wound infections^[185].

These findings suggest that visceral adiposity is a more useful parameter than BMI when identifying patients at increased risk of adverse postoperative outcomes. However it is important to note that much of the research examining visceral adiposity and postoperative outcomes has been carried out in Japanese populations who, due to their ethnicity, have a different body composition and use BMI $\geq 25 \text{ kg/m}^2$ as the cut-off for obesity^[187]. In addition, different techniques for quantifying visceral adiposity and different cut-offs for classifying visceral obesity were used in the various studies. Despite this, there is still compelling evidence that visceral adiposity is a more specific tool for investigating the association between obesity and risk of postoperative complications and further studies in different populations are warranted.

1.3.3 The Metabolic Syndrome and Risk of Postoperative Complications

A number of studies have been carried out examining the impact of the metabolic syndrome on operative mortality and risk of complications post CABG surgery ^[188-192]. Echahidi *et al.* ^[189] retrospectively analyzed the data of 5,304 consecutive patients who underwent an isolated CABG procedure, of which 46% met the National Cholesterol Education Program-Adult Treatment Panel III criteria ^[193] for the metabolic syndrome. This study found that the metabolic syndrome was independently associated with increased operative mortality with a relative risk (RR) of 3.04, whereas BMI $\geq 25\text{kg/m}^2$ or diabetes alone were not identified as risk factors for mortality. In addition, the metabolic syndrome was found to be independently associated with stroke, renal failure, mediastinitis and pneumonia ^[189].

Following on from these studies, Girerd *et al.* ^[194] sought to determine which feature of the metabolic syndrome is associated with postoperative atrial fibrillation (POAF) following CABG. The data of 2214 male patients <65 years who had undergone elective isolated CABG was retrospectively analysed. The study found that waist circumference (WC) $> 102\text{ cm}$ was an independent predictor of POAF (OR 1.40, $p=0.04$) whereas BMI $\geq 30\text{kg/m}^2$ was not. As the metabolic syndrome is associated with a pro-inflammatory state, the researchers also examined postoperative white cell count (WCC) count and inflammatory markers in blood and their association with POAF in a nested case-control study. Multivariate analysis found that WC $> 102\text{ cm}$, but not BMI $\geq 30\text{ kg/m}^2$, was a predictor of higher postoperative WCC count and higher WCC counts were associated with greater incidence of POAF. When WC and levels of inflammatory markers were analysed together, only patients having both a WC $> 102\text{ cm}$ and elevated C-reactive protein or IL-6 were at increased risk of POAF ^[194]. The findings of these studies indicate that it is the presence of the metabolic syndrome rather than obesity that increases the risk of complications post CABG and, with respect to POAF, it is the visceral obesity element of the metabolic syndrome and its associated pro-inflammatory state that promotes this risk.

In non-cardiac surgery, the metabolic syndrome has also been shown to increase risk of adverse postoperative outcomes. In a study of colorectal cancer patients by Lohsiriwat *et al.* ^[195] the presence of the metabolic syndrome was an independent risk

factor for complications following colorectal resection. In the cohort of 114 patients, complications occurred 40.5% of the time in those with the metabolic syndrome versus 11.1% in those without the metabolic syndrome ($p < 0.001$)^[195]. In other studies of joint arthroplasty, spinal surgery and liver resection, the presence of the metabolic syndrome has also been associated with increased morbidity and mortality^[196-198].

Whilst there is not the same volume of research specifically examining the association between visceral obesity, the metabolic syndrome and postoperative complications, the findings have significant implications. These results highlight the importance of adipose tissue distribution in the body, and indicate that it is centrally located adiposity with its associated state of inflammation that leads to the increased risk of postoperative morbidity and mortality. Further research is warranted in this area and would benefit from more standardised methods for quantifying and classifying visceral obesity.

1.4 THE NUTRITIONAL MANAGEMENT OF POST-OPERATIVE OUTCOMES IN OESOPHAGECTOMY PATIENTS – A ROLE FOR IMMUNONUTRITION?

Surgical resection is the curative approach to oesophageal cancer, with neoadjuvant chemoradiotherapy considered when the disease is locally advanced^[199]. However, this major surgical procedure is associated with significant risks, with nearly half of all patients experiencing a postoperative complication^[200]. Furthermore, the physical impact of the surgery has a profound and long term impact on health related quality of life and functional capacity^[201, 202].

The anatomical changes that occur following an oesophagectomy necessitate the use of enteral nutrition postoperatively (Figure 1.3). Postoperative early enteral nutritional support via a jejunostomy tube is now considered a standard element of care following oesophagectomy^[203-205]. Commencing enteral nutrition early limits the hypermetabolic response to major surgery, preserves nutritional status, enhances gut function, decreases the risk of morbidity and promotes recovery^[203, 206]. As oesophagectomy patients are fed exclusively through enteral nutrition in the immediate postoperative phase and are maintained on supportive enteral nutrition for

a number of weeks following surgery, they represent an interesting cohort in which to investigate the potential of immunonutrition. Enteral nutrition has already been proven to enhance postoperative recovery in oesophagectomy patients when compared to parenteral nutrition or fasting ^[206-208]. An emerging research area is the possibility of delivering an enteral feed to a patient that not only provides their macro- and micronutrient requirements but that contains other dietary compounds which can alter the postoperative inflammatory response to further aid recovery. Eicosapentaenoic acid (EPA) is a long chain polyunsaturated fatty acid (PUFA) of the omega-3 family which has immunomodulatory properties. Its mechanism of action and potential role in post-operative immunonutrition is discussed below.

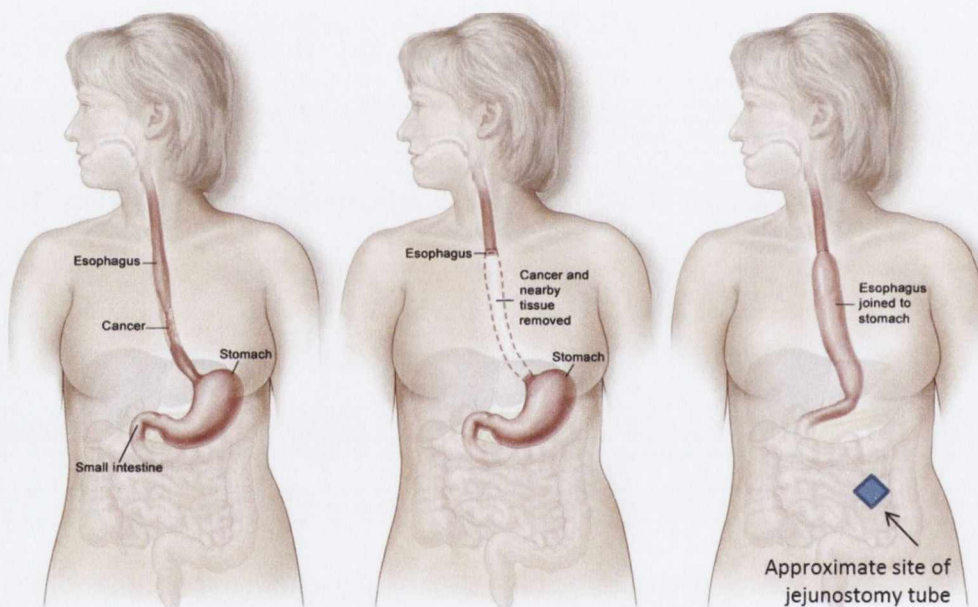


Figure 1.3 The anatomical changes following oesophagectomy

The curative treatment of oesophageal cancer is resection of the tumour. Following the removal of the oesophagus, the stomach is pulled up and an anastomosis is formed with the upper remaining portion of the oesophagus. At the time of resection a needle catheter jejunostomy tube is positioned in the jejunum for post-operative enteral feeding. (Adapted from Terese Winslow Medical and Scientific Illustration, VA, USA)

1.4.1 The Anti-Inflammatory Properties of Eicosapentaenoic Acid

Eicosapentaenoic acid (EPA) is an essential fatty acid which humans require from diet, the richest source being marine oils. The association between omega-3 PUFA and health was first reported in 1980's following numerous epidemiological studies which observed a much lower incidence of inflammatory and cardiovascular disorders amongst populations with high consumption of omega-3 PUFA from marine sources, e.g. the Japanese and Greenland Eskimos^[209-211].

Omega-3 PUFA are incorporated into the phospholipid membrane of many cells, along with omega-6 PUFA, the predominant source of PUFA in Western diets. Upon activation, e.g. in response to tissue damage, omega-3 and omega-6 PUFA are metabolised to form the lipid mediators of inflammation, prostaglandins, thromboxanes, leukotrienes and lipoxins, via the action of cyclooxygenases (COX) and lipoxygenases (LOX). The downstream metabolites of omega-3 PUFA (EPA) and omega-6 PUFA (arachidonic acid) differ in their inflammatory effects^[212].

Arachidonic acid (AA) metabolism gives rise to the series-2 prostaglandins (PGE₂), prostacyclins (PGI₂), thromboxanes (TXA₂) and the series-4 leukotrienes (LTA₄, LTB₄), and lipoxins (LXA₄, LXB₄); whereas metabolism of EPA gives rise to series-3 prostaglandins (PGE₃), prostacyclins (PGI₃), thromboxanes (TXA₃) and series-5 leukotrienes (LTA₅, LTB₅) and lipoxins (LXA₅, LXB₅) (Figure 1.4). The metabolites of AA are considered more potent mediators of inflammation than those of EPA^[212, 213]. PGE₂ exerts a wide range of pro- and anti-inflammatory effects on macrophages, T cells and dendritic cells. It suppresses the production of IL-12 by monocytes and dendritic cells and suppresses interferon- γ (IFN- γ) production in natural killer cells. PGE₂ also suppresses IL-2 production and IL-2 responsiveness in T cells, shifting the pattern of T cell response from the pro-inflammatory Th1 towards the Th2 response that mediates less tissue-destructive forms of immunity^[214]. It has also been shown that PGE₂ can amplify its own production by inducing COX-2 expression. This PGE₂-dependent amplification of COX-2 is hypothesized to be an important part of sustained proliferative and chronic inflammatory conditions. However PGE₃, whilst still possessing pro-and anti-inflammatory properties, is less potent and has been shown to be less efficient in inducing COX-2 gene expression and IL-6 synthesis^[215]. Similarly, AA

derived LTB_4 is a more powerful inducer of leukocyte chemotaxis and adherence than the EPA derived equivalent, LTB_5 , and TXA_3 is a weaker vasoconstrictor and platelet aggregator than TXA_2 [212, 216].

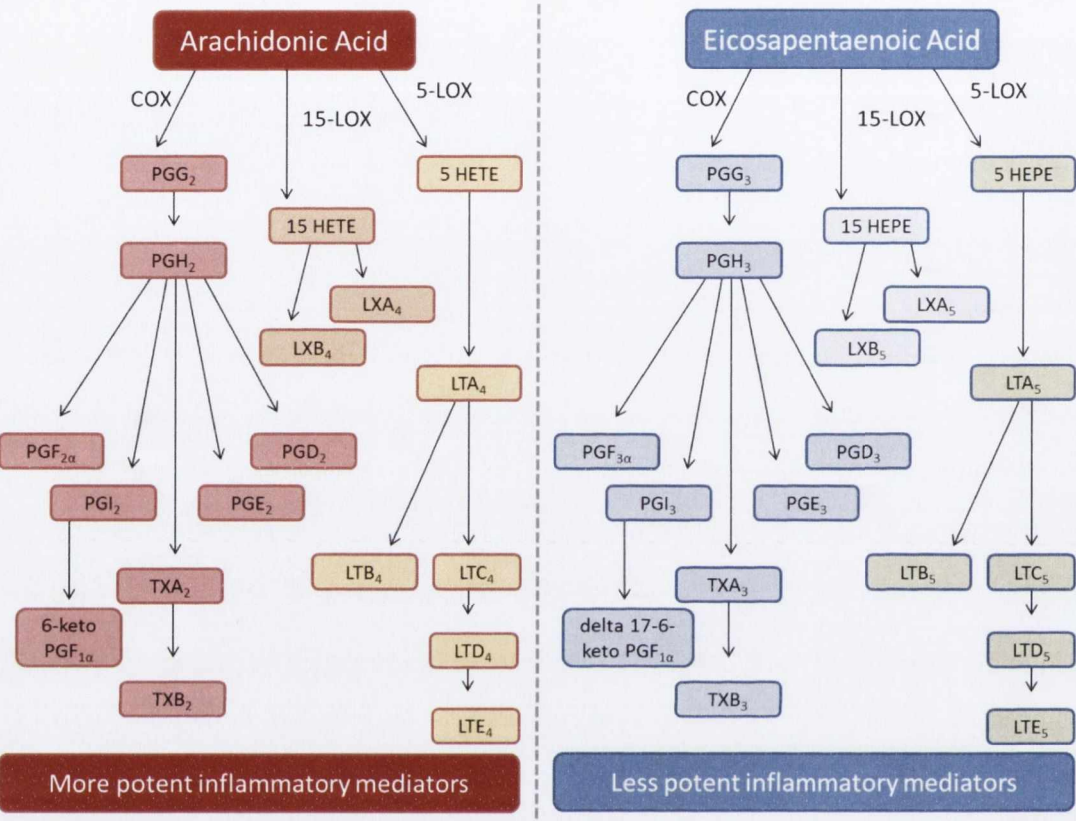


Figure 1.4 Metabolism of arachidonic acid and eicosapentaenoic acid

Arachidonic acid (AA) and eicosapentaenoic acid (EPA) reside in phospholipid membranes. Upon activation, the fatty acids are metabolised by the action of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. Arachidonic acid (AA) metabolism gives rise to the series-2 prostaglandins (PGE_2), prostacyclins (PGI_2), thromboxanes (TXA_2) and the series-4 leukotrienes (LTA_4 , LTB_4), and lipoxins (LXA_4 , LXB_4) whereas metabolism of EPA gives rise to series-3 prostaglandins (PGE_3), prostacyclins (PGI_3), thromboxanes (TXA_3) and series-5 leukotrienes (LTA_5 , LTB_5) and lipoxins (LXA_5 , LXB_5). The downstream metabolites of AA and EPA differ in their inflammatory effects, with AA derived eicosanoids deemed to be more potent inflammatory mediators than EPA derived eicosanoids.

AA, arachidonic acid; EPA, eicosapentaenoic acid; COX, cyclooxygenase; LOX, lipoxygenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HEPE, hydroperoxyeicosapentaenoic acid; LT, Leukotriene; LX, lipoxin; PG, prostaglandin; TX, thromboxane.

As acute inflammation progresses, intravascular platelet-leukocyte interactions evoke the formation of lipoxin A₄ and lipoxin B₄, which stop further recruitment of neutrophils^[217]. Alongside lipoxins, a new EPA derived lipid compound has been found to have an important role in inflammation resolution. Resolvin E1 is generated when aspirin acetylates COX-2 in vascular endothelial cells forming 18R-hydroperoxyeicosapentaenoic acid which is further converted via 5-LOX in leukocytes to form resolvin E1. Resolvin E1 directly activates ChemR23 receptor on macrophages and dendritic cells and directly inhibits BLT1 that is expressed by human neutrophils, thus blocking the pro-inflammatory effect of LTB₄. This enhances resolution of inflammation by regulating the chemotaxis and transmigration of neutrophils to the site of tissue injury, promoting neutrophil apoptosis and their phagocytic removal by macrophages and decreasing IL-12 production by dendritic cells^[217] (Figure 1.5). In a murine model of colitis, administering EPA gave rise to increased levels of resolvin E1 which in turn was associated with decreased leukocyte infiltration, decreased production of the pro-inflammatory cytokines IL-12 and TNF- α and reduced colitis-associated mortality^[218]

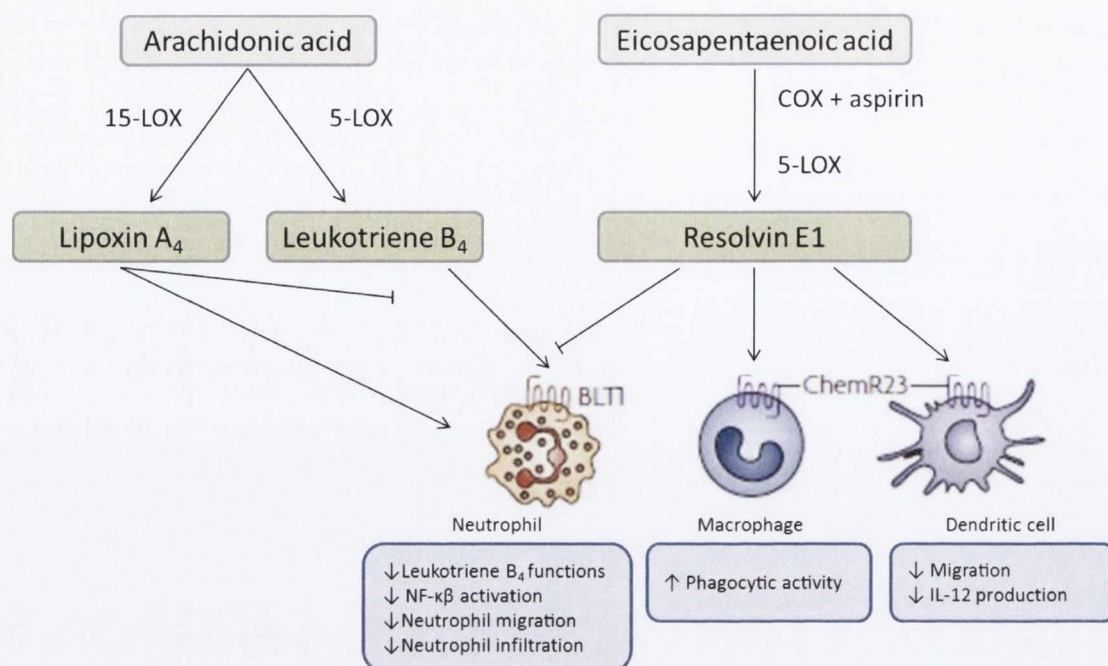


Figure 1.5 Role of resolvin E1 in inflammation resolution

Along with lipoxin A₄ and lipoxin B₄, resolvin E1 plays an important role in inflammation resolution. Resolvin E1 blocks the BLT1 receptor in neutrophils, inhibiting the effects of LTB₄. Lipoxins and resolvin E1 reduce neutrophil chemotaxis and transmigration to the site of tissue injury and promote neutrophil apoptosis. Resolvin E1 binds to the ChemR23 receptor on macrophages and dendritic cells, increasing phagocytic activity by macrophages and reducing IL-12 production by dendritic cells.

Adapted from Serhan *et al.*^[217].

When humans increase their consumption of EPA, there is increased incorporation of EPA into cell membranes and the ratio of omega-6:omega-3 is altered. Competition then occurs between EPA and AA for COX and LOX enzymes, leading to a decreased production of AA derived eicosanoids and an increase in EPA derived eicosanoids. This manipulation of the eicosanoid pathway can then affect the pathogenesis of numerous chronic diseases, such as cardiovascular disease and arthritis ^[219, 220]. In cardiovascular disease, increased consumption of omega-3 PUFA reduces the rates of all-cause mortality, cardiac and sudden death, and possibly stroke ^[219]. In the study of cancer cachexia, supplementation with omega-3 PUFA has been shown to have both anti-inflammatory and anabolic effects. EPA supplementation was shown to promote weight stabilisation in cancer patients with cachexia by attenuation of the acute phase response via suppressed IL-6 production ^[221-223].

Given the positive effects of EPA on inflammatory processes in many diseases, it may prove an effective agent in attenuating the inflammatory response following major surgery and promote recovery.

1.4.2 Postoperative Inflammation Response and Recovery Following Oesophagectomy – A Role For EPA?

Intra-operatively and in the immediate postoperative phase, the inflammatory response is characterised by an increase in IL-6, which positively correlates with the magnitude of the surgery performed. This increase in IL-6 then influences the production of other inflammatory mediators such as CRP and fibrinogen. Hepatic production of other proteins, such as albumin, decreases during this time ^[224]. This period is referred to as the acute-phase response and its function is to achieve the three primary goals of stimulating antimicrobial functions, reducing tissue damage and promoting wound healing ^[225]. To facilitate the increase in the synthesis of acute phase proteins in the liver, there is a shift in protein metabolism with an increase in protein loss from skeletal muscle, connective tissue, and the gut ^[225]. There is an increased rate of whole body protein turnover with up to 4-5 times greater nitrogen excretion leading to negative nitrogen balance ^[226, 227]. If uncontrolled and excessive, this loss of lean body mass may be associated with reduced muscle, immune and pulmonary function

and increased mortality ^[228-230]. In response to this initial acute pro-inflammatory environment that arises following surgery, the body attempts to launch an anti-inflammatory response to restore homeostasis. The increased levels of cortisol that occur following surgery, as a result of stimulation by adrenocorticotrophic hormone (ACTH) and in response to rising IL-6, are believed to be responsible for this ^[224]. Cortisol inhibits IL-12 production by macrophages and suppresses the production of Th1 type pro-inflammatory cytokines such as IFN- γ whilst promoting production of Th2 type anti-inflammatory cytokines such as IL-10 and IL-4 ^[231-233]. If this increased stimulation of the Th2 response persists, the imbalance gives rise to an immunosuppressive state, also referred to as the catabolic anti-inflammatory response, which can lead to increased morbidity and mortality ^[234] (Figure 1.6).

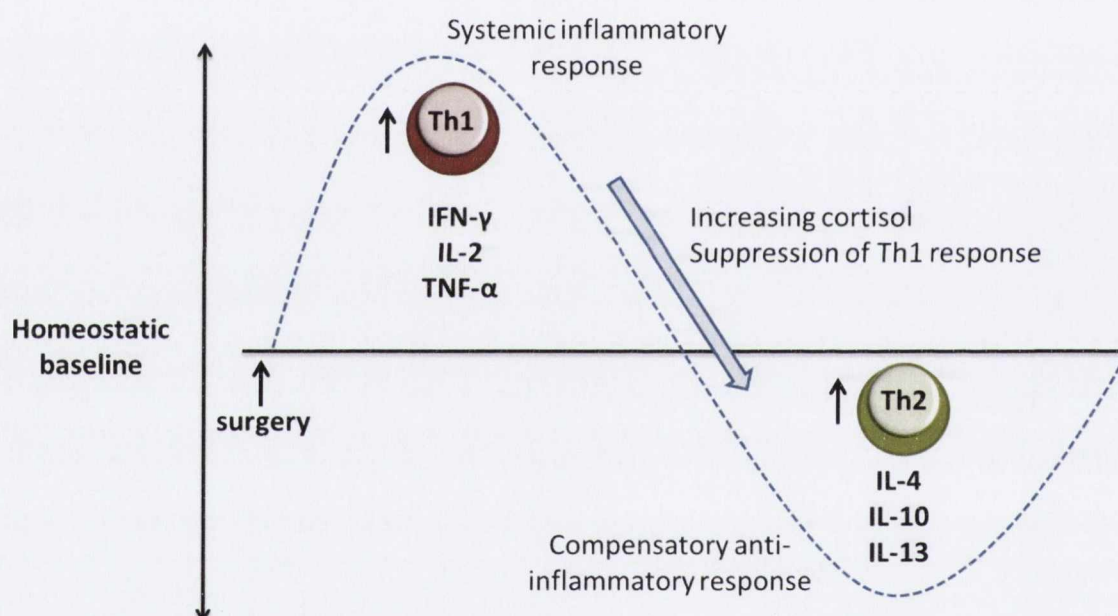


Figure 1.6 The biphasic inflammatory response

Following trauma or surgery, there is an initial state of hyper-inflammation, characterised by a Th1 type immune response with increased production of pro-inflammatory cytokines such as IFN- γ , IL-2 and TNF- α . This precipitates a delayed compensatory anti-inflammatory response. It is believed that this is driven by cortisol which suppresses the production of Th1 type pro-inflammatory cytokines whilst promoting the production of Th2 type cytokines IL-4 and IL-10. A persistence of this immunosuppressive state increases the risk of morbidity and mortality.

As described above, EPA supplementation impacts on inflammatory processes and resolution of inflammation. Furthermore, in healthy populations, supplementation with EPA can reduce circulating levels of pro-inflammatory cytokines and can also reduce cortisol levels ^[235, 236]. These immunomodulatory properties of EPA have raised interest in its potential use in attenuating the inflammatory response in patients undergoing major surgery, such as oesophagectomy. A previous double-blind randomised controlled trial carried out by this research group examined the effects of an EPA supplemented enteral feed versus a standard enteral feed on outcomes in 53 oesophagectomy patients. Feed was provided for 5 days pre-operatively and 21 days post-operatively, with those in the treatment arm receiving 2.2g/day of EPA. The results demonstrated that supplementation with EPA was associated with a more attenuated inflammatory response and a reduced maximal body temperature. In addition, patients who received the EPA enriched enteral feed preserved their lean body mass compared to the standard feed group who experienced an average loss of 1.9kg of fat free mass ^[237].

1.5 HYPOTHESIS AND AIMS

This work has two hypotheses. Firstly that visceral obesity influences oesophageal adenocarcinoma progression and impacts on recovery following surgery; and secondly that supplementation with eicosapentaenoic acid attenuates inflammation following oesophagectomy.

The specific aims of the research are as follows:

1. Determine a pathologically relevant threshold for visceral fat area in male and female gastrointestinal cancer patients, to allow for correct classification of visceral obesity status.
2. Investigate the role of visceral obesity and the insulin-like growth factor axis in oesophageal cancer.

3. Determine the impact of visceral obesity on the postoperative inflammatory response in patients undergoing upper gastro-intestinal resection.
4. Investigate the impact of an eicosapentaenoic acid enriched enteral feed on the postoperative immune response and recovery in oesophagectomy patients.

Chapter 2 Materials and Methods

2.1 REAGENTS

All laboratory chemicals and reagents were purchased from Sigma Chemical Company (MO, USA) unless otherwise stated. Solid reagents were weighed using an Explorer Pro fine electronic balance (Ohaus Corporation, NJ, USA). The pH of solutions was measured using a pH 211 microprocessor pH metre (Hanna Instruments, RI, USA). Gilson pipettes (Gilson S.A., France) and Labmate Pro multichannel pipettes (HTL Lab Solutions, Warsaw Poland) were used to transfer liquid volumes up to 1ml, electronic pipette aids (Drummond, PS, USA) and disposable Pasteur pipettes (Sarstedt Ltd., Wexford, Ireland) were used for volumes greater than 1ml and graduated cylinders were used for volumes in excess of 10ml.

2.1.2 Cell Culture Medium

Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza, Basel, Switzerland), supplemented with 10% heat inactivated foetal bovine serum (FBS), 100mM L-glutamine (Gibco, Biosciences, Dublin, Ireland), 100 u/ml penicillin/100 µg/ml streptomycin (Gibco, Biosciences, Dublin, Ireland). Complete RPMI (c-RPMI) was used to culture oesophageal cell lines.

2.1.3 Hank's Balanced Salt Solution

5.33mM Potassium chloride (400 mg)

0.44mM Potassium phosphate monobasic (60 mg)

4.0mM Sodium bicarbonate (350 mg)

138mM Sodium chloride (8000 mg)

0.30mM Sodium phosphate dibasic anhydrous (48 mg)

5.6mM D-Glucose (1000 mg)

Made up to 1L with deionised H₂O (dH₂O)

2.1.4 Dextran Solution

3% w/v Dextran-500

0.9% w/v Sodium chloride

Made up to 1L with dH₂O

2.1.5 Monocyte Isolation Buffer

2% FBS

1mM Ethylenediaminetetraacetic acid (EDTA)

Made up in phosphate buffered saline (PBS)

2.1.6 Hypotonic Buffer

20mM HEPES, pH 7.50,

0.1mM EDTA

10μL protease inhibitor cocktail/5ml

Made up in dH₂O

2.1.7 Phosphate Citrate Buffer

10.19g Anhydrous citric acid

36.69g di-Sodium hydrogen orthophosphate dodecahydrate (Na₂HPO₄·12H₂O)

Make up to 1L with dH₂O, pH 5.0

2.1.8 ELISA Developing Solution

1 x10 mg *o*-Phenylenediamine dihydrochloride (OPD) tablet

25mls Phosphate citrate buffer

7μl 1M H₂O₂

2.1.9 ELISA Stopping Solution

26.74mls 18M H₂SO₄

473.26mls dH₂O

2.1.10 ELISA Washing Buffer

1L PBS

0.5ml Tween 20

2.1.11 Fluorescence-Activated Cell Sorting (FACS) Buffer

2% FBS

0.1% Sodium azide (NaN_3), 10% w/v solution in dH_2O

PBS

2.1.12 FACS Blocking Buffer

50% FBS

50% FACS Buffer

2.1.13 RIPA Lysis Buffer

50mM Tris-HCl pH 7.4

150mM NaCl

1mM EDTA

1% Triton-X-100

1% Sodium deoxycholate

0.1% Sodium dodecyl sulphate (SDS)

1mM Phenylmethanesulphonyl fluoride

200µl Protease inhibitor cocktail (5µg/ml aprotinin and 5µg/ml leupeptin)

2.1.14 Separating Gels

8% separating gel:

6.13ml 30% acrylamide:bisacrylamide (37.5:1)

4.5ml 1.875 M Tris HCl (pH 8.8)

10.3ml water

176µl 10% (w/v) SDS

120µl 10% (w/v) fresh ammonium persulfate

10µl N,N,N',N'-tetramethylethylenediamine (TEMED)

2.1.15 5% (w/v) Stacking Gel

1ml 30% (w/v) acrylamide:bisacrylamide (37.5:1)

2ml 0.6 M Tris HCl (pH 6.8)

6ml water

100μl 10% (w/v) SDS

150μl 10% (w/v) fresh ammonium persulfate

10μl TEMED

2.1.16 Laemmli Buffer

2ml Tris HCl pH 6.8

5ml 10% (w/v) SDS

1ml 2-β-mercaptoethanol

2ml glycerol

0.05 g (w/v) bromophenol blue

2.1.17 Electrode Buffer

50mM Trizma base

384mM Glycine

0.1% (w/v) SDS

2.1.18 Cold Transfer Buffer

0.15M Glycine

20mM Tris

0.1% (w/v) SDS

20% (v/v) Methanol

2.1.19 Tris-Buffered Saline (TBS)

25mM Tris HCl (pH 7.6)

150mM NaCl

2.1.20 Tris-Buffered Saline Tween (TBST)

Tris-buffered saline

0.1% Tween-20 TBST

2.1.21 Tris-Acetate-EDTA (TAE) Buffer

40mM Tris HCl (pH 8.3)

20mM Acetic acid

1mM EDTA

2.2 CELL CULTURE

All cell culture media was purchased from Lonza (Basel, Switzerland) and all cell culture plastics were purchased from Starstedt Ltd. (Wexford, Ireland) unless otherwise indicated. Two oesophageal squamous carcinoma cell lines, KYSE 410 and OE21; and two oesophageal adenocarcinoma cell lines, OE19 and OE33 were used in this research. OE19 was obtained from American Type Culture Collection and OE33, OE21 and KYSE 410 were sourced from the European Collection of Cell Cultures. Cells were cultured and maintained in c-RPMI media at 37°C in humidified air containing 5% CO₂.

2.2.1 Cell Subculture

Cell lines were examined daily using an inverted phase contrast Nikon microscope (Nikon Corporation, Tokyo, Japan) and subcultured upon reaching 80-90% confluency. Growth medium was decanted and cells were washed in phosphate buffered saline (PBS) to remove residual media. 1ml trypsin EDTA was added to the flask and incubated at 37 °C for several minutes or until the cells detached from the surface. 10ml of c-RPMI was then added to inactivate the trypsin and cells were centrifuged at 180 x *g* for 3 minutes. Re-suspended cells were then seeded at appropriate densities into new flasks.

2.2.2 Cell Counting

Cells were trypsinised as described (section 2.2.1), added to 10ml maintenance medium and centrifuged at 180 x *g* for 3 minutes. The supernatant was decanted and

the cell pellet resuspended by pipetting in 1ml of medium. 20µl of the cell suspension was added to 180µl trypan blue (0.4% w/v) solution and allowed to sit at room temperature for one minute. Viable cells were unstained due to their active exclusion of trypan blue, whereas dead cells were stained blue, due to their disrupted membranes. 20µl of this cell suspension was added to the counting chamber of a disposable KOVA haemocytometer (HYCOR Biomedical, IN, USA) and the number of viable cells in each of the nine squares of the grid was counted. The number of cells per ml was calculated using the following equation:

$$(total\ number\ of\ cells/9) \times 10^4 \times 10\ (dilution\ factor) = no.\ cells/ml$$

2.3 BRDU CELL PROLIFERATION ASSAY

Oesophageal cancer cells were seeded at a dilution of 5×10^3 cells per well in c-RPMI medium in 96 well plates and allowed to adhere for a minimum of 6 hours. They were then serum starved (media containing 0.5% FBS) overnight. Cells were treated with various concentrations ranging from 0.1-1000ng/ml of recombinant human IGF-1 (R&D Systems, MN, USA) for 24 hours. A BrdU cell proliferation assay was carried out following manufactures guidelines (Roche Diagnostics Ltd, Sussex, UK). The pyrimidine analogue BrdU (5-bromo-2'-deoxyuridine) is incorporated in the place of thymine in the newly synthesised DNA of proliferating cells, and is bound by the BrdU anti-POD antibody. The antibody complexes are then detected by a subsequent substrate reaction. BrdU label was added to each well (except no BrdU label control) for 3 hours at 37 °C, following which the media was tapped and flicked off. Cells were fixed by adding 200µl of fixative solution for 30 minutes at room temperature. 100µl anti-BrdU-POD was added to each well and the plate was incubated for 90 minutes at room temperature. Cells were then washed in PBS and 100µl of substrate solution was added to each well. The plate was incubated at room temperature for 5-10 minutes or until colour was sufficient for photometric detection. 25µl H₂SO₄ was then added to stop the reaction and the plate absorbance was read at 450 nm using a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, CA, USA). Wells containing cells but no BrdU label were used to subtract background absorbances and percentage increase/decrease in proliferation was calculated relative to untreated cells.

2.4 WESTERN BLOTTING

2.4.1 Protein Extraction

Cells were washed three times in PBS and lysed by the addition of 800µl ice-cold RIPA lysis buffer directly into the T75 flask. Cells were incubated on ice for 5 minutes and the resulting cell lysate was collected using a cell scraper. Insoluble protein was sheared using a 29 gauge needle (BD Micro-Fine, NJ, USA) and the sample was centrifuged at $4,000 \times g$ for 2 minutes to pellet any remaining insoluble fraction the supernatant was stored at -20°C .

2.4.2 Protein Quantification

Protein quantification was carried out using the commercially available Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, IL, USA). A standard curve was prepared from supplied stock solution of bovine serum albumin (BSA) (2000 µg/ml), creating standards of 1000 µg/ml, 800 µg/ml, 600 µg/ml, 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml and 0 µg/ml. 10µl of each standard concentration and of each sample was added to a 96-well plate in triplicate. A 50:1 solution was prepared of BCA Reagent A and BCA Reagent B and 200µl was added to each well of the 96-well plate which was then incubated at 37°C for 30 minutes. The absorbance of each well was read on a VersaMax microplate reader with SoftMax Pro software at 490nm and protein concentrations were determined by interpolation from the standard curve of known concentrations of BSA.

2.4.3 Denaturing Polyacrylamide Gel (SDS-PAGE)

Gels were cast between upright glass plates (Bio-Rad Laboratories, Hercules, CA, USA). 70% ethanol was gently layered on top of the gels to exclude air and aid polymerisation; this was removed once polymerisation occurred. A 5% (w/v) stacking gel was prepared and added to the top of the polymerised gel and a 12-well comb was inserted before allowing the gel to set.

2.4.4 Protein Electrophoresis and Transfer

Protein samples were diluted to a final volume of 30µl with 2x Laemmli buffer and denatured by heating to 95°C for 10 minutes. Samples were separated by electrophoresis at 50 mA per gel in electrode buffer for 90 minutes, or until the dye

front reached the bottom of the gel. 10 μ l of a tri-chrom pre-stained protein marker (Pierce, Rockford, IL, USA) was also loaded onto each gel. Separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Pall Corp., Pensacola, FL, USA) using a wet transfer apparatus (Bio-Rad, CA, USA). The PVDF membrane was pre-activated in 100% methanol for one minute and the PVDF and Whatman filter paper (Whatman Laboratory Division, Maidstone, Kent, UK) were soaked in transfer buffer before transfer. The transfer apparatus was covered in cold transfer buffer and polyacrylamide gels were placed in the transfer apparatus on top of the PVDF membrane in a sandwich of double layered 3mm Whatman filter paper. Bubbles were carefully removed from between layers and proteins were transferred for 2 hours at 225 mA and maximum voltage.

2.4.5 Antibody Probing of Membranes

Following the transfer of proteins, membranes were blocked with 5% (w/v) non-fat dry milk (Marvel) in TBST on a shaker (Bibby Sterilin Ltd., Staffordshire, UK) for one hour at room temperature. The membrane was then incubated in the primary antibody, diluted in 5% (w/v) Marvel in TBST on a shaker overnight at 4°C. Membranes were washed three times for 5 minutes each in TBST before incubation in horseradish peroxidase (HRP) conjugated secondary antibody (1:2,000 dilution in TBST) (Dako, Glostrup, Denmark) for one hour on a shaker at room temperature (RT). Six further 5 minute washes in TBST were then carried out. The Supersignal West Pico Chemiluminescent substrate kit (Pierce, Rockford, IL, USA) was then used to detect bound antibody complexes. Working reagent was prepared just prior to use and added to the membrane for 1 minute. Membranes were then exposed for 2 minutes to scientific X-Ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan), which was developed using a medical film processor (Agfa-Gevaert, Mortsel, Belgium). Specific antibodies and reagents used in Western blotting are detailed in Table 2.1 below.

Table 2.1 Antibodies used in Western blotting

Antibody	Supplier	Isotype	Type	Block	Primary Ab
IGF-1R	R&D Systems	Goat	Polyclonal	5% Marvel	1/500
				1 hour, RT	o/n, 4°C
β-actin	Calbiochem	Mouse	Monoclonal	5% Marvel	1/20,000
				1 hour, RT	1 hour, RT

2.4.6 Densitometry

Densitometric analysis was carried out using TINA (version 4.0) software (Raytest, Straubenhardt, Germany). Values were represented as a ratio of sample band intensity to that of the corresponding β-actin control.

2.5 INVESTIGATION OF RNA EXPRESSION

2.5.1 RNA Isolation from Tumour Cell Lines using TriReagent Method

RNA to be used for reverse transcriptase polymerase chain reaction (RT-PCR) was isolated from oesophageal cancer cell lines using the TriReagent method (Molecular Research Center, Montgomery Road, OH, USA). 1ml of TriReagent was added to cell pellets which were then lysed by pipetting up and down. 100μl of 1-bromo-2-chloropropane (BCP) was added to samples which were then vortexed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. Following centrifugation at $12,000 \times g$ at 4 °C for 15 minutes, the upper aqueous phase was carefully removed to a new tube, and subsequent steps were carried out at room temperature. 500μl isopropanol was added and the tube incubated for 10 minutes followed by centrifugation at $12,000 \times g$ for 8 minutes. The supernatant was decanted and the pellet washed in 1ml ethanol (75% v/v) by brief vortexing followed by centrifugation at $12,000 \times g$ for 5 minutes. The ethanol was decanted and the pellet allowed to air dry for 3-5 minutes. The pellet was resuspended in 60μl RNase free water.

2.5.2 RNA Quantification and Purity Analysis

RNA quantification was determined spectrophotometrically, using a Nanodrop 1000 spectrophotometer (version 3.1.0, Nanodrop technologies, DE, USA). 1μl of RNase free

water was used to blank the instrument. 1µl of each sample of isolated RNA was loaded onto the instrument and concentration was measured in ng/µl. 260:280 and 260:230 purity ratios were also recorded. A 260:280 ratio greater than 1.65 was indicative of a relatively pure RNA yield, while a 260:230 ratio greater than 1.7 indicated that the sample was free of phenol contamination.

2.5.3 cDNA Synthesis

All reverse transcription reagents were purchased from Bioline (Bioline, Kilkenny, Ireland). cDNA was made by adding random primers (0.5 µg/µl) to 1 µg total RNA and heating the sample to 70°C for 10 minutes in order to denature the RNA. A master mix containing reverse transcription components was then added to each sample containing: RNaseOUT recombinant ribonuclease inhibitor (1 Unit/µl), deoxyribonucleotide triphosphates (dNTPs) (10mM, prepared as a 1:1:1:1 ratio of dATP, dTTP, dCTP, dGTP), dithiothreitol (DTT) (0.01mM) and Bioscript reverse transcriptase (200 Units/µl) in 5× Bioscript reaction buffer (Table 2.2). This mixture was incubated at 37°C for 1 hour. The resulting cDNA was stored at -20 ° C and 1µl of cDNA template was used to investigate gene expression.

Table 2.2 Reagents and volumes used in cDNA synthesis

Reagent	Volume (µl) per sample
Random Primers	1
250 ng-1 µg RNA + RNase free H ₂ O	11
Heated for 10 min at 70°C and cooled on ice for 1 min then following mix added:	
5X Bioscript reaction buffer	4
H ₂ O	2
DNTPs	1
RNase out	0.5
Bioscript Reverse Transcriptase	0.5
Total Volume	20

2.5.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA was used as a template for reverse transcriptase polymerase chain reaction (RT-PCR). The following PCR master mix was added to each sample: GoTaq Green Master Mix (2× GoTaq Reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3mM MgCl₂, GoTaq DNA polymerase) (Promega, WI, UK), forward

primer (20 μ M), reverse primer (20 μ M) and RNase free water (Table 2.3). The reaction was initialised by heating the samples to 94°C for 5 minutes. This was followed by denaturation at 95°C for 1 min, primer annealing at 55°C for 1 minute and elongation at 72 °C for 1 minute. These 3 temperature cycles were repeated 40 times followed by a final elongation step at 72 °C for 10 minutes.

Table 2.3 Reagents and volumes used in RTPCR

Reagent	Volume (μ l) per sample
GoTaq Master Mix	10
Forward primer	1
Reverse primer	1
RNase free H ₂ O	7
cDNA	1
Total Volume	20

All reverse transcriptase primers were purchased in lyophilised form from Operon (Eurofins MWG Operon, Ebersberg, Germany) and rehydrated to a stock concentration of 400 μ M in RNase free water. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an endogenous control and HepG2, a liver carcinoma cell line, served as a positive control for expression of IGF-1R, IGF-1 and IGF-2. Specifically designed primer sequences are detailed below in Table 2.4 below

Table 2.4 Primer sequences in RT-PCR

Gene	Forward Primer	Reverse Primer
IGF-1R	TGGAGTGCTGTATGCCTCTG	ACGCCAATAAGTTCGTCCAC
IGF-1	ATGCACACCATGTCCTCCTGGCAT	CTACATCCTGTAGTTCTTGTT
IGF-2	TCCTGGAGACGTACTGTGCTA	GGGTTGTTGCTATTTTCGGAT
GAPDH	CAAATTCATGGCACCCTCA	GGAGTGGGTGTCGCTGTTGA

2.5.5 Agarose Gel Electrophoresis

1% agarose gels were prepared by dissolving agarose in TAE buffer and heating in a microwave for several minutes. It was allowed to cool to approximately 55 °C before adding SYBR safe DNA gel stain (10,000 $\times$ concentrate in DMSO, Invitrogen

Corporation, CA, USA) to a final concentration of 1 µg/ml. The solution was poured into a gel tray to a depth of 3-5mm and allowed to set.

The DNA samples, containing loading buffer present in the GoTaq[®] reagent, were loaded along with a 100 base pair DNA ladder (Promega Corporation, Madison, WI, USA). Electrophoresis of the DNA samples was carried out at 100 volts in an EC-360 Maxicell gel system (EC Apparatus Corp., FL, USA), using 1 × TAE as a running buffer. The bands were visualised and photographed under UV light using a BioSpectrum Imaging System (Ultra Violet Products, Cambridge, UK).

2.6 IMMUNOHISTOCHEMISTRY

Resected tumour from oesophageal cancer patients was fixed in 10% formalin overnight and embedded in paraffin wax on a Leica EG 114OH embedder (Laboratory Instruments and Supplies, Dublin, Ireland) in the Central Pathology Laboratory at St. James's Hospital. Sections 5 µm in thickness were cut from paraffin embedded sections of oesophageal tumour using a Leica RM 2235 microtome (Leica, Meath, Ireland) and floated onto Superfrost Plus poly-L-lysine coated glass slides (Thermo Fisher Scientific, IL, USA). Cut sections were baked overnight at 37 °C in a tissue-drying oven (Binder, Tuttlingen, Germany) and processed immediately.

2.6.1 Immunohistochemical Staining

Antigen retrieval was carried out using Trilogy (Cell Marque Corporation, Rocklin, CA, USA) which combines three pre-treatment steps: deparaffinisation, rehydration and unmasking. Slides were incubated in Trilogy (1:20 dilution in distilled water) in a Princess DVB350 programmable pressure cooker on low pressure for 10 minutes. Vectastain Elite kits (Vector Labs, Burlingame, CA, USA) were used for all immunohistochemical staining. Tissue sections were incubated in 3% H₂O₂ in methanol for 30 minutes to quench endogenous peroxidase activity. The sections were then washed three times for 5 minutes each in PBS. For the detection of IGF-1R, slides were blocked for 30 minutes with diluted rabbit serum then incubated in a 2µg/ml solution of anti-human IGF-1R antibody (R&D Systems) overnight at 4°C. Human placenta served as a positive control and negative sections were treated with rabbit serum in place of IGF-1R antibody. For CD68 staining, sections were blocked with diluted horse serum then incubated with a ready to use solution of mouse anti-human CD68

antibody (Dako, Glostrup, Denmark) overnight at 4°C. Sections were washed 3 times for 5 minutes each in PBS then incubated with a 1:400 dilution of relevant biotinylated secondary antibody. Sections were washed 3 times for 5 minutes each in PBS then incubated in avidin-biotin complex reagent (supplied). Following this, the sections were washed three times for 5 minutes each in PBS and incubated for 2-15 minutes (depending on the level of protein expression in the tissue) in the dark in diaminobenzidine (DAB) peroxidase solution. The sections were rinsed in tap water and counterstained in Harris's haematoxylin for 30 seconds. The slides were placed in a PBS bath for 5 minutes and subsequently rinsed in gently running tap water for 5 minutes. The slides were then dipped in two separate baths of 100% methanol (up and down 10 times) and two separate baths of xylene (up and down 10 times) before being transferred to a separate container of xylene for at least 4 hours. Coverslips (VWR International, West Chester, PA, USA) were mounted onto the slides using DPX mountant (B.D.H. Ltd., Poole, Dorset, UK) and left to dry in a fume hood. Images were taken using Image Pro-Plus 4.1 software (Media Cybernetics, Gelichen, Germany).

2.6.2 Immunohistochemical Staining Quantification

Stained sections were graded independently by three individuals, including a consultant pathologist, using a validated technique ^[238]. Intensity of IGF-1R staining was graded on a scale from 0 to 3. A score of 0 corresponded to no staining, a score of 1 to weak staining, a score of 2 to moderate staining and a score of 3 to strong staining. The percentage of tumour cells stained was graded in quartiles of 0%, 25%, 50%, 75% and 100%. The average grade for each patient was calculated by multiplication of the intensity score by the percentage of tumour score, giving an overall score for each section area of 0-300. Areas of tumour and tumour stroma were graded separately. Where present, the invasive edge at the junction of tumour and normal tissue was identified and marked by a pathologist and this section was graded separately. For CD68, positively stained cells were counted in three different areas of tumour, stroma and at the invasive edge. Statistical analysis was carried out as described in section 2.18.

2.7 ADIPOSE TISSUE BIOBANK

An adipose tissue biobank was established in the Department of Surgery in July 2007. Patients undergoing resective surgery for oesophageal adenocarcinoma, oesophageal squamous cell carcinoma and colorectal carcinoma, in addition to non-cancer patients undergoing surgery for laparoscopic Nissen fundoplication, cholecystectomy and hernia repair, are recruited to the adipose tissue biobank. All recruited patients gave informed consent for use of their adipose tissue in this study, and the study obtained ethical approval from St James's Hospital Ethical Review Board. Extensive data pertaining to the patients' clinical status, medical management and treatment outcomes is recorded on the Upper Gastrointestinal Database which is maintained by a dedicated database manager.

2.7.1 Anthropometry

All measurements are carried out preoperatively on the morning of surgery by a clinician. Waist circumference (WC) was measured to the nearest 0.1 cm using a tape measure at the midpoint between the lowest rib and ileac crest on the mid-auxiliary line after respiratory exhalation whilst patient was in a standing position ^[239]. Height and weight were measured to the nearest 0.1 cm and 0.1 kg using SECA Alpha 770 digital scale (CMS Weighing Equipment Ltd, London, UK) and portable stadiometer (SECA, Vogel and Halke, Germany). Body mass index (BMI) was calculated as weight (kg) / height (m)².

All patients had a preoperative diagnostic CT scan performed. All patients were scanned on a Siemens Emotion single slice or a multislice Somatom Sensation scanner (Siemens, Erlangen, Germany), with individual scans analysed on a Siemens Leonardo workstation. The cross-sectional surface areas of the different abdominal fat compartments were calculated at the disc space between the L3 and L4 vertebrae, using a previously validated method ^[240, 241]. Firstly, to quantify total fat area (TFA), a line was drawn around the skin surface with a cursor. Then an automatic algorithm and a Hounsfield threshold value of -50 to -150 was used to determine the cross-sectional fat content within that area. Visceral fat area (VFA) was calculated by repeating the technique with an area delineated by a line drawn around the inner layer of the abdominal wall musculature. Subcutaneous fat area (SFA) was calculated by subtracting VFA from TFA ^[36]. All areas were measured in square centimetres.

2.7.2 Clinical Measurements

Systolic and diastolic blood pressure were measured, using a digital sphygmomanometer (UA-767 *Plus* A&D Medical, Tokyo, Japan), on the left arm after the subject had rested while seated for 10 minutes ^[242]. Blood pressure measurements are taken by ward nursing staff and documented in the Adipose Tissue Biobank as elevated or normal.

2.7.3 Blood Sampling and Biochemical Measurements

Pre-operative fasting venous blood samples are taken for the determination of serum triglycerides, high density lipoprotein cholesterol (HDL-C) and fasting glucose. All samples are processed in the Central Pathology Laboratory at St. James’s Hospital, Dublin using enzymatic assay based techniques. Additional serum samples are collected and stored at -80°C for use in obesity related research projects.

2.7.4 Definition of the Metabolic Syndrome

In this centre, the International Diabetes Federation (IDF) 2006 guidelines are used to diagnose the presence of the metabolic syndrome ^[32]. The criteria for diagnosis are outlined in Table 2.5 below.

Table 2.5 Criteria for Metabolic Syndrome defined by IDF

Visceral Obesity	Waist Circumference > 94cms in males Waist Circumference > 80cms in females
Plus any two of the following:	
Raised Triglycerides	≥1.7 mmol/l Or on specific treatment for this lipid abnormality
Reduced HDL	<1.03 mmol/l in males <1.29 mmol/l in females Or on specific treatment for this lipid abnormality
Raised Blood Pressure	Systolic ≥130 mmHg or diastolic ≥85 mmHg Or treatment of previously diagnosed hypertension
Raised Fasting Plasma Glucose	Fasting plasma glucose ≥5.6 mmol/l Or previously diagnosed Type 2 Diabetes

2.8 PATIENT RECRUITMENT FOR STUDY OF OBESITY AND POSTOPERATIVE INFLAMMATION

Patients scheduled to undergo resective surgery for oesophageal and gastric cancer were invited to participate in a study investigating the postoperative inflammatory response in obese and non-obese patients. Recruitment was conducted by a research dietitian and the study took place between October 2009 and December 2010. All patients provided informed consent preoperatively to have serum and plasma samples collected on the morning of surgery and on days 1, 3, 7 and 14 postoperatively. Ethical approval was obtained from St James's Hospital Ethical Review Board. Anthropometry, including height, weight, waist circumference and abdominal fat quantification was performed as described in section 2.7.1

2.9 DOUBLE BLIND RANDOMISED CONTROLLED TRIAL DESIGN

A double blinded randomised controlled trial to investigate the long term effects of an enteral nutritional supplement enriched with eicosapentaenoic acid (EPA) on body composition, immune function and quality of life in patients undergoing treatment of localized oesophageal cancer began in January 2011. Ethical approval from the St. James's Hospital Institutional Review Board was obtained, with informed written consent required from all patients. Patients were consented pre-treatment (neoadjuvant therapy/surgery) by a research dietitian.

2.9.1 Trial Endpoints

Primary Endpoints:

- Quality of life as measured by European Organisation for Research and Treatment of Cancer (EORTC) Questionnaires
- Lean body mass

Secondary Endpoints:

- Effects on the immuno-inflammatory response postoperatively
- Postoperative clinical outcome including SIRS, sepsis and organ failure

The research surrounding the secondary endpoints of this clinical trial are presented in this thesis

2.9.2 Inclusion and Exclusion Criteria

Inclusion:

- Adult (male & female) patients >18 years with resectable gastro-oesophageal cancer:

Exclusion Criteria:

- Patients with metastatic disease
- Non-operable cases
- Patients requiring chemotherapy/radiotherapy early following surgery
- Patients with known immunological disorder
- Emergency oesophagectomy cases
- Patients with cardiac, liver or renal failure
- Active small intestinal disease eg Crohns disease
- Allergy to any of the ingredients
- Uncontrollable Diabetes
- Use of fish oil/n-3 fatty acids
- Drug abuse
- Unable to take preparation for 5 days preoperatively
- Pregnant women
- Cessation of enteral feeding for longer than 3 consecutive days post operatively for medical/surgical reasons (e.g. Chyle leaks)

2.9.3 Randomisation

Participants were randomly assigned to 1 of 2 treatment groups using a computerised random number generator.

2.9.4 Test and Comparator Product

The test product is a commercially available nutritional supplement called Prosure (Abbott Laboratories) that is enriched with EPA and licensed for use in cancer cachexia

patients. The 480ml/day provided to patients in the test group pre-operatively and the 500ml/day provided post-operatively equates to 2.16g/day and 2.25g/day of EPA respectively. The comparator product is a standard nutritional supplement called Ensure Plus (Abbott Laboratories) that is routinely used in the postoperative setting to provide nutritional support. The full nutritional composition of both the test and the standard feed is detailed in Table 2.6 below.

Table 2.6 Composition of Test Product (Prosure) and Comparator (Ensure Plus)

Nutritional Information	Unit	Per 100ml Prosure	Per 100ml Ensure Plus
Energy	kJ	526	630
	kcal	125	150
Protein	g	6.65	6.3
Fat	g	2.56	4.91
Eicosapentaenoic Acid	g	0.45	0
Carbohydrate	g	19.4	20
Water	g	79.4	77
Vitamin A	mcg RE	205	160
Vitamin D	mcg	1.7	1.0
Vitamin E	mg TE	20	3.02
Vitamin K	mcg	10	7.6
Vitamin C	mg	43	15
Folic acid	mcg	169	40
Vitamin B ₁	mg	0.25	0.27
Vitamin B ₂	mg	0.29	0.31
Vitamin B ₆	mg	0.34	0.4
Vitamin B ₁₂	mcg	0.5	0.57
Niacin	mg NE	2.5	2.9
Pantothenic acid	mg	1.1	1.4
Biotin	mcg	5.0	6.5
Choline	mg	51	55
Sodium	mg	150	140
Potassium	mg	200	165
Chloride	mg	152	145
Calcium	mg	148	100
Phosphorus	mg	105	100
Magnesium	mg	42	31
Iron	mg	1.7	2.2
Zinc	mg	2.5	1.9
Manganese	mg	0.42	31
Copper	mcg	23	250
Iodine	mcg	16	15
Selenium	mcg	7.9	7.0
Chromium	mcg	10	10
Molybdenum	mcg	14	15

2.9.5 Treatment Administration

Preoperatively patients must consume the supplement orally. Patients receive either the test or the standard product in the form of a 225ml bottle of a vanilla flavoured milkshake drink. They are advised to consume 2 bottles (480ml) each day for 5 days preoperatively.

Postoperatively the supplement is delivered enterally via a jejunostomy tube sited at the time of resection. Both the test product and the standard product are provided as 500ml ready-to-hang enteral feed bottles. Feeding commences on day 1 postoperatively at a starter rate of 30mls/hour increasing as tolerated by the patient, to reach 1L of nutritional feed. On day 1 postoperatively the 500mls of either test product or standard product is delivered first. The remaining 500mls of nutrition is provided in the form of standard feed to all patients.

By day 2 postoperatively most patients have progressed to full feeding at a rate of approximately 100mls/hour over 20 hours. Again, the first 500mls of feed is delivered as either test product or standard product with the remaining ~1.5 litres of feed delivered as standard product, ensuring the full nutritional requirements of the patient are met.

A gastrograffin swallow is performed on approximately day 5 postoperatively and, if the anastomosis is intact, the patient can begin on sips of free fluid, progressing as tolerated to oral diet. During this phase, as oral intake increases the need for enteral nutrition support decreases. Enteral feeding is tapered down gradually until only 500mls of either test product or standard product is being delivered enterally overnight. This is the feeding regimen the patient is discharged on. A one month supply of either test product or standard product is supplied to the patient on discharge and supplementation with the test product is discontinued at the 1 month outpatient appointment. Patients are reviewed again at 3 and 6 month outpatient appointments

2.9.6 Method of Blinding

Both the test product and comparator are presented in identically sized packaging and have the same taste, smell and viscosity. The manufacturers place a number on the packaging to indicate whether the product is “group 1” or “group 2”. This ensures that patients and the investigators remain blinded as to which group is the test group.

2.9.7 Anthropometric Measures

At the time points specified below (Figure 2.1) anthropometric assessments are performed including weight, height, abdominal fat (as described above in 2.7.1) and BMI. Usual weight, % weight loss and usual pre illness BMI are also determined.

Segmental body composition is analysed using the Tanita BC 418 MA bioelectrical impedance analyzer (Tanita UK Ltd, Middlesex, UK) which gives relative information on the amount of lean and fat tissue in the trunk area and in each limb, as well as overall body composition and hydration status.

2.9.8 Nutritional Assessment

Preoperatively and postoperatively, nutritional assessments are performed on the patient by a research dietitian. This entails quantification of nutritional intake and estimation of nutritional requirements using the Schofield equation ^[243] and appropriate stress factors ^[244].

2.9.9 Routine laboratory measures of biochemical & immune function

At the time points specified below in figure 2.1 blood samples are collected on the patient. Routine biochemical and immunological tests are performed by the Central Pathology Laboratory (CPL) in St. James’s Hospital, detailed in Table 2.7 below. Advanced investigation of immune cell function is described in section 2.10 below.

Table 2.7 Routine Laboratory Tests Carried out by CPL in St. James’s Hospital

Hematology	Chemistry Clinical	Immunology
Hemoglobin	Urea	C-Reactive Protein
Neutrophil Count	Creatinine	Erythrocyte Sedimentation Rate
Lymphocyte Count	Total Bilirubin	
White Cell Count	Sodium	
Platelets	Potassium	
Albumin	Calcium	
	Inorganic Phosphorus	
	Total Protein	

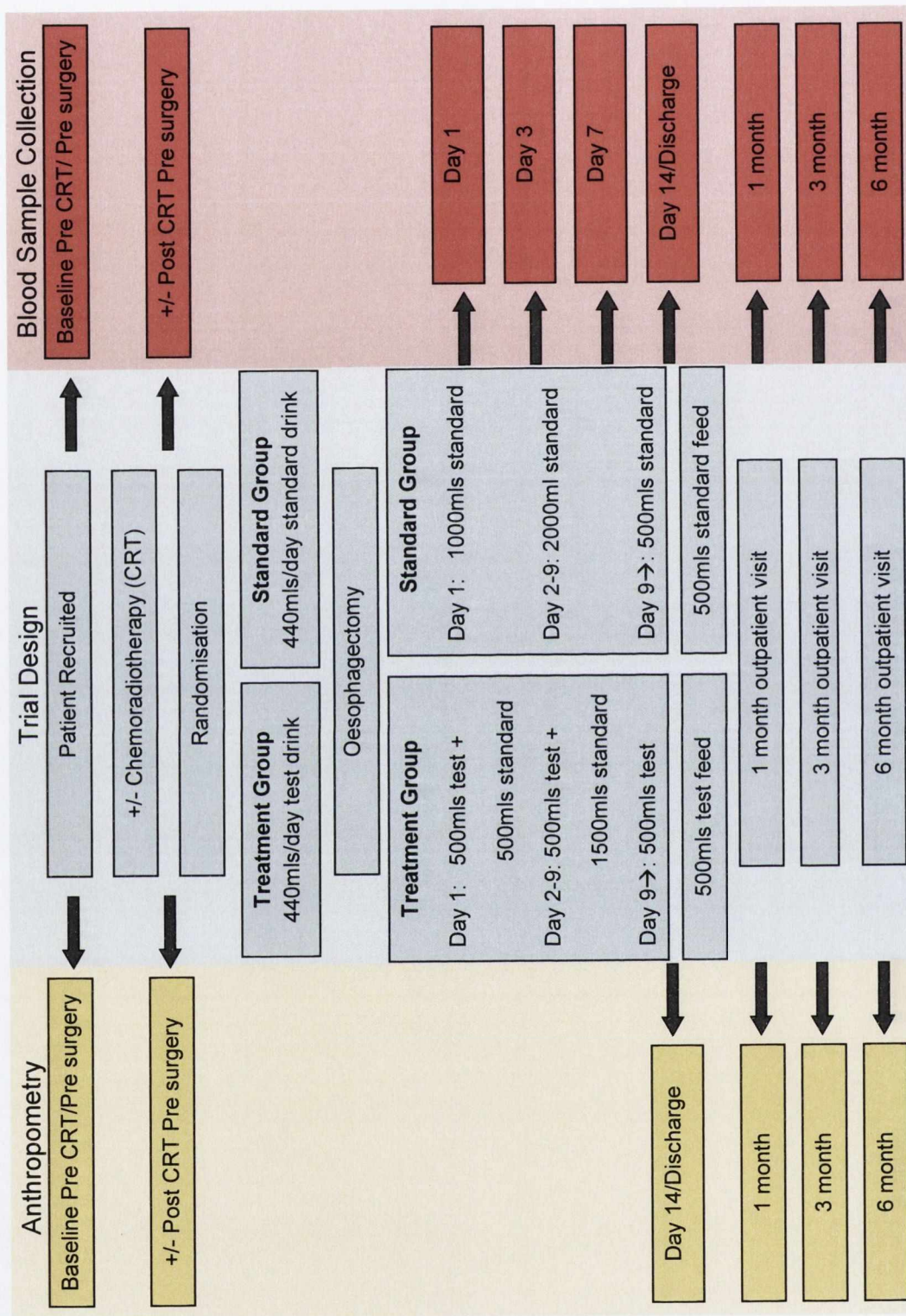


Figure 2.1 Design of randomised controlled trial and collection of anthropometric measures and blood samples

2.10 BLOOD SAMPLE PROCESSING

2.10.1 Serum and Plasma Isolation

Blood for serum isolation was collected in Z serum clot activator tubes and blood for plasma isolation was collected in lithium heparin tubes (Grenier Bio-One, Frickenhausen, Germany). Blood tubes were centrifuged at $3000 \times g$ for 10 mins at 4°C . Upper layers of serum and plasma were then transferred to 2ml cryotubes and stored at -80°C .

2.10.2 Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using the Ficoll-Plaque Plus (GE Healthcare, Buckinghamshire, UK) technique in accordance to manufacturer's guidelines. Briefly, blood collected in a K_2EDTA tube (Grenier Bio-One, Frickenhausen, Germany) was diluted with an equal volume of phosphate buffered saline (PBS) and layered over Ficoll-Plaque in a ratio of 4:3. Samples were spun at room temperature for 30 minutes at $400 \times g$ with no brake. Following centrifugation, the plasma above the mononuclear band was aspirated and discarded. The PBMC layer was then extracted and transferred to a 50ml tube. Cells were washed with PBS at $600 \times g$ for 6 minutes. Supernatant was discarded, the pellet was re-suspended in PBS and cells were counted. The PBMC were divided into aliquots of 1×10^7 cells/ml, RLT lysis buffer (Qiagen, West Sussex, UK) was added and cells were sheared using 21 gauge needles (Grenier Bio-One, Frickenhausen, Germany). Cell lysates were then stored at -80°C for subsequent RNA isolation.

2.10.3 Neutrophil Isolation

Neutrophils were isolated as previously described by Nauseef *et al* ^[245]. Following centrifugation with Ficoll-Plaque and removal of PBMC layer, the remaining gradient above the granulocyte/erythrocyte pellet was aspirated and discarded. The granulocytes resting on the surface of the erythrocyte pellet were then extracted and transferred to a 50ml tube and re-suspended to 25ml with Hank's Balanced Salt Solution (HBSS). 25mls of dextran solution was added and the tube was inverted 10 times and set aside for 20-25minutes to allow erythrocytes to pellet. The granulocyte

rich upper layer was aspirated and transferred to a new 50ml tube and centrifuged at 500 x g for 10 minutes at 4°C. Supernatant was discarded and the pellet was re-suspended in 25mls of sterile water and mixed for 28 seconds to lyse residual erythrocytes. 25mls of 1.8% w/v NaCl was added to restore tonicity and solution was centrifuged at 500 x g for 5 minutes at 4°C. Supernatant was discarded and cells were re-suspended in 1ml HBSS and counted. Neutrophils were divided into aliquots of 1×10^7 cells/ml, RLT lysis buffer was added and cells were sheared. Cell lysates were then stored at -80°C.

2.10.4 Monocyte Isolation

In a subset of patients, monocytes were extracted from isolated PBMC (1×10^7 cells/ml) using an Easysep Monocyte Enrichment Kit without CD16 depletion (#19058 Stemcell Technologies, Grenoble, France), following manufacturers guidelines. Briefly, cells were re-suspended in 500µL of monocyte isolation buffer in a 5mL polystyrene tube. 25µl of Human Monocyte Enrichment Cocktail was added and solution was incubated at 4°C for 10 minutes. The EasySep D Magnetic Particles for Monocytes were vortexed for 30 seconds, 25µl was added and the solution was incubated for a further 5 minutes at 4°C. The cell suspension was brought up to a total volume of 2.5ml by adding recommended medium and cells were mixed gently. The tube was placed into Purple Easysep Magnet and set aside for 2.5 minutes at RT. In one continuous motion the magnet and tube were inverted, pouring off the desired fraction into a 15ml tube. Cells were centrifuged for 3 minutes at 300 x g. The pellet was re-suspended; cells were counted and stored in RLT lysis buffer at -80°C.

2.10.5 Flow Cytometry

Flow cytometry was performed to confirm the effectiveness of neutrophil and monocyte isolation techniques. All antibodies were sourced from Becton Dickson (NJ, USA). A sample of isolated neutrophils and monocytes was pelleted at $180 \times g$ for 3 minutes and the supernatant discarded. 1ml FACS buffer was added to each tube, vortexed for a few seconds, and centrifuged at $180 \times g$ for 3 minutes. The supernatant was discarded and 1ml FACS blocking buffer (50% FCS, 50% FACS buffer) was added to

each tube and incubated at room temperature for 5 minutes. 1ml FACS blocking buffer was added and tubes centrifuged at $180 \times g$ for 3 minutes. Supernatant was discarded and the required volume of antibody (detailed in Table 2.8 below) was added to appropriate tubes. Samples were vortexed to resuspend cells and incubated in the dark for 20 minutes at 4°C. 1ml FACS buffer was added to each tube, vortexed and centrifuged at $180 \times g$ for 3 minutes. Cells were resuspended in 300µl FACS buffer and levels of specific cell surface markers were measured using a CyAn ADP Analyzer (Beckman Coulter, CA, USA). A minimum of 20,000 events were collected per sample in the live cell gate. Data analysis was carried out using FlowJo Software, version 7.6.1 (FlowJo, OR, USA) by a trained user of the instrument, Dr Joanne Lysaght.

Table 2.8 Antibodies Used for FACS Analysis

Surface Marker	Detecting	Fluorochrome	Volume
CD14	Monocyte	FITC	5µl
CD66b	Neutrophil	FITC	3µl
CD16	Neutrophil	PeCy5	10µl

2.10.6 Preparation of Nuclear Extracts

In a subset of patients, nuclear extracts were isolated from 1×10^7 PBMC. Briefly, cells were washed in 1ml of ice-cold PBS. The pellet was re-suspended in 1ml of ice-cold hypotonic buffer and cells were allowed to swell on ice for 15 minutes. 50µl of 10% Igepal CA-630 was added to cell suspension and mixed gently. The homogenate was centrifuged at $12000 \times g$ for 30 seconds at 4°C. The nuclear pellet was re-suspended in 50µl of complete lysis buffer [supplied in commercial NFκβ kit (Active Motif, Belgium)] and incubated on ice for 30 minutes on a shaking platform. Nuclear extracts were centrifuged at $12000 \times g$ for 10 minutes at 4°C after which the supernatant was extracted and stored at -80°C for subsequent NF-κβ analysis.

2.11 RNA ISOLATION FROM IMMUNE CELLS USING QIAGEN RNEASY KIT

RNA was isolated from PBMC, neutrophils and monocytes using Qiagen RNeasy Mini Kit (Qiagen Inc., CA, USA). Frozen cell lysates were allowed to thaw and then 600µl of ethanol (70% v/v) was added, mixed immediately by pipetting and transferred in

volumes of 700µl to an RNeasy spin column placed in a 2ml collection tube. The spin column was centrifuged at $12,000 \times g$ for 15 seconds and the flow through was discarded. The spin column was washed by first applying 700µl buffer RW1 (supplied), then 500µl buffer RPE (supplied) with centrifugation at $12,000 \times g$ for 15 seconds at room temperature between each step. In a final wash step, 500µl of buffer RPE was added and samples were centrifuged for 2 minutes at $12,000 \times g$ in order to dry the membrane and ensure no ethanol was carried over. RNA was eluted in 30µl of RNase free water by centrifugation for 1 minute at $12,000 \times g$ at room temperature. RNA quality and yield was assessed by using a Nanodrop® ND-1000 spectrophotometer as described in section 2.5.2.

2.12 INFLAMMATORY GENE ARRAYS

To assess alterations in PBMC inflammatory gene expression, pathway focused Inflammatory Response & Autoimmunity PCR Arrays were used (#PAHS 3083, SABiosciences, MD, USA).

2.12.1 RT² First Strand cDNA Synthesis

cDNA was synthesized using the RT² First Strand Kit (#330401, SABiosciences, MD, USA). Briefly, for each sample 500ng of RNA was combined with 2µl of genomic DNA Elimination Mixture (supplied) and RNase free H₂O to a final volume of 10µl. Samples were incubated at 42°C for 5 min then chilled on ice for 1 minute. An “RT Cocktail” was prepared as follows per sample: 4µl BC3 (5X RT Buffer 3), 1µl P2 (Primer and External Control Mix), 2µl RE3 (RT Enzyme Mix 3) and 3µl H₂O. 10µl of RT Cocktail was added to each sample which were incubated at 42°C for 15 min. The reaction was immediately stopped by heating at 95°C for 5 minutes. 91µl of H₂O was added to each 20µl of cDNA and samples were stored at -20°C.

2.12.2 Quantitative Polymerase Chain Reaction (qPCR) for Inflammatory Arrays

The qPCR components were mixed as follows: 2100µl of 2x RT² SYBR Green Mastermix, 102µl cDNA synthesis reaction and 1998µl RNase-free water, leading to a total volume of 4200µl per sample. Each sample was loaded onto an individual 384 well plate

(10µl/well), which contained primers for 370 key genes involved in immune response during autoimmunity and inflammation and 14 housekeeping control genes. Plates were run on an ABI 7900HT FAST block real-time thermal cycler using the following PCR programme: 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, with the latter 2 temperature changes repeated for 40 cycles. Genes measured by this array are listed in Appendix I. Results were analysed using RT² Profiler PCR Array Data Analysis version 3.4 software (SABiosciences, MD, USA), based on the $2^{-\Delta\Delta CT}$ method [246]. Genes that had >1.5 fold increase/decrease and a p value <0.1 were considered to be significantly altered and selected for validation by quantitative PCR (qPCR).

2.13 QUANTITATIVE PCR (qPCR)

All reagents were purchased from ABI Biosystems (CA, USA). A master mix containing forward and reverse primer, probe and Taqman® Gene Expression Master Mix was added to 1µl cDNA template (described in section 2.5.3). A final volume of 20µl was pipetted into wells of a MicroAmp™ Optical 96 well reaction plate (ABI Biosystems, CA, USA) and qPCR was performed on the ABI Prism 7900 FAST block (ABI Biosystems, CA, USA) real-time thermal cycler using the same cycle as above (section 2.12.2). Samples were assayed in triplicate. The threshold cycle (Ct) for each well was calculated using the instrument software and data analysis was carried out the $2^{-\Delta\Delta CT}$ method with raw data normalised by the 18S housekeeping genes included on the plate. As this analytical method examines samples in a relative manner, the preoperative timepoint from one of the non-obese patients was chosen as an arbitrary calibrator sample for use across all plates.

2.14 PBMC GENE EXPRESSION

PBMC gene expression of novel inflammatory genes, including significantly altered genes identified from the inflammatory array, was assessed by qPCR. cDNA was synthesised as described in section 2.5.3 using 500ng of RNA from isolated PBMC and qPCR was performed as described in section 2.13. Primers used are listed in Table 2.9.

2.15 NEUTROPHIL ACTIVATION

The expression of the neutrophil activation markers myeloperoxidase, L-selectin and integrin alpha-M was assessed in isolated neutrophils. cDNA was synthesised as described in section 2.5.3 using 500ng of RNA from isolated neutrophils and qPCR was performed as described in section 2.13. Primers used are listed in Table 2.9.

2.16 MONOCYTE ACTIVATION

The expression of the monocyte activation markers arginase-1 and IL-10 (M2 phenotype) and IL-12 and TNF- α (M1 phenotype) was assessed in isolated macrophages. cDNA was synthesised as described in section 2.5.3 using 250ng of RNA from isolated macrophages and qPCR was performed as described in section 2.13. Primers used are listed in Table 2.9.

Table 2.9 qPCR Primer Probes Used to Detect Gene Expression in Immune Cells

Immune cell	Gene	Inventory number
PBMC		
	TNFSF-14	Hs 00542477-m1
	LT β	Hs00242739-m1
	LT β R	Hs00158922-m1
	CCL3L1	Hs03407473-uH
	INHBA	Hs01081598-m1
	IFNK	Hs00737883-m1
	MGLL	Hs00200752-m1
	CCL4	Hs01031494-m1
	IL-10	Hs00961622-m1
Neutrophil		
	SELL	Hs00174151-m1
	ITGAM	Hs00355885-m1
	MPO	Hs00924296-m1
Monocyte		
	IL-10	Hs00961622-m1
	ARG1	Hs00968979-m1
	IL12a	Hs01073447-m1
	TNF	Hs01113624-g1

2.15 MEASUREMENT OF NUCLEAR FACTOR- $\kappa\beta$ (NF- $\kappa\beta$) p65 SUBUNIT

NF- $\kappa\beta$ p65 was assessed in nuclear extracts taken from whole blood PBMC isolated preoperatively and on days 1, 3, 7 and 14 postoperatively from 4 non-obese and 3 obese oesophagectomy patients. A TransAM NF- $\kappa\beta$ p65 transcription factor assay kit (Active Motif, Belgium) was used, as per manufacturer's guidelines. Briefly, 30 μ l of complete binding buffer (supplied) was added to each well. 15 μ g of each sample (as determined by Bradford assay (Sigma-Aldrich, MO, USA)) diluted in complete lysis buffer was added to sample wells in duplicate. 2.5 μ g of Jurkat nuclear extract diluted in complete lysis buffer served as a positive control. The plate was then sealed incubated for 1 hour at room temperature with mild agitation. Wells were then washed 3 times with 200 μ l of washing buffer. 100 μ L of diluted NF- $\kappa\beta$ p65 antibody was added to wells and the plate was incubated for 1 hour at room temperature. Wells were washed 3 times, 100 μ l of diluted horseradish peroxidase (HRP) antibody was added to each well and the plate was incubated for 1 hour at RT. Wells were then washed 4 times and 100 μ l of developing solution (supplied) was added to each well and the plate was covered to protect from light. When sufficient colour change had occurred, the reaction was stopped by the addition of 100 μ l of stop solution (1M H₂SO₄) to each well. Absorbance was read at 450nm using a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, CA, USA).

2.16 ELISA

Enzyme-linked immunosorbent assay (ELISA) DuoSet assays (R&D systems, Inc., MN, USA) were used to determine the following cytokine concentrations in patient serum: IGF-1, IL-6, IL-8, IL-10, IL-12p70, TNF- α , transforming growth factor- β (TGF- β) and IFN- γ . NUNC High binding ELISA plates (Cruinn Diagnostics, Dublin, Ireland) were coated with 50 μ l capture antibody overnight at room temperature, and washed three times in wash buffer. 50 μ l of sample was applied to each well in triplicate with the exception of blanks which contained only reagent diluent. A serial dilution was made from the standard supplied and 50 μ l of each concentration were applied to the plate in triplicate. Samples were incubated for 2 hours at room temperature and the plate was washed three times in wash buffer. 50 μ l of detection antibody was added and the

plate incubated at room temperature for two hours followed by five washes in wash buffer. A 50µl volume of streptavidin-HRP (1:200 dilution in reagent diluent) was added to each well and incubated for 20 minutes at room temperature. 100µl of 3,3',5,5'-Tetramethylbenzidine (TMB) or ELISA developing solution was added to each well and covered with tinfoil until the colour developed (5-20 minutes). 50µl of stop solution was added to each well and the plate was read immediately at 450nm or 492 nm using a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, CA, USA). Protein concentrations were determined by interpolating from the standard curve of known concentrations. Kit specific antibodies and reagents are listed in Table below 2.10.

For the TGF-β ELISA samples were activated from latent TGF-β1 to immunoreactive TGF-β1 prior to assaying as follows. To 40µl of serum, 20µl of 1N HCl was added and samples were mixed well and incubated at room temperature for 10 minutes. 20µl of 1.2N NaOH/0.5M HEPES was added to each sample and mixed well. Samples were diluted 1:20 in reagent diluent and assayed.

ELISA MAX Deluxe Sets (BioLegend, CA, USA) were used to measure serum levels of IL-4 and IL-17A. Maxisorp ELISA plates (supplied) were coated with 100µl of capture antibody diluted 1:200 in 1x coating buffer (supplied). Plates were incubated overnight at 4°C. Plates were washed 4 times with wash buffer then 200µl of 1x assay diluent A (supplied) was added to each well and plates were incubated at room temperature for 1 hour on a plate shaker at 200 rpm. Plates were washed 4 times and 100µl of sample was added in triplicate to wells with 1x assay diluent A serving as a blank control. A seven point standard curve was prepared by serially diluting the standard supplied 1:2 with 1x assay diluent A and 100µl of each concentration was applied to the plate in triplicate. Plates were incubated at room temperature for 2 hours at 200 rpm. Plates were washed 4 times and 100µl of detection antibody, diluted 1:200 in 1x assay diluent A, was added to each well and plates were incubated for 1 hour at 200 rpm at room temperature. Plates were washed and 100µl of avidin-HRP solution, diluted 1:1000 in 1x assay diluent A, was added to each well. Plates were incubated at room temperature for 30 minutes at 200 rpm. Plates were washed 5 times, allowing wash buffer to remain in wells for 1 min with each wash. The supplied TMB substrate

solution was prepared by mixing equal parts of substrate solution A with substrate solution B. 100µl was added to each well and plates were incubated in the dark for 15-30 minutes until sufficient colour change occurred. The reaction was stopped by the addition of 100µl of stop solution to each well and plates were read at 450nm on a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, CA, USA). Protein concentrations were determined by interpolating from the standard curve of known concentrations. Kit specific antibodies and reagents are listed in Table below 2.10.

Table 2.10 Antibody Concentrations and Reagents Used in ELISA

ELISA	Capture Antibody	Top Standard	Detection Antibody	Reagent Diluent	Blocking Buffer	Developed With	Wavelength
DuoSet ELISA (R&D Systems)							
IGF-1	4µg/ml	2ng/ml	80ng/ml	0.05% Tween PBS, 0.05% N _a N ₃	5% Tween in PBS, 0.05% N _a N ₃	TMB (Sigma)	450nm
IL-6	2µg/ml	600pg/ml	200ng/ml	1% BSA in PBS	1% BSA in PBS	Developing solution	492nm
IL-8	4µg/ml	2ng/ml	20ng/ml	0.1% BSA, 0.05% Tween in TBS	1% BSA in PBS, 0.05% N _a N ₃	Developing solution	492nm
IL-10	2µg/ml	2ng/ml	150ng/ml	1% BSA in PBS	1% BSA in PBS	Developing solution	492nm
IL-12p70	4µg/ml	2ng/ml	100ng/ml	1% BSA in PBS	1% BSA in PBS	Developing solution	492nm
TNF-α	4ng/ml	1ng/ml	250ng/ml	1% BSA in PBS	1% BSA in PBS	Developing solution	492nm
TGF-β	2µg/ml	2ng/ml	300ng/ml	Reagent Diluent Concentrate, R&D Systems #DY997	5% Tween in PBS, 0.05% N _a N ₃	Developing solution	492nm
IFN-γ	4µg/ml	1ng/ml	50ng/ml	0.1% BSA, 0.05% Tween in TBS	1% BSA in PBS, 0.05% N _a N ₃	Developing solution	492nm
ELISA MAX Deluxe (Biolegend)							
IL-17A	1:200	250pg/ml	1:200	1x Assay diluent A	1x Assay diluent A	TMB substrate solution (supplied)	450nm
IL-4	1:200	250pg/ml	1:200	1x Assay diluent A	1x Assay diluent A	TMB substrate solution (supplied)	450nm

2.17 MEASUREMENT OF EICOSANOID METABOLITES

2.17.1 6-keto-Prostaglandin F_{1α} and Leukotriene B₄

The circulating levels of prostacyclin I₂ (PGI₂) were measured indirectly due to its short half-life by measuring 6-keto-Prostaglandin F_{1α} (PGF_{1α}), a stable molecule derived from the hydration of PGI₂, using a PGF_{1α} EIA kit. LTB₄ was measured directly using an LTB₄ EIA kit. All kits were sourced from Enzo Life Sciences, Exeter, UK and kit specific variations to the protocol are detailed below in Table 2.11. The supplied standards were serially diluting with assay buffer (supplied) to generate standard curves. 100μl of assay buffer was added into the non-specific binding (NSB) and the maximum binding (Bo) wells. 100μl of standards and samples (diluted if necessary with assay buffer) was added in duplicate to appropriate wells and 50μl of assay buffer was added to the NSB wells. 50μl of blue conjugate (supplied) was added into each well, except the Total Activity (TA) and Blank wells. 50μl of yellow antibody (supplied) was added into each well, except the Blank, TA and NSB wells. The plate was incubated at room temperature on a plate shaker for 2 hours at ~500 rpm. The content of the wells was discarded and wells were washed with 400μl of wash solution a total of three times. 5μl of the light blue conjugate (supplied) was added to the TA wells and 200μl of pNpp substrate solution (supplied) was added to every well. The plate was incubated as per kit specific instructions. 50μl of Stop Solution (supplied) was added to every well and the plate was read at 405nm on a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, CA, USA). Sample concentrations were determined by interpolating from the standard curve of known concentrations.

Table 2.11 EIA to detect 6-keto-Prostaglandin F_{1α}, Leukotriene B₄ and Thromboxane B₂

Kit	Sample (dilution)	Top Standard	No. of standards	Incubation with pNpp substrate solution
6-keto-Prostaglandin F _{1α}	Serum (1:100)	50ng/ml	7	Room temperature, 45 min
Leukotriene B ₄	Plasma (neat)	3ng/ml	5	37°C, 2 hours

2.17.2 Prostaglandin E₂

A PGE₂ EIA kit (Cayman Chemical, MI, USA) was used to detect PGE₂ levels in plasma. 100µl of EIA buffer (supplied) was added to the NSB wells and 50µl of EIA buffer was added to the Bo wells. An eight point standard curve was prepared by serially diluting the standard supplied 1:2 with EIA buffer, giving a top standard of 1ng/ml. 50µl of standards and samples (diluted 1:100 with EIA buffer) was added in duplicate to appropriate wells. 50µl of Prostaglandin E₂ Monoclonal Antibody (supplied) was added to each well except the TA, NSB and blank wells. 50µl of Prostaglandin E₂ AChE Tracer (supplied) was added to each well except the TA and blank wells. The plate was covered and incubated for 18 hours at 4°C. Ellman's reagent (supplied) was reconstituted in molecular grade H₂O immediately before use. The content of the wells was discarded and wells were washed with 400µl of wash solution a total of five times. 200µl of Ellman's Reagent was added to each well and 5µl of tracer was added to the TA wells. The plate was covered and incubated in the dark at room temperature on an orbital shaker for 60 minutes. The plate was read at 405nm on a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, CA, USA). Sample concentrations were determined by interpolating from the standard curve of known concentrations.

2.18 STATISTICAL ANALYSIS

Statistical analysis was computed using PASW Statistics 18 for Windows (© IBM Corporation 2010, NY 10589) and GraphPad Prism 5 software (GraphPad, CA, USA). Unless otherwise specified, data are expressed as mean ± standard error of the mean (SEM). SEM is calculated as the standard deviation (SD) of the original sample divided by the square root of the sample size.

Unpaired *t*-tests and the Mann-Whitney U test were used to examine statistical significance between unpaired groups of parametric and non-parametric data respectively. Categorical variables were analysed by Chi-square and Fisher's exact tests. Data analysis was performed by one way analysis of variance (ANOVA), where the number of groups in the experiment was three or more. A Tukey multiple

comparisons post hoc test was necessary following ANOVA in order to determine which groups were significantly different to each other.

Survival statistics were calculated using the Kaplan-Meier method and the log rank test was used to assess differences in survival between patients with high and low IGF-1R tumour expression. Survival time was measured from the date of first treatment to the date of death or last follow-up.

Receiver operating characteristics (ROC) analyses were used to determine gender-specific discriminate cut-off values for total fat area (TFA), visceral fat area (VFA) and subcutaneous fat area (SFA) that were associated with the metabolic syndrome. In addition, ROC analysis was used to establish cut-off values for TFA, VFA and SFA above which there was an increased prevalence of the individual factors of the metabolic syndrome, i.e., high triglycerides, low HDL-C, hypertension or hyperglycemia. In the case of these separate ROC analyses, when dichotomizing the cohort into those with high or low triglycerides, HDL-C, blood pressure or fasting glucose, only those subjects with unadulterated clinical parameters could be included. Hence if a subject was taking medication to control triglyceride, HDL-C, glucose or blood pressure levels they were excluded from that specific ROC analysis to avoid data skewing.

The sensitivity and specificity of each anthropometric cut-off was determined using the Youden Index. Linear regression was used to determine the waist circumference values associated with the new anthropometric cut-off values.

For all statistical analysis, a probability (p) of ≤ 0.05 was considered to represent a significant difference between groups.

Chapter 3 Establishing CT-Defined Visceral Fat Area Thresholds for Use in Obesity Related Cancer Research

3.1 INTRODUCTION

Obesity is a known risk factor for chronic diseases including cardiovascular disease and type 2 diabetes mellitus ^[247]. In recent years, there has been an increased acknowledgement of the impact of obesity on the incidence and mortality of a wide range of cancer types, including adenocarcinoma of the oesophagus, pancreas, colorectum, breast, endometrium and kidney ^[248]. What drives the association between obesity and cancer is unclear, and emerging research has targeted the study of different fat compartments, with visceral fat considered to be more pro-inflammatory and pro-tumourigenic than subcutaneous fat depots ^[112].

Visceral adipose tissue has multiple endocrine, metabolic and immunological functions ^[31] and is central to the pathogenesis of the metabolic syndrome (MetSyn), a pro-inflammatory, pro-coagulant state associated with insulin resistance ^[249]. The multiple risk factors that commonly appear together as the MetSyn include abdominal obesity, atherogenic dyslipidemia (raised triglycerides and reduced high-density lipoprotein cholesterol), elevated fasting plasma glucose and hypertension ^[32]. The ratio of visceral fat area (VFA) to subcutaneous fat area (SFA) as a relative index of intra-abdominal fat accumulation has been shown to be strongly related with disorders of glucose and lipid metabolism in obese subjects ^[33]. These metabolic parameters have been shown to be significantly dysregulated in individuals with a greater proportion of visceral fat (VFA/SFA ratio of ≥ 0.4) compared to those with a greater proportion of subcutaneous fat (VFA/SFA ratio of < 0.4) ^[33].

As increasing evidence points to the specific role of visceral obesity in disease, it is important to be able to identify individuals with excessive amounts of visceral adipose tissue, as opposed to increased overall mass. The widely used BMI criteria for overweight and obesity ^[250] is not suitable for determining visceral obesity as within each category of BMI there can be substantial individual variation in total and visceral adiposity ^[239]. Commonly used BMI cut-off values to diagnose obesity have high specificity, but low sensitivity to identify adiposity, as they fail to identify those with excess body fat ^[251]. Furthermore, VFA is superior to both waist circumference measurements and body mass index (BMI) for discriminating individuals with two or

more features of dysmetabolism ^[252]. Hence, when researching the metabolic implications of excess adiposity, reliable methods to discriminate between visceral and subcutaneous adipose tissue depots are essential. Computed tomography (CT) imaging and magnetic resonance imaging (MRI) are the gold standard for the quantification of total, visceral and subcutaneous adipose tissue ^[253].

The current hypothesis in obesity related cancer research is that visceral obesity increases cancer risk via its adverse impact on inflammation and metabolism ^[254]. Numerous studies have employed these gold standard techniques to quantify visceral fat area and have confirmed that an increased volume of visceral adipose tissue is deleterious to metabolic function, promotes inflammation and promotes tumourigenesis ^[33, 126, 255-258]. Furthermore, recent studies have suggested that CT-determined visceral fat area may influence response to therapy in obesity-associated malignancies ^[259, 260]. However what is not known is the acceptable threshold level of visceral adipose tissue, above which it begins to detrimentally affect metabolic and inflammatory processes, ultimately increasing cancer risk. There is a paucity of studies attempting to identify pathologically relevant and gender specific cut-off values for VFA in Caucasian populations. Studies carried out in the 1990's determined that in both men and women, a VFA above 100 cm² was associated with moderate disturbances in the risk profile, whereas a VFA greater than 130 cm² was associated with a further deterioration of metabolic variables predictive of type 2 diabetes mellitus and cardiovascular disease ^[261, 262].

However neither of these studies took into consideration the gender difference in adipose tissue distribution ^[263, 264] and dysmetabolism ^[265]. Moreover, studies that have determined separate optimal thresholds for VFA in males and females have been carried out in Asian populations who, due to ethnic differences in fat distribution and metabolism, are not representative of Caucasian populations ^[266-268]. Whilst established cut-offs exist for BMI and waist circumference, no such cut-offs exist for VFA. In the absence of a cut-off, many researchers dichotomise their cohort into those above and below the median of VFA which in turn results in a range of different quantities of visceral fat area being reported in the literature as "high" ^[259, 269, 270].

The absence of appropriate VFA thresholds is hampering the field of cancer research as it hinders our ability to make useful associations between visceral obesity and markers of tumourigenesis. CT scanning is generally limited to individuals undergoing medical investigation; nevertheless an appropriate threshold should be generated for use in obesity research when CT scanning is available. In the absence of healthy individuals, gastrointestinal cancer patients represent an appropriate cohort for the study of VFA thresholds. One of the strongest obesity-cancer links exists between gastrointestinal cancer and visceral obesity and all patients undergo a diagnostic CT scan. Thus, in this study we focused on oesophageal and colorectal adenocarcinoma cohorts and used the collective factors of the MetSyn as a pathological indicator(s) for visceral obesity.

In this study it was hypothesised that when associating total, visceral and subcutaneous fat area with the metabolic syndrome, different CT derived cut-off values would be determined for Caucasian male and female gastrointestinal cancer patients. For the first time, this study generated pathologically relevant thresholds for VFA that associate with MetSyn in Caucasian males and females. This new anthropometric measure is of paramount importance for determining the accurate pathological obesity status of cancer patients, a cornerstone in study of obesity-associated cancer research.

3.1.1 Specific Aims

The aim of this study was to identify pathologically relevant thresholds for the classification of visceral obesity in Caucasian males and females for use in gastrointestinal cancer research. Utilising CT quantified abdominal fat area measures the following parameters will be assessed:

The association between specific abdominal fat depots and separate markers of dysmetabolism in males and females, determined by receiver operator characteristics analysis.

The association between specific abdominal fat depots and the metabolic syndrome in males and females, determined by receiver operator characteristics analysis.

The correlation between identified upper thresholds of abdominal obesity and waist circumference.

3.2 RESULTS

3.2.1 Subject Characteristics

An Adipose Tissue Biobank was established in the Department of Surgery in 2007, as described in section 2.7, and it was from this database that subjects for this study were selected. The study cohort consisted of 236 consecutively recruited subjects, 170 male and 66 females, who underwent a gastrointestinal resection for malignancy with curative intent between July 2007 and January 2011 (Table 3.1). Excluded from the study were individuals who were pregnant, HIV or Hepatitis C positive or had diagnosed metastatic disease. The cohort ranged in age from 29-94 years, with a mean age of 65 years. Anthropometry and clinical measurements were performed as described in section 2.7.1 and 2.7.2 and MetSyn was diagnosed as per International Diabetes Federation criteria ^[271] (section 2.7.4).

Selected subject characteristics, separated by gender and the MetSyn status, are summarised in Table 3.1 below. The male and female cohorts were matched in terms of age and their MetSyn status. Males had significantly higher BMI ($p=0.001$) and WC ($p<0.0001$) than females. However both groups demonstrated similar overall prevalence of obesity as measured by BMI ($\geq 30\text{kg/m}^2$) or WC ($\geq 94\text{cm}$ in males and $\geq 80\text{cm}$ in females). As expected, a significant difference was observed in abdominal fat distribution between males and females, with males having higher amounts of visceral fat ($p<0.0001$) and lower amounts of subcutaneous fat ($p=0.005$) compared to females. Within the male and female cohorts, those with the MetSyn had significantly higher TFA, VFA and SFA ($p<0.001$) than those without the MetSyn. In terms of tumour characteristics, within the male cohort there was 170 oesophageal adenocarcinoma and 50 colorectal adenocarcinoma cases and within the female cohort there was 22 oesophageal adenocarcinoma and 44 colorectal adenocarcinoma cases. There was significantly more T₁ and significantly less T₃ tumours in the males with the MetSyn. There was no significant difference in tumour stage or nodal status between females with or without the MetSyn.

Table 3.1 Patient demographics according to gender and metabolic syndrome status

	Males n = 170 (120 OAD, 50 CRC)				Females n = 66 (22 OAD, 44 CRC)			
	Range	No MetSyn (n=95)	MetSyn (n=75)	p	Range	No MetSyn (n=41)	MetSyn (n=25)	p
Age (years)	29.0-88.0	61.8±11.9	67.9±9.1	0.005	42.0-94.0	67.2±12.4	68.3±8.6	0.712
BMI (kg/m ²)	18.3-39.0	25.5±3.9	28.9±3.3	<0.0001	15.4-36.1	22.8±4.1	28.6±3.2	<0.0001
WC (cm)	66.0-130.0	92.5±11.3	104.7±7.7	<0.0001	61.0-116.0	79.9±10.3	95.3±9.3	<0.0001
TFA (cm ²)	22.7-771.3	291.0±149.8	416.7±102.7	<0.0001	7.2-725.3	232.5±151.4	417.0±97.3	<0.0001
VFA (cm ²)	13.1-397.1	140.7±79.7	228.6±69.7	<0.0001	6.0-261.0	67.7±54.3	146.8±53.0	<0.0001
SFA (cm ²)	3.9-479.7	150.3-85.6	188.1±59.1	0.001	1.2-496.4	164.9±105.3	271.5±88.3	<0.0001
HDL-C (mmol/L)	0.25-2.82	1.28±0.43	1.01±0.34	<0.0001	0.66-2.38	1.58±0.40	1.26±0.40	0.003
Triglycerides (mmol/L)	0.35-6.84	1.34±0.09	1.83±0.12	0.001	0.44-4.90	1.18±0.53	1.55±0.90	0.046
Glucose (mmol/L)	3.2-15.9	5.16±0.64	6.24±0.24	<0.0001	3.5-9.1	4.90±0.48	5.77±1.21	0.001
Chemoradiotherapy, n (%)		46 (48.4%)	30 (40%)	0.273		9 (22%)	9 (36%)	0.214
Tumour Stage, n (%) [#]		9 (9.5%)	7 (9.3%)	0.912		4 (9.8%)	1 (4%)	0.424
T ₀ /T _{is}								
T ₁		13 (13.7%)	22 (29.3%)	0.017		8 (19.5%)	3 (12%)	0.484
T ₂		14 (14.7%)	16 (21.3%)	0.313		5 (12.2%)	4 (16%)	0.593
T ₃		51 (53.7%)	24 (32%)	0.002		20 (48.8%)	10 (40%)	0.618
T ₄		2 (2%)	4 (5.3%)	0.278		3 (7.3%)	5 (20%)	0.102
Nodal Status, n (%) [#]		51 (53.7%)	39 (52%)	0.621		18 (43.9%)	12 (48%)	0.583
N ₀								
N ₁		32 (33.7%)	30 (40%)	0.503		15 (36.6%)	7 (28%)	0.571
N ₂		4 (4.2%)	2 (2.6.7%)	0.556		5 (12.2%)	4 (16%)	0.593
N ₃		2 (2.1%)	2 (2.6.7%)	0.841		2 (4.9%)	0 (0%)	0.276

Continuous variables are presented as means ± SD; categorical variables are presented as numbers and percentages. Data analysed using student t-tests and chi-squared tests. [#] Tumour and nodal information was unavailable on 8 males and 3 females. OAD, oesophageal adenocarcinoma; CRC, colorectal adenocarcinoma; MetSyn, The Metabolic Syndrome; BMI, body mass index; WC, waist circumference; TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area; HDL-C, high density lipoprotein-cholesterol

3.2.2 Determination of Abdominal Adiposity Thresholds Using Individual Markers of Dysmetabolism

Receiver operating characteristics (ROC) analysis was used to establish cut-off values for total fat area (TFA), visceral fat area (VFA) and subcutaneous fat area (SFA) above which there was an increased prevalence of the individual factors of the metabolic syndrome, i.e., high triglycerides, reduced HDL-C, hypertension or elevated fasting glucose. Subjects that were taking medications to control blood pressure, cholesterol or glucose levels were excluded from respective analyses, to avoid data skewing. Results are shown in Table 3.2 and the cut-off values shown represent a combination of the greatest sensitivity and specificity, calculated by the Youden Index.

With the exception of hypertension in females, all markers of dysmetabolism demonstrated a stronger association with VFA than SFA, as indicated by the area under the curve values. The association between visceral adiposity and dysmetabolism was more evident in males compared to females as the VFA cut-offs generated for identifying risk of reduced HDL-C, elevated triglycerides, hypertension and elevated fasting glucose proved to be significant predictors. The strongest association was observed between a VFA cut-off of 212.2cm^2 and hypertension [AUC, 0.789 (0.661-0.917), $p=0.001$]. Using an optimal threshold for VFA of 212.2cm^2 in males identifies hypertensive subjects with a sensitivity of 70.6% and a specificity of 83.3%. In females, the VFA cut-offs generated for identifying risk of elevated triglycerides and elevated fasting glucose proved to be significant predictors. The strongest association was observed between a VFA cut-off of 65.1cm^2 and elevated triglycerides [AUC, 0.738 (0.593-0.882), $p\ 0.023$]. Using an optimal threshold for VFA of 65.1cm^2 in females identifies subjects with elevated triglycerides with a sensitivity of 100% and a specificity of 50%.

Table 3.2 Abdominal fat thresholds associated with increased risk of dysmetabolism

	Males					Females				
	Cut-off Value	AUC (95% CI)	p value	Sensitivity (%)	Specificity (%)	Cut-off Value ^a	AUC (95% CI)	p value	Sensitivity (%)	Specificity (%)
Lowered HDL-C										
(n=101 males, 47 females)										
TFA (cm ²)	380.4	0.624 (0.509-0.738)	0.036*	53.7	75.0	446.5	0.586 (0.410-0.762)	0.330	35.3	86.7
VFA (cm ²)	201.0	0.622 (0.509-0.734)	0.039*	53.7	68.3	124.0	0.642 (0.476-0.809)	0.108	58.8	76.7
SFA (cm ²)	210.1	0.591 (0.474-0.708)	0.121	39.0	85.0	71.4	0.569 (0.397-0.740)	0.438	100	23.3
Elevated Triglycerides										
(n=101 males, 47 females)										
TFA (cm ²)	328.3	0.597 (0.478-0.716)	0.145	76.0	53.9	186.8	0.647 (0.467-0.828)	0.158	100	33.3
VFA (cm ²)	180.4	0.632 (0.509-0.754)	0.049*	72.0	61.8	65.1	0.738 (0.593-0.882)	0.023*	100	50.0
SFA (cm ²)	125.8	0.554 (0.432-0.675)	0.422	76.0	40.8	292.7	0.594 (0.388-0.801)	0.365	40.0	88.9
Hypertension										
(n=83 males, 36 females)										
TFA (cm ²)	365.2	0.751 (0.622-0.880)	0.001**	76.5	74.2	253.3	0.585 (0.386-0.784)	0.401	69.2	56.5
VFA (cm ²)	212.2	0.789 (0.661-0.917)	0.001**	70.6	83.3	69.9	0.560 (0.357-0.764)	0.553	61.5	60.9
SFA (cm ²)	194.4	0.709 (0.581-0.838)	0.008**	52.9	81.8	267.0	0.579 (0.375-0.782)	0.439	46.2	82.6
Raised Fasting Glucose										
(n=137 males, 62 females)										
TFA (cm ²)	359.8	0.616 (0.521-0.711)	0.025*	58.3	65.2	305.7	0.648 (0.483-0.814)	0.113	75.0	58.0
VFA (cm ²)	194.6	0.662 (0.569-0.755)	0.002**	62.5	68.5	111.5	0.688 (0.523-0.854)	0.044*	75.0	66.0
SFA (cm ²)	140.0	0.578 (0.480-0.676)	0.134	70.8	47.2	101.5	0.577 (0.403-0.750)	0.412	91.7	28.0

Data analysed using receiver operating characteristics analysis, cut-off value obtained using Youden Index. AUC, area under the curve; CI, confidence interval; HDL-C, high-density lipoprotein cholesterol; TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area. * $p<0.05$; ** $p<0.01$

3.2.3 Determination of Abdominal Adiposity Thresholds Using the MetSyn

In addition to individual markers of dysmetabolism, the collective IDF criterion for the MetSyn was also employed to detect threshold levels of abdominal adiposity. All subjects were included in this analysis and results are shown in Table 3.3 and Figure 3.1. In both males and females, abdominal fat area proved to be robust indicator of altered metabolism, with the cut-offs generated for TFA, VFA and SFA proving to be significantly associated with the presence of the MetSyn. In both males and females, VFA demonstrated a strong association with the MetSyn, as indicated by the AUC values. A VFA cut-off of 163.8cm² identified males with the MetSyn with 83.6% sensitivity and 62.5% specificity and a VFA cut-off of 80.1cm² identified females with the MetSyn with 96% sensitivity and 73.2% specificity. There was a marked gender difference in anthropometric cut-offs generated with males having a higher optimal threshold for VFA but a lower optimal threshold for SFA compared to females.

Table 3.3 Abdominal fat thresholds associated with increased risk of the MetSyn in males and females

	Cut-off Value	AUC (95% CI)	<i>p</i> value	Sensitivity (%)	Specificity (%)
Men (n=170)					
TFA (cm ²)	364.2	0.758 (0.686-0.830)	<0.0001	71.2	74.0
VFA (cm ²)	163.8	0.788 (0.721-0.856)	<0.0001	83.6	62.5
SFA (cm ²)	132.3	0.693 (0.614-0.773)	<0.0001	86.3	50.0
Women (n=66)					
TFA (cm ²)	318.8	0.885 (0.807-0.964)	<0.0001	88.0	78.0
VFA (cm ²)	80.1	0.880 (0.802-0.959)	<0.0001	96.0	73.2
SFA (cm ²)	261.8	0.833 (0.732-0.933)	<0.0001	68.0	85.4

Data analysed using receiver operating characteristics analyses, cut-off value obtained using Youden Index. AUC, area under the curve; CI, confidence interval; TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area

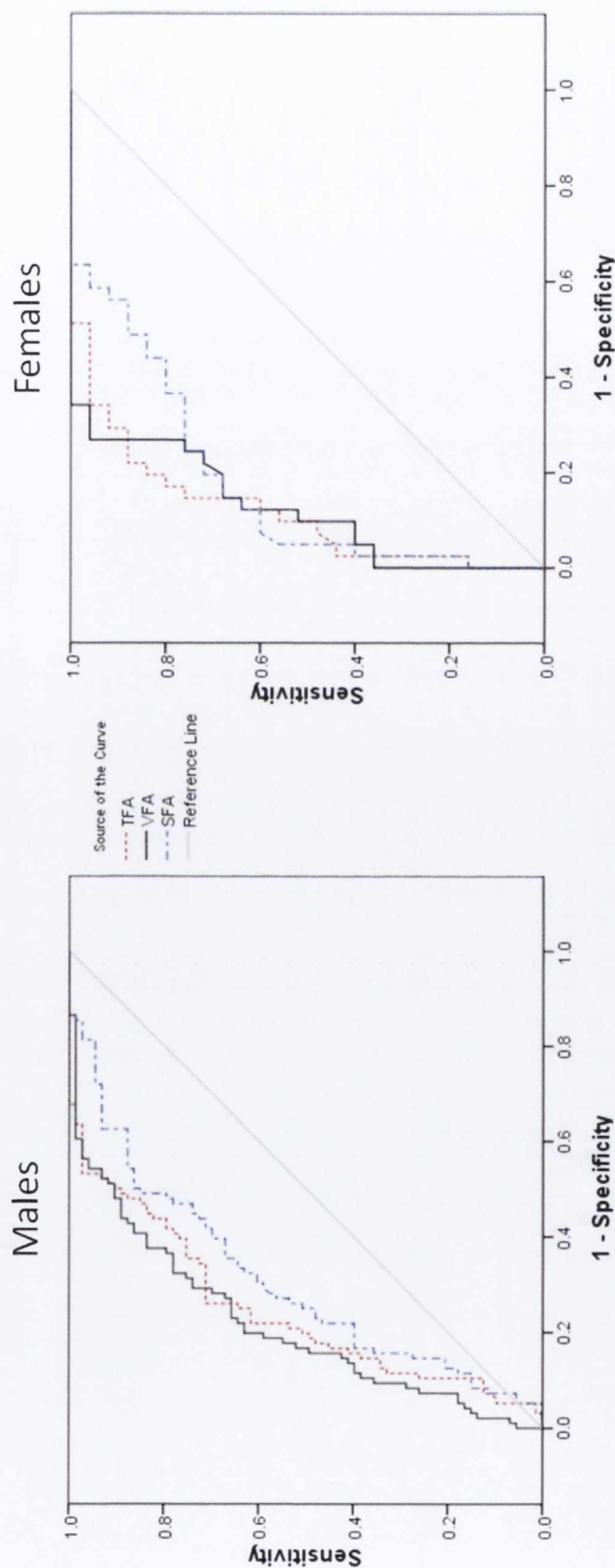


Figure 3.1 Abdominal fat areas associated with the presence of the MetSyn in males and females

ROC analysis and the Youden Index were used to determine cut-off values for abdominal fat associated with increased presence of the MetSyn. In both males ($n=170$) and females ($n=66$), VFA demonstrated a strong association with the MetSyn. A VFA cut-off of $\geq 163.8\text{cm}^2$ identified males with the MetSyn with 83.6% sensitivity and 62.5% specificity and a VFA cut-off of $\geq 80.1\text{cm}^2$ identified females with the MetSyn with 96% sensitivity and 73.2% specificity.

TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area; ROC, receiver operating characteristics; MetSyn, The Metabolic Syndrome.

3.2.3.1 Cancer-specific variations in abdominal thresholds associated with the MetSyn

Within the larger male cohort, the ROC analysis was repeated for the oesophageal and colorectal adenocarcinoma patients separately to assess cancer specific variations in the association between VFA and dysmetabolism. Minimal variation in optimal abdominal fat thresholds was observed between to the two cancer cohorts, as shown in Table 3.4. Males with oesophageal adenocarcinoma had a slightly lower threshold value for VFA of 156.8cm² [AUC, 0.746 (0.659-0.833), *p* <0.0001] compared to males with colorectal adenocarcinoma who had an optimal VFA threshold of 164.0cm² [AUC, 0.873 (0.774-0.971), *p* <0.0001]. In both cancer types, VFA remained strongly associated with the presence of the MetSyn.

Table 3.4 Abdominal fat thresholds associated with increased risk of the MetSyn in males with oesophageal and colorectal adenocarcinoma

	Cut-off Value	AUC (95% CI)	<i>p</i> value	Sensitivity (%)	Specificity (%)
OAD (n=120)					
TFA (cm ²)	262.7	0.700 (0.607-0.793)	<0.0001	98.0	42.0
VFA (cm ²)	156.8	0.746 (0.659-0.833)	<0.0001	84.0	55.1
SFA (cm ²)	132.3	0.653 (0.556-0.751)	0.004	86.0	84.9
CRC (n=50)					
TFA (cm ²)	312.5	0.894 (0.800-0.988)	<0.0001	95.7	74.1
VFA (cm ²)	164.0	0.873 (0.774-0.971)	<0.0001	91.3	77.8
SFA (cm ²)	137.0	0.795 (0.671-0.920)	<0.0001	87.0	66.7

Data analysed using receiver operating characteristics analyses, cut-off value obtained using Youden Index. AUC, area under the curve; CI, confidence interval; OAD, oesophageal adenocarcinoma; CRC, colorectal adenocarcinoma; TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area

3.2.3.2 Determination of abdominal adiposity thresholds using the Harmonized criteria for the MetSyn

As disease related weight loss is common in gastrointestinal malignancy, the ROC analyses of abdominal fat and the MetSyn were repeated using the Harmonized Metabolic Syndrome Criteria which does not require abdominal obesity as a definitive, but rather the presence of three of the five criteria used in the IDF definition ^[272]. All patients classified as MetSyn positive as per the IDF guidelines met the Harmonized criteria for the MetSyn. Four of the males, but none of the females, that were classified as MetSyn negative by the IDF criteria became classified as MetSyn positive as per the Harmonized criteria. The results are illustrated in Table 3.5. Again, abdominal fat area proved to be robust indicator of altered metabolism, with VFA demonstrating the strongest association with the Harmonized MetSyn. Compared with using the IDF criteria, similar pathological thresholds for abdominal fat were obtained in the males. In the males, a VFA of 177.9cm² identified males with the Harmonized MetSyn with 76% sensitivity and 67.7% specificity [AUC, 0.775 (0.704-0.836), *p* <0.0001]. In females, as there was no difference in the cohorts when using either set of criteria for MetSyn, no difference was observed in the ROC analyses. A VFA cut-off of 80.1cm² identified females with the MetSyn with 96% sensitivity and 73.2% specificity.

Table 3.5 Abdominal fat thresholds associated with increased risk of the MetSyn in males and females (Harmonized Metabolic Syndrome Criteria)

	Cut-off Value	AUC (95% CI)	<i>p</i> value	Sensitivity (%)	Specificity (%)
Men (n=170)					
TFA (cm ²)	361.4	0.739 (0.666-0.804)	<0.0001	68.0	73.1
VFA (cm ²)	177.9	0.775 (0.704-0.836)	<0.0001	76.0	67.7
SFA (cm ²)	130.4	0.680 (0.604-0.750)	<0.0001	85.3	48.4
Women (n=66)					
TFA (cm ²)	318.8	0.885 (0.807-0.964)	<0.0001	88.0	78.0
VFA (cm ²)	80.1	0.880 (0.802-0.959)	<0.0001	96.0	73.2
SFA (cm ²)	261.8	0.833 (0.732-0.933)	<0.0001	68.0	85.4

Data analysed using receiver operating characteristics analysis, cut-off value obtained using Youden Index. AUC, area under the curve; CI, confidence interval; TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area

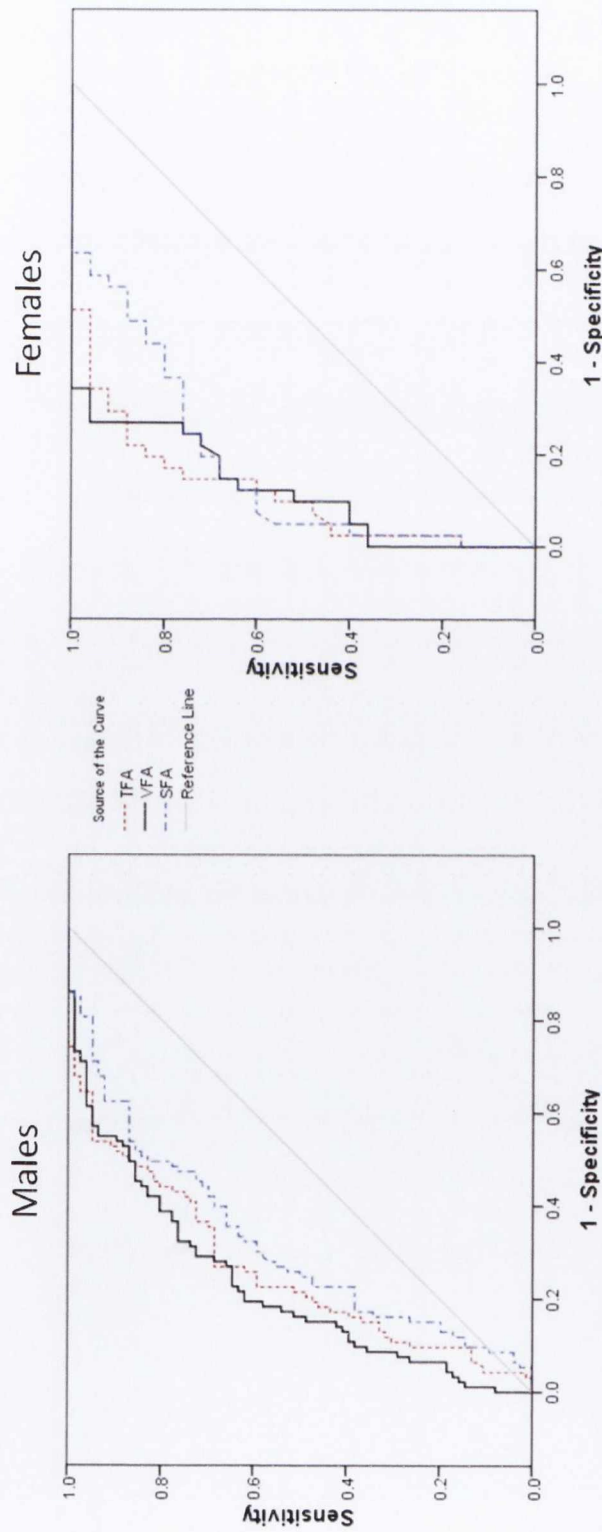


Figure 3.2 Abdominal fat areas associated with presence of the MetSyn in males and females using Harmonized Metabolic Syndrome

Criteria

ROC analysis and the Youden Index were used to determine cut-off values for abdominal fat associated with increased presence of the MetSyn using the Harmonized Metabolic Syndrome Criteria. In both males (n=170) and females (n=66), VFA was strongly associated with the presence of the Harmonized MetSyn. A VFA cut-off of $\geq 177.9\text{cm}^2$ identified males with the Harmonized MetSyn with 76% sensitivity and 67.7% specificity and a VFA cut-off of $\geq 80.1\text{cm}^2$ identified females with the Harmonized MetSyn with 96% sensitivity and 73.2% specificity.

TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area; ROC, receiver operating characteristics; MetSyn, The Metabolic Syndrome.

3.2.4 Correlating Optimal Thresholds for Abdominal Adiposity with Waist Circumference

Following identification of gender specific optimal thresholds for metabolic health, linear regression was used to determine the corresponding waist circumference (WC) cut-off values (Table 3.6, Figure 3.3). In all cases, WC correlated strongly with abdominal fat area, indicated by the *r* values. In both males and females WC correlated most strongly with TFA, as expected. In males a VFA threshold value of 163.8cm² correlated to a WC of 96.1cm, while in females a VFA threshold value of 80.1cm² correlated to a WC of 83.2cm.

Table 3.6 Corresponding waist circumference to new TFA, VFA and SFA cut-off values

	Cut-off Value	Corresponding WC (cm)	<i>p</i> value	<i>r</i> value
Men (n =170)				
TFA (cm ²)	364.2	98.9	<0.0001	0.717
VFA (cm ²)	163.8	96.1	<0.0001	0.599
SFA (cm ²)	132.3	94.1	<0.0001	0.520
Women (n =66)				
TFA (cm ²)	318.8	87.0	<0.0001	0.756
VFA (cm ²)	80.1	83.2	<0.0001	0.570
SFA (cm ²)	261.8	91.0	<0.0001	0.676

Cut-off value obtained using Youden Index, corresponding waist circumference determined by linear regression. WC, waist circumference; TFA, total fat area; VFA, visceral fat area; SFA, subcutaneous fat area

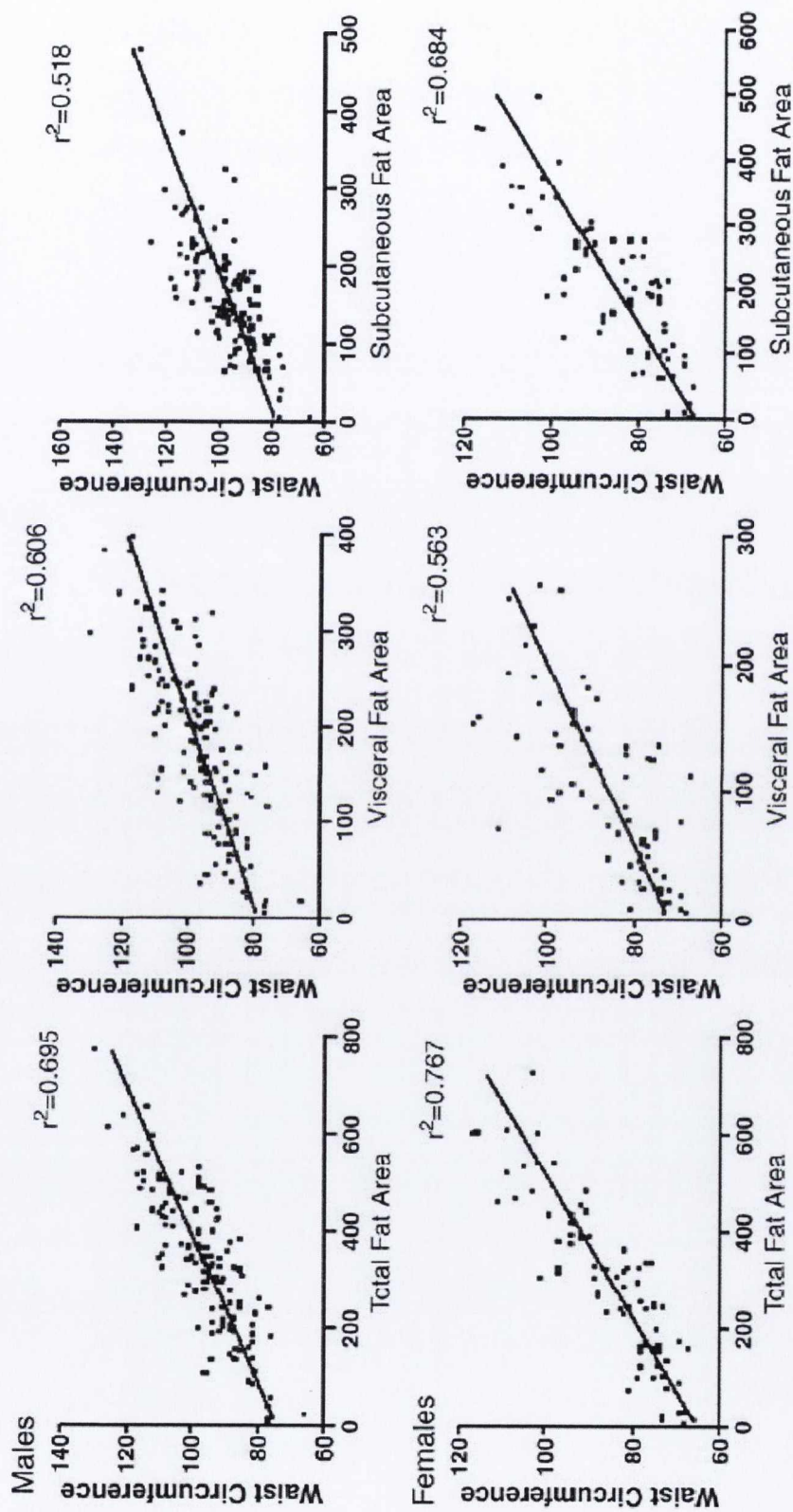


Figure 3.3 Correlation of optimal adiposity thresholds to waist circumference in males and females.

Linear regression was used to determine the WC values that correlated to the newly generated abdominal fat cut-off values. WC correlated strongly to all measures of abdominal fat in both males and females ($p<0.001$). In males ($n=170$) a VFA cut of 163.8cm^2 correlated to a WC of 96.1cm ($r=0.599$). In females ($n=66$) a VFA of 80.1cm^2 correlated to a WC of 83.2cm ($r=0.570$).

3.3 DISCUSSION

The current doctrine in obesity related cancer research is that excess visceral adipose tissue fuels a pro-inflammatory state with the associated MetSyn which ultimately creates a pro-tumourigenic environment ^[254]. CT staging is the standard of care for diagnosing patients with gastrointestinal cancer and there is an emerging interest in CT-determined VFA as a marker of altered biology in patients with obesity-associated cancer ^[126, 259, 269, 273-275].

This study sought for the first time to generate cut-offs for VFA that were pathologically relevant and reflective of the gender differences in visceral adipose tissue distribution in Caucasians. Gender greatly influences fat distribution, with men storing approximately 20-30% of their total body fat in the visceral compartment, irrespective of their obesity status ^[264]. Conversely, women tend to amass excess adipose tissue peripherally, and do not accumulate significant amounts of visceral fat until a moderate level of obesity is reached ^[263]. Other reports show that men have a higher prevalence of obesity-related metabolic diseases ^[252, 276]. This study identified significantly different mean visceral fat areas ($p < 0.0001$) for men (228.6cm^2) and women (146.8cm^2) with the MetSyn, highlighting profound differences between men and women and the absolute requirement for gender-specific abdominal fat area cut-offs. Although previous studies have used the gold standard techniques of CT or MRI scanning for the quantification of fat compartments in obesity, and report a VFA $\geq 130\text{cm}^2$ was associated with obesity related pathologies, no study heretofore has provided gender specific VFA cut-off values in Caucasians ^[261, 262, 277, 278].

The potential error in using the currently accepted VFA value of $\geq 130\text{cm}^2$ to associate obesity with metabolic dysfunction is evident in this research and the findings support the study hypothesis that when associating total, visceral and subcutaneous fat area with the MetSyn, different CT derived cut-off values would be determined for Caucasian males and females. Using the combined criteria for the diagnosis of the MetSyn, the optimal visceral fat area cut-off values were determined as $\geq 163.8\text{cm}^2$ for men and $\geq 80.1\text{cm}^2$ for women. This suggests that previously identified cut-off values of $\geq 130\text{cm}^2$ may result in the misclassification of men and women as viscerally obese,

using the dysmetabolism as the outcome. A study by Oka *et al.* reported gender-specific VFA cut-off associated with the MetSyn as $<132.6\text{cm}^2$ for men and $<91.5\text{cm}^2$ for females in a Japanese population ^[252]. However, Japanese and Asian populations traditionally have a greater VFA:SFA ratio than Caucasians, thus these optimal VFA thresholds are not suitable for use in Caucasian populations ^[279].

The much lower threshold for VFA in females supports the hypothesis that an excessive amount of visceral adipose tissue is more detrimental to health in females compared to males ^[263, 280]. In previous studies where the relative risk of cardiovascular disease has been assessed in those with the highest WC or waist-to hip ratio compared to those with the lowest, a greater fold increase has been observed within the female cohort compared to the male ^[265, 281, 282]. The cause of this gender difference is uncertain but may be related to the higher amount of hepatic free fatty acid delivery derived from lipolysis of VFA that has been observed in women than in men ^[30]. With the exception of hypertension in females, VFA had the strongest association with all individual markers of dysmetabolism, compared with SFA. In addition a much stronger association was observed between VFA and the MetSyn in males and females rather than SFA, reflected in the AUC values. VFA also demonstrated a stronger association with the MetSyn rather than individual markers of dysmetabolism, validating the use of the MetSyn as a marker of whole body dysfunction when generating optimal thresholds for VFA. These data strongly support the theory that visceral adipose tissue is pathologically distinct and quantification of this depot is essential for research in obesity and its associated morbidities.

It is acknowledged that a potential influence of cancer related weight loss associated with gastrointestinal malignancy is an important consideration when using the IDF criteria for the MetSyn diagnosis as abdominal obesity is a prerequisite. To assess this further, the ROC analyses of abdominal fat and the MetSyn were repeated using the Harmonized Metabolic Syndrome Criteria which does not require abdominal obesity as a definitive, but rather the presence of three of the five criteria used in the IDF definition ^[272]. Using the Harmonized criteria for the MetSyn, similar pathological thresholds for TFA (361.4 cm^2), VFA (177.9cm^2) and SFA (130.4cm^2) were obtained in

males. In the female cohort those designated as MetSyn positive or negative using the IDF criteria remained classified as MetSyn positive or negative using the Harmonized criteria, thus there was no difference in the thresholds obtained for TFA, VFA and SFA. Hence in this study, weight loss, which is an important consideration in gastrointestinal cancer, did not appear to influence MetSyn status or the significant association between VFA and dysmetabolism.

It is now well established that waist circumference correlates with CT generated VFA^[283]. Regression analysis was therefore used to determine the corresponding waist circumference to these newly generated abdominal fat cut-off values. The strongest correlation was observed between TFA and WC in both males and females. This finding is unsurprising, given that WC is a composite measure of both the subcutaneous and visceral adipose tissue depots. The World Health Organization (WHO) proposed that a WC ≥ 94 cm in men and ≥ 80 cm in women is a predictor of an increased risk to health, and that a WC of ≥ 102 cm in men and ≥ 88 cm in women is a predictor of a substantial risk to health for the Caucasian population^[27]. Regression analysis found that a WC of 96.1cm for men and 83.2cm for women corresponds to the gender specific discriminate VFA values associated with the MetSyn established in this study. The corresponding WC to VFA values obtained in this study for males and females are closely related to the WHO WC values proposed as an increased risk to health.

This study confirms that, although both SFA and VFA have been shown to correlate with metabolic risk factors, VFA remains more strongly associated with an adverse metabolic risk profile^[284]. In the current study, the new VFA cut-off value was superior to SFA in identifying patients with the MetSyn. Measurement of VFA provides a complete understanding of obesity related pathologies associated with variation in fat distribution^[285]. Therefore, the quantitative assessment of VFA by CT, as an independent risk factor for obesity-related diseases, is a powerful research tool.

A limitation of this study is that cut-offs were derived solely in a cancer population. It would be worthwhile to study these values in a non-cancer cohort, but enrolling such non-cancer patients for unnecessary abdominal CT imaging presents challenges in

terms of both ethics and resources. Nevertheless, these cut-offs are a powerful research tool for the study of obesity associated malignancies, which are convincingly fuelled by visceral fat ^[254, 286, 287]. In research where CT derived VFA is available, such as obesity-associated gastrointestinal malignancy, cut-offs values for the classification of visceral obesity are long overdue and urgently required to allow for more consistent and comparable research.

In conclusion, this study is the first to generate ethnic and gender specific cut-offs for visceral, subcutaneous and total fat areas in a cohort of Caucasian gastrointestinal cancer patients. Using the metabolic syndrome as a quantifiable marker of the whole body dysfunction associated with excess visceral adiposity, we have identified pathologically relevant criteria for classifying visceral obesity. This study highlights the current lack of appropriate VFA cut-off values for use in clinical research and also demonstrates the importance of assessing the relationship between adipose tissue distribution and dysmetabolism separately in men and women. VFA thresholds of $\geq 160\text{cm}^2$ for men (82.7% sensitivity and 62.1% specificity) and $\geq 80\text{cm}^2$ for women (95.8% sensitivity and 71.4% specificity) are proposed as pathologically relevant cut-off values for use in obesity-associated cancer research.

**Chapter 4 IGF-1 and its Receptor in Oesophageal Cancer: Association
with Adenocarcinoma and Visceral Adiposity**

4.1 INTRODUCTION

There is strong epidemiological evidence linking obesity to oesophageal adenocarcinoma ^[12]. As the prevalence of obesity continues to rise in society and the incidence of oesophageal adenocarcinoma increases ^[2-5], there is a drive to understand the molecular mechanisms by which obesity increases the risk of oesophageal adenocarcinoma.

The observation that obesity is a risk factor for oesophageal adenocarcinoma independent of reflux status indicates that other systemic factors aside from the mechanical impact of excess abdominal fat are at play ^[11]. Furthermore, the fact that obesity has differing effects on the histological subtypes of oesophageal cancer suggests specific tumour/host interactions are occurring. Thus oesophageal adenocarcinoma may be a paradigm model of obesity and cancer, and current molecular research is focused on mechanisms that underpin this association.

Visceral obesity is associated with a proinflammatory, procoagulant and insulin resistant state, collectively known as the metabolic syndrome ^[288]. The dysmetabolism observed in visceral obesity is believed to create an environment that promotes tumourigenesis, and this has been described in patients with colorectal and breast cancer ^[10, 55, 289]. In patients with Barrett's oesophagus, the pre-malignant condition, a high prevalence of visceral adiposity and metabolic syndrome was identified by this research group. Furthermore, it was found that visceral obesity was associated with the length of Barrett's ^[125], an important finding as longer length of Barrett's is associated with an increased risk of developing oesophageal adenocarcinoma ^[290].

Insulin, insulin resistance, and the insulin-like growth factor (IGF) system are interesting targets for cellular studies linking obesity and cancer. The IGF axis is involved in cell proliferation, differentiation and apoptosis ^[88, 291]. The IGF system may be highly dysregulated in obesity and the metabolic syndrome; with insulin resistance giving rise to hyperinsulinaemia and increased free IGF-1 ^[292]. The IGF system consists of two ligands, insulin like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-2); three cell-membrane receptors, IGF-1 receptor (IGF-1R), IGF-2 receptor (IGF-2R) and insulin receptor (IR); and six high-affinity IGF binding proteins, IGFBP-1 to IGFBP-6 ^[88]. IGF-1R is a heterotetrameric tyrosine kinase receptor consisting of two α and two β

subunits. Binding of the ligands IGF-1 and IGF-2 to IGF-1R results in receptor autophosphorylation and activation of multiple signalling pathways, including the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositide 3-kinase (PI3K)/anti-apoptotic serine threonine kinase (Akt-1) pathways, leading to cell proliferation and protection from apoptosis ^[293, 294]. Dysregulation of this pathway can give rise to malignancy. In oesophageal cancer, over expression of IGF-1R has been associated with the malignant progression of Barrett's to oesophageal adenocarcinoma ^[291]. In addition, blockade of IGF-1R leads to apoptosis in oesophageal cancer cells ^[293, 294]. IGF-1 has been shown to stimulate the growth of oesophageal cancer cells *in vitro* ^[295, 296]. The source of IGF-1 may be systemic as high circulating levels have been observed in oesophageal cancer patients ^[297, 298]. Alternatively, macrophages have been demonstrated to produce IGF-1 and are a likely source of this growth factor within the tumour stroma acting in a paracrine fashion ^[299-302].

4.1.1 Specific Aims

This aim of this study is to investigate the role of the IGF-1 axis in oesophageal cancer tumourigenesis, both *in vitro* and *in vivo*, and its relationship with obesity by

- Determining the expression of IGF-1R at the gene and protein level in a panel of oesophageal cell lines and the impact of IGF-1 on proliferation
- Measuring the circulating levels of IGF-1 in a cohort including oesophageal adenocarcinoma, oesophageal SCC, Barretts oesophagus and non cancer control patients and relate levels to anthropometric and clinical status.
- Assessing the expression of IGF-1R and CD68⁺ cells in resected oesophageal tumour specimens and relate findings to tumour and patient characteristics

4.2 RESULTS

4.2.1 *In Vitro* mRNA and Protein Expression of IGF-1 and its Receptor

mRNA expression of IGF-1R, IGF-1 and IGF-2 was assessed by reverse transcriptase PCR in 2 oesophageal adenocarcinoma cell lines (OE19 and OE33) and 2 oesophageal

squamous cell carcinoma cell lines (KYSE 410 and OE21), as described in section 2.5. The hepatic carcinoma cell line HepG2 served as a positive control. All 4 cells lines expressed mRNA for IGF-1R. IGF-1 mRNA was expressed in the SCC cell line OE21 and the adenocarcinoma cell line OE19, and IGF-2 was expressed in the SCC cell lines, KYSE 410 and OE21 (Figure 4.1, A). Western blot analysis identified the IGF-1R as a 150kDa and 120 kDa protein, representing α and β -subunits respectively, in all cell lines. There was visibly higher expression of IGF-1R in both of the SCC cell lines, with OE21 having the highest expression, compared with the endogenous control β -actin, as illustrated by densitometric analysis (Figure 4.1, B).

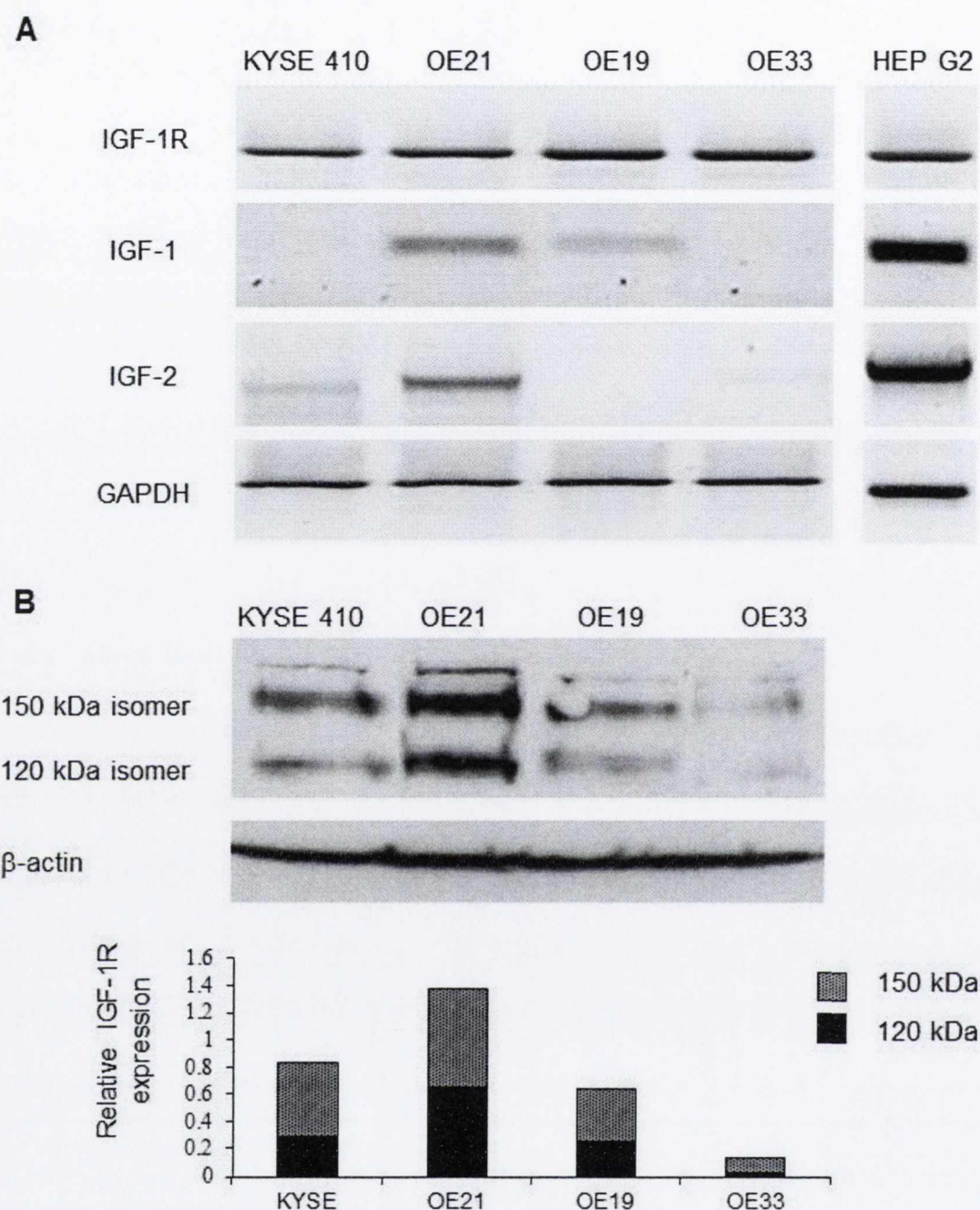


Figure 4.1 Expression of IGF-1, IGF-2 and IGF-1R in oesophageal cancer cell lines

A) mRNA expression of IGF-1, IGF-2 and IGF-1R in KYSE 410, OE21, OE19 and OE33 cell lines was assessed by RT-PCR. HepG2 hepatic carcinoma cell line served as a positive control and GAPDH was used as the endogenous control gene. **B)** Expression of IGF-1R at the protein level was determined by Western Blotting in KYSE 410 and OE21 SCC cell lines, and OE19 and OE33 adenocarcinoma cell lines. β -actin expression was also assessed to ensure equal sample loading. Densitometric analysis was carried out to determine differential IGF-1R expression across the cell lines relative to β -actin.

4.2.2 Changes in Cell Proliferation in Response to Exogenous IGF-1

A BrdU cell proliferation assay was used to assess the proliferation of oesophageal cancer cells in response to various concentrations of human recombinant IGF-1 (0.1ng/ml, 1ng/ml, 10ng/ml, 100ng/ml, 1000ng/ml) as described in section 2.3. A dose dependent increase in cell proliferation was observed in the adenocarcinoma cell lines in response to IGF-1. A significant increase in cell proliferation was observed in the OE33 and OE19 cell lines at a concentration of >10ng/ml and >100ng/ml respectively ($p<0.05$). The effect was most pronounced in the OE19 cell line, with 100ng/ml IGF-1 leading to a 25% increase in cell proliferation ($p<0.01$). In contrast, no dose dependent increase in cell proliferation was observed in the SCC cell lines when treated with IGF-1. The results are illustrated below in Figure 4.2.

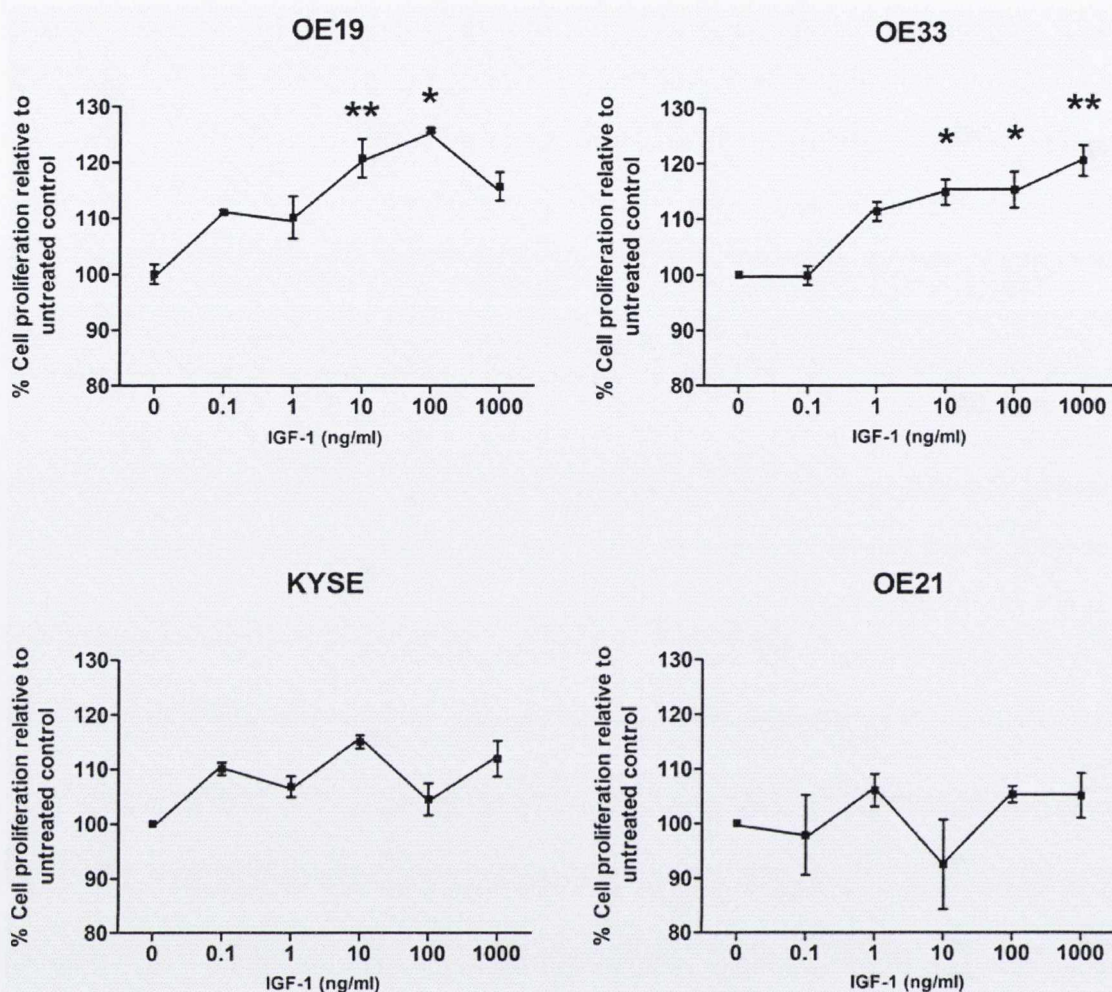


Figure 4.2 IGF-1 stimulates oesophageal adenocarcinoma cell line growth in a dose-dependent fashion

Oesophageal adenocarcinoma (OE19, OE33) and SCC (KYSE 410, OE21) cell lines were treated with varying concentrations of IGF-1 (0.1-1000ng/ml) for 24 hours. Cell proliferation was measured by BrdU incorporation assay, all samples were assayed in triplicate. Differences between timepoints was assessed by ANOVA with Tukey post hoc test. Data represents mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$.

4.2.3 Patient Characteristics

The study population consisted of 124 patients, 91 males and 33 females, who underwent an oesophagectomy at this centre between 2007 and 2010. Oesophageal tumour samples were collected as per the standard protocols of the Central Pathology Laboratory in St. James's Hospital. Serum samples were collected on these patients as part of the Adipose Tissue Biobank studies and anthropometry was performed as detailed in section 2.7.1. Ninety-six oesophageal adenocarcinoma and 28 oesophageal squamous cell carcinoma (SCC) patients were included in this study. The age at time of surgery ranged from 30-86yrs, with a mean age of 63yrs in both the adenocarcinoma and SCC groups. There were significantly more males than females in the oesophageal adenocarcinoma cohort compared to SCC. Increased BMI ($p<0.001$) and VFA ($p<0.001$) and a higher prevalence of the metabolic syndrome ($p=0.011$) was observed in the adenocarcinoma patients. In both tumour types the most common pathological stage was stage 3. Patient and tumour characteristics are summarized in Table 4.1.

Table 4.1 Oesophageal cancer patient demographics

	Oesophageal Adenocarcinoma (n=96)	Oesophageal SCC (n=28)	<i>p</i>
Male, n (%)	81 (84%)	10 (36%)	0.0001
Age (y), mean (range)	63 (30-86)	63 (45-78)	0.85
Pathological Stage, n (%)			
Stage 0	5 (5.2%)	4 (14.3%)	0.12
Stage I	11 (11.5%)	5 (17.9%)	0.35
Stage II	34 (35.4%)	7 (25%)	0.37
Stage III	36 (37.5%)	9 (32.1%)	0.66
Stage IV	1 (1%)	1 (3.6%)	0.40
Unknown	9 (9.3%)	2 (7.1%)	1.0
BMI(kg/m²), median (range)	26.95 (18.64 – 39.0)	23.23 (16.7-31.9)	<0.001
*VFA(cm²), median (range)	168.7 (9.17-383.8)	60.0 (5.8-381.1)	<0.001
#Metabolic syndrome, n (%)	38 (42.2%)	4 (14.8%)	0.011

*CT scans for VFA calculation available on 101 patients. BMI, body mass index; VFA, visceral fat area. #Metabolic syndrome status available on 117 patients.

4.2.4 Serum IGF-1 levels

The level of free IGF-1 in serum was assessed in 82 oesophageal adenocarcinoma patients and 17 SCC by ELISA, as described in section 2.16. In order to further assess the relationship between circulating IGF-1 and oesophageal cancer, free IGF-1 levels were measured in 25 Barrett's oesophagus patients and 27 non-cancer controls on whom serum was collected as part of on-going research studies within this unit. Results were correlated with tumour type, gender, obesity and metabolic syndrome status. Obesity was determined by BMI and VFA. For the classification of visceral obesity the published threshold of VFA $>130\text{cm}^2$ was used as well as the newly generated gender specific VFA thresholds of $\geq 160\text{cm}^2$ for males and $\geq 80\text{cm}^2$ for females, referred to as "high VFA" (Chapter 3).

Significantly higher circulating levels of IGF-1 were found in patients with oesophageal adenocarcinoma compared to those with SCC ($p<0.05$), Barrett's oesophagus ($p<0.001$) or controls ($p<0.001$). Gender did not affect circulating IGF-1 levels (Figure 4.3, A). No association was found between obesity and IGF-1 levels when patients were classified by BMI. However, in the patients on whom VFA was available, significantly higher circulating IGF-1 levels were found in those with elevated VFA ($p<0.05$), suggesting that excess visceral adipose tissue influences serum IGF-1 levels. In addition, higher levels of IGF-1 were observed in patients with the metabolic syndrome ($p<0.05$) (Figure 4.3, B).

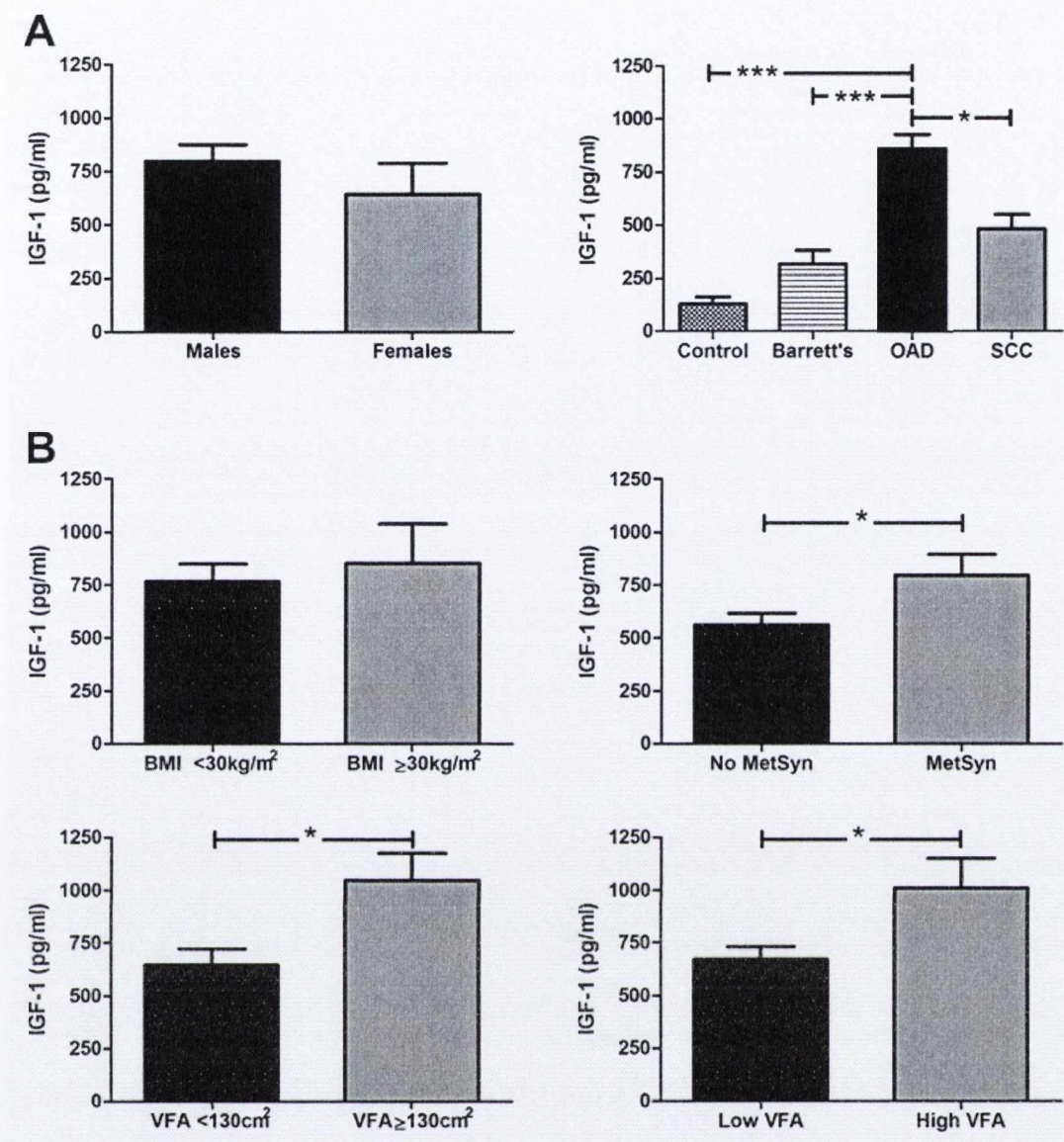


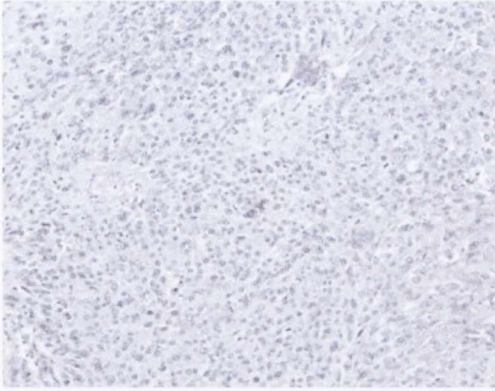
Figure 4.3 Serum IGF-1 levels are increased in patients with oesophageal adenocarcinoma, visceral obesity and the metabolic syndrome

Serum IGF-1 levels were measured in 82 oesophageal adenocarcinoma patients, 17 oesophageal SCC patients, 25 Barrett’s oesophagus patients and 27 healthy controls by ELISA. All samples were assayed in triplicate. Results are correlated to clinical status, tumour type, gender, obesity and metabolic syndrome status. Visceral obesity was classified using 3 different anthropometric indicators of obesity, BMI ≥30kg/m²; VFA ≥130cm²; or VFA ≥160cm² for males and ≥80cm² for females (referred to as “high VFA”). Data represent mean ± SEM; * p<0.05, ***p<0.001. OAD, oesophageal adenocarcinoma; SCC, oesophageal squamous cell carcinoma; BMI, body mass index; MetSyn, the metabolic syndrome; VFA, visceral fat area.

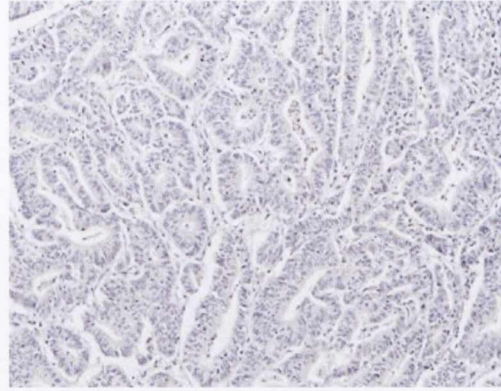
4.2.5 Tumour Expression of IGF-1R and CD68⁺ cells

IGF-1R expression in oesophageal tumour tissue was assessed using immunohistochemistry in 96 patient resections as described in section 2.6.1. Staining intensity was graded by three independent observers, including a consultant pathologist, using the grading system detailed in section 2.6.2. Representative images of the different grades of IGF-1R staining intensity are shown in Figure 4.4. No association was observed between IGF-1R expression and obesity status, metabolic syndrome, tumour stage, nodal status or survival (Figure 4.5 and 4.6). Differences were observed in the pattern of staining throughout the tissues, with the highest level of IGF-1R expression observed at the basal layer of normal squamous epithelium. In both adenocarcinoma and SCC sections, increased levels of IGF-1R expression were observed in areas of tumour and at the invasive tumour edge compared to tumour associated stroma, ($p < 0.05$) (Figure 4.7). As macrophages have been shown to be a source of IGF-1 both physiologically and at tumour sites, CD68, a marker for human macrophage infiltration, was assessed by immunohistochemistry in 50 oesophageal tumour sections (section 2.6). Positively stained cells were counted in three different areas: tumour, stroma and at the invasive edge by three independent observers, including a consultant pathologist. Representative images of positive and negative CD68⁺ staining are shown in Figure 4.8. No overall difference was found in CD68⁺ cell numbers between oesophageal adenocarcinoma and oesophageal SCC sections, or between obese and non-obese patients (Figure 4.9). There was a significant variation in CD68⁺ cell numbers based on location within the tumour tissue, with increased levels in tumour associated stroma compared to the tumour island ($p < 0.001$), and further increased levels observed in the stroma surrounding the invasive tumour edge ($p < 0.05$) (Figure 4.10).

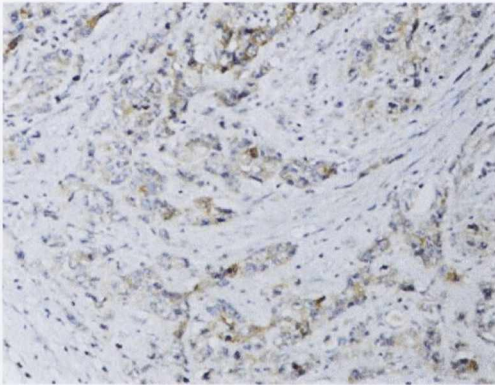
Grade 0



Grade 1



Grade 2



Grade 3

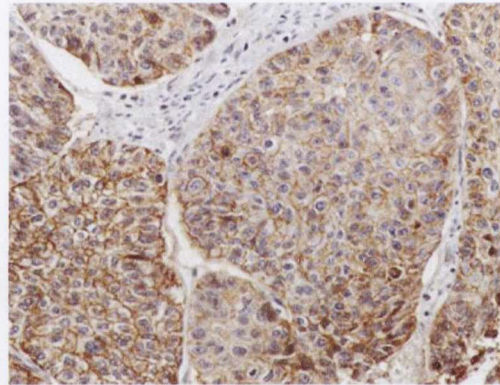


Figure 4.4 Representative images of IGF-1R staining intensity.

IGF-1R expression was assessed by immunohistochemistry in resected oesophageal tumour specimens. Staining intensity was graded by three independent observers, including a consultant pathologist. Intensity of IGF-1R staining was graded on a scale from 0 to 3. A score of 0 corresponded to no staining, a score of 1 to weak staining, a score of 2 to moderate staining and a score of 3 to strong staining. Percentage of tumour cells stained was graded in quartiles of 0%, 25%, 50%, 75% and 100%. The average grade for each patient was calculated by multiplication of the intensity score by the percentage of tumour score, giving an overall score for each section area of between 0-300. Areas of tumour and tumour stroma were graded separately. Where present, the invasive edge at the junction of tumour and normal tissue was identified and marked by a pathologist and this section was graded separately

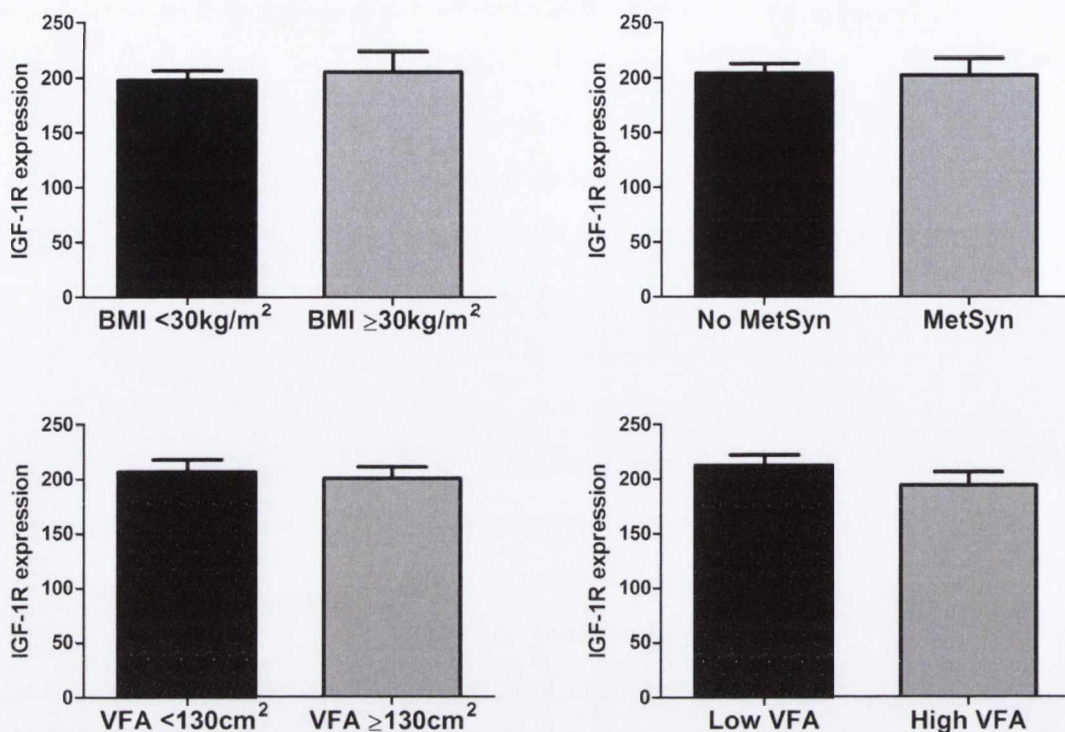


Figure 4.5 IGF-1R tumour expression is not associated with obesity or the metabolic syndrome

The tumour expression of IGF-1R was determined by immunohistochemistry. Intensity of IGF-1R staining was graded by three independent observers on a scale from 0 to 3 and percentage of tumour cells stained was graded in quartiles. The average grade for each patient was calculated by multiplication of the intensity score by the percentage of tumour score, giving an overall score for each section area of 0-300. Expression was assessed in relation to obesity and metabolic syndrome status by Mann Whitney U test. IGF-1R expression was not associated with obesity as classified by BMI or VFA (using $\geq 130\text{cm}^2$ or the gender-specific thresholds). Data represents mean $\pm$ SEM. BMI, body mass index; MetSyn, the metabolic syndrome; VFA, visceral fat area.

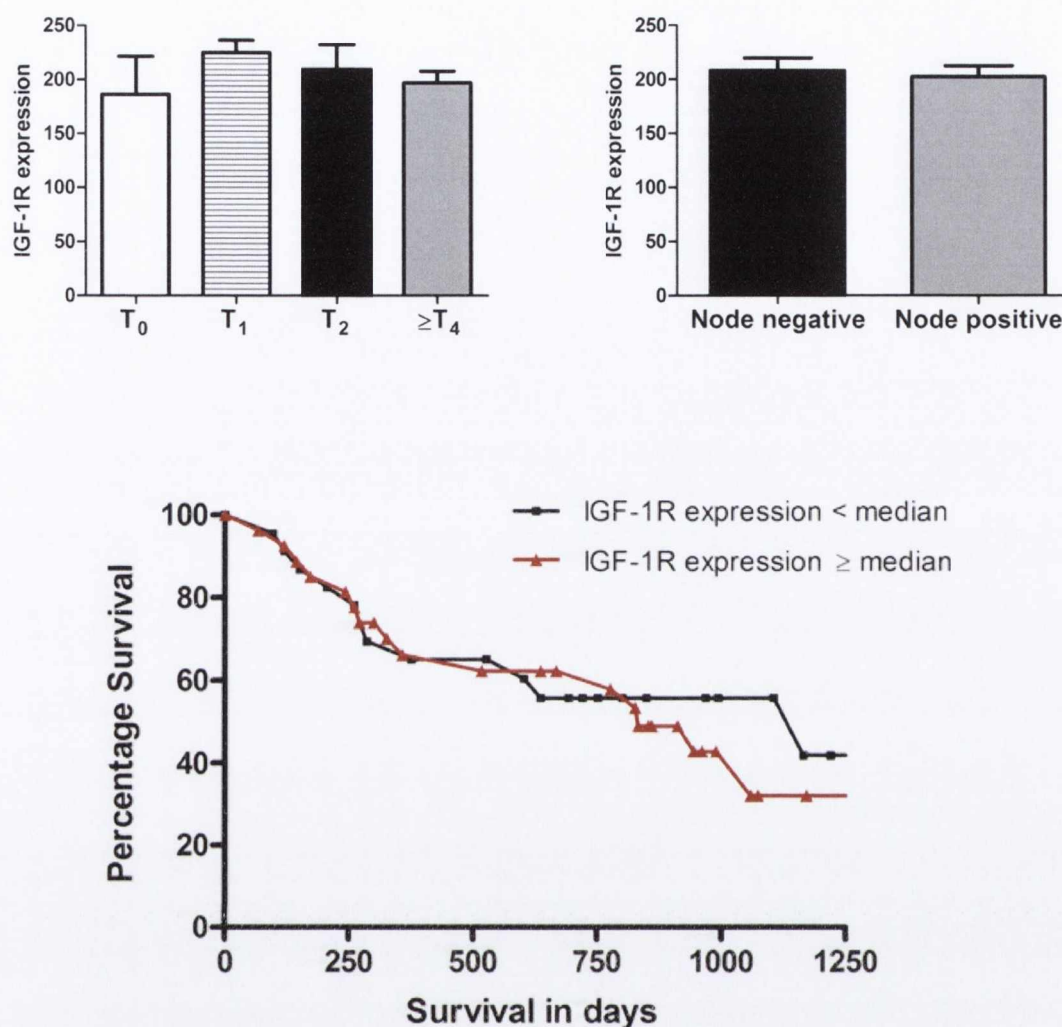
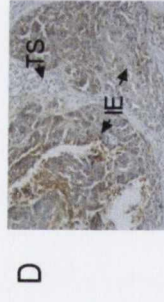
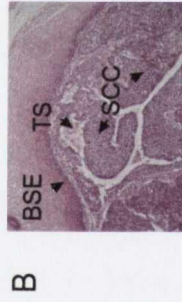
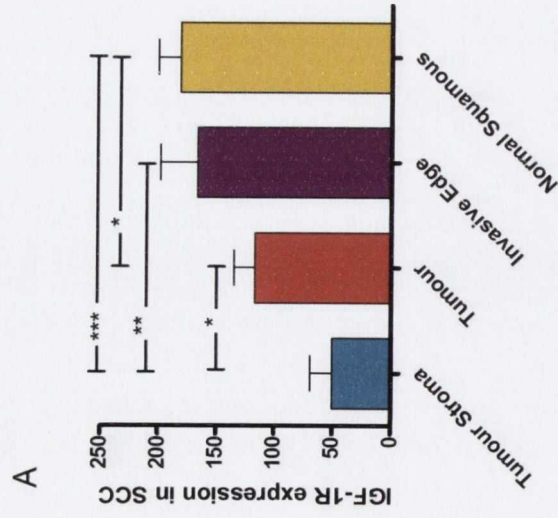


Figure 4.6 IGF-1R expression is not associated with T-stage, nodal status and survival

Tumour expression of IGF-1R was determined by immunohistochemistry and expression was analysed in relation to pathological T-stage and nodal status by ANOVA and Mann Whitney U test. IGF-1R expression was not associated with a more advanced T-stage or nodal positivity. IGF-1R expression was dichotomised by the median into high and low expression and overall survival was calculated by Kaplan Meier. The median survival for patients with high or low tumour IGF-1R expression was 833 and 1165 days respectively. Data represents mean $\pm$ SEM.

Oesophageal Squamous Cell Carcinoma



Oesophageal Adenocarcinoma

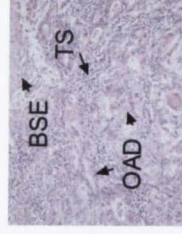
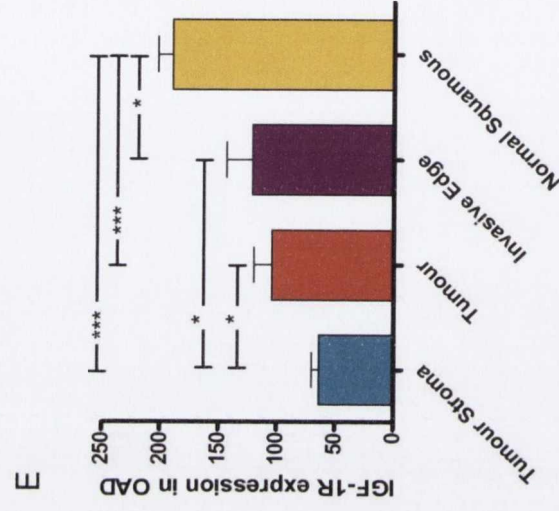


Figure 4.7 Pattern of IGF-1R expression in oesophageal adenocarcinoma and squamous cell carcinoma tumour sections

There was heterogeneous expression of IGF-1R, denoted by brown staining, within oesophageal SCC (A) and oesophageal adenocarcinoma (OAD) and sections (E). Representative images of IGF-1R and Haematoxylin and Eosin staining of SCC and OAD sections are shown in images A-C and F-H respectively. In both OAD and SCC sections the highest level of IGF-1R expression was observed at the basal layer of normal squamous epithelium (BSE) (B, F). Increased levels of IGF-1R expression observed in areas of tumour compared to tumour associated stroma (TS), $p < 0.05$ (C, G) and at the invasive tumour edge (IE) compared to TS, $p < 0.05$ (D, H). Data represents mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

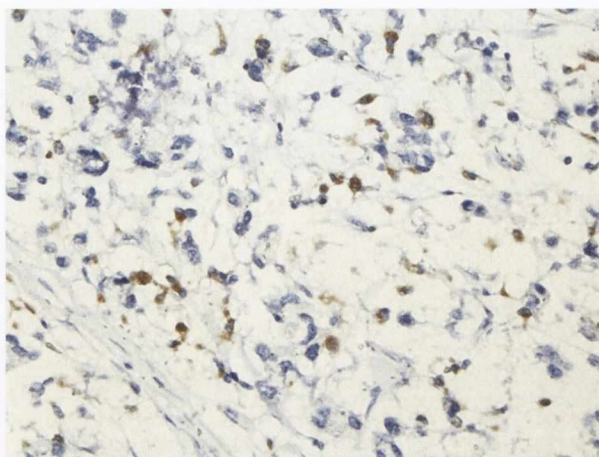


Figure 4.8 Representative image of staining for CD68⁺ cells in resected oesophageal tumour.

Resected oesophageal sections were stained for the expression of CD68⁺ cells by immunohistochemistry. Positively stained cells, identified by the brown staining, were counted in three different areas of tumour, stroma and at the invasive edge by three independent observers.

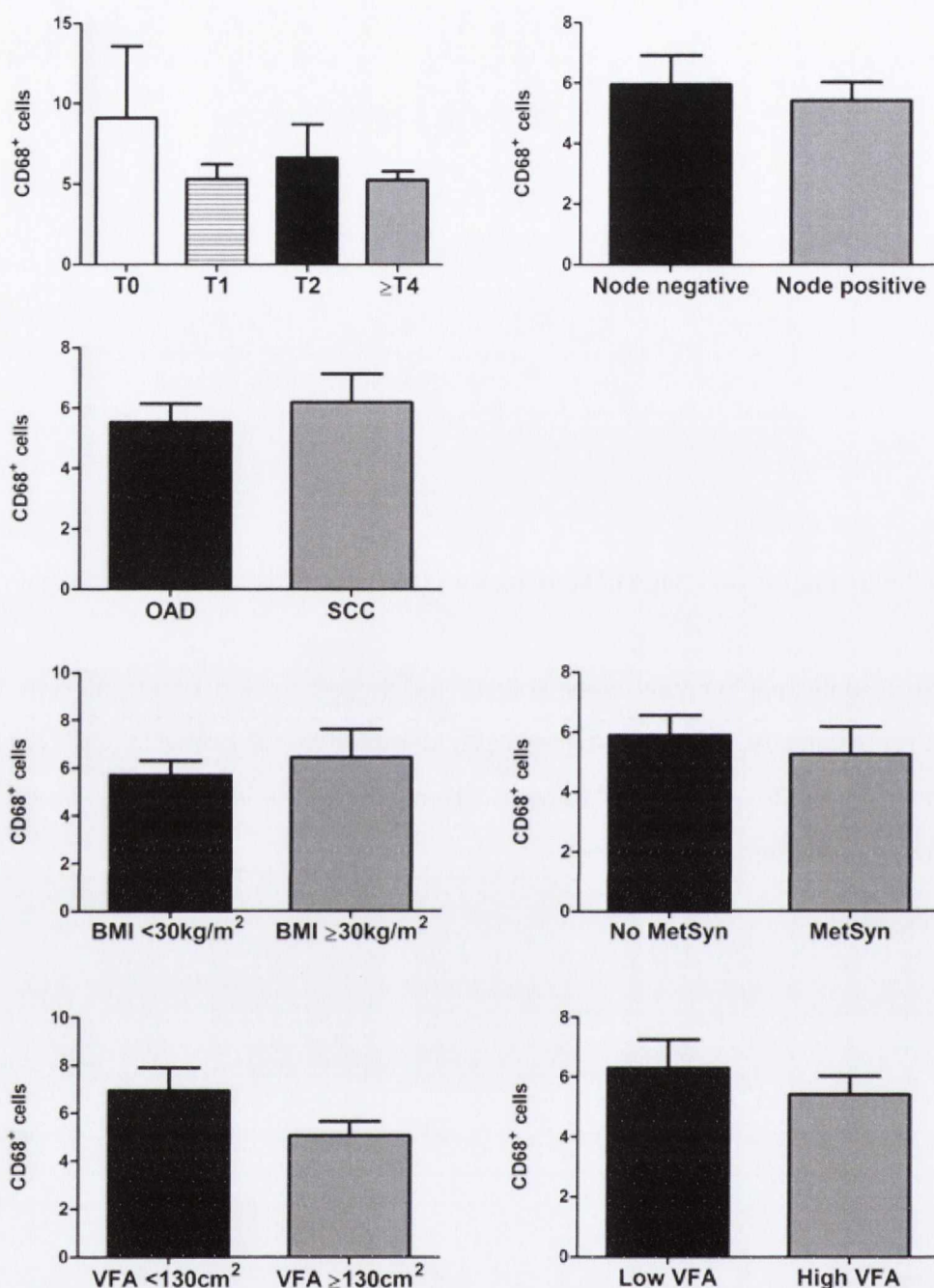


Figure 4.9 Presence of CD68⁺ cells in oesophageal tumour is not associated with obesity or the metabolic syndrome

The expression of CD68⁺ cells in oesophageal tumour sections was assessed by immunohistochemistry and analysed in relation to T-stage, nodal status, obesity and metabolic syndrome status using Mann Whitney U and ANOVA. Data represents mean $\pm$ SEM. OAD oesophageal adenocarcinoma; SCC, oesophageal squamous cell carcinoma; BMI, body mass index; MetSyn, metabolic syndrome; VFA, visceral fat area.

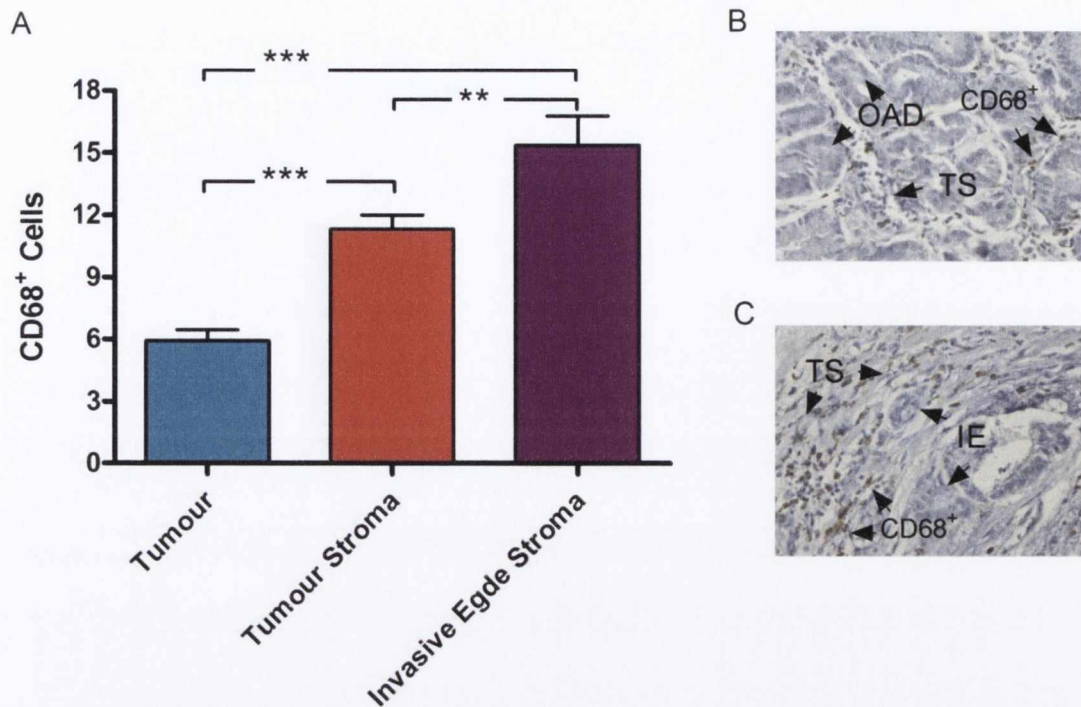


Figure 4.10 Increased CD68⁺ cell numbers are present in the tumour associated stroma compared to the tumour island

In oesophageal tumour sections there was a significant variation in CD68⁺ cell numbers, denoted by brown staining, based on location within the tumour tissue **(A)**, with increased levels observed in the tumour associated stroma (TS) compared to the tumour ($p < 0.001$) **(B)**, and further increased levels observed in the stroma surrounding the invasive tumour edge (IE) ($p < 0.05$) **(C)**. Statistical analysis was performed using ANOVA. Data represents mean $\pm$ SEM; ** $p < 0.01$, *** $p < 0.001$. OAD, oesophageal adenocarcinoma

4.3 DISCUSSION

The IGF-1 axis has well-described effects on tumour cell proliferation, differentiation and apoptosis, and an anticipated dysregulation in obesity particularly where it is associated with insulin resistance and metabolic syndrome ^[88, 292]. The current study presents novel findings that position the IGF-1 axis as key to oesophageal tumour growth and a plausible link between visceral obesity and gastrointestinal malignancy.

Obesity and excess visceral adiposity have been identified as strong, independent risk factors for oesophageal adenocarcinoma ^[11, 248]. Our observation that circulating IGF-1 levels are upregulated in adenocarcinoma compared to oesophageal SCC suggests that obesity and its influence on the IGF-1 pathway may be a critical factor for oesophageal adenocarcinoma development. The relationship between BMI and circulating IGF-1 and IGF binding proteins is complex and nonlinear. Whilst chronic energy deficiency leads to a reduction in IGF-1, chronic energy excess does not lead to increased IGF-1 ^[93, 292, 303]. However, elevated levels of free IGF-1 have been reported in obese individuals and are believed to be a consequence of hyperinsulinaemia inhibiting production of IGFBP-1 and -2 ^[93-95, 304, 305]. Obesity is now considered to be a state of chronic systemic inflammation, a state that is thought to contribute to tumour development and progression ^[10]. A study by Wu *et al.* investigated the role IGF-1 plays in enhancing tumour growth and progression in a murine model of obesity and chronic IGF-1 gene deficiency ^[96]. They reported that in diet-induced obese mice, both local tumour growth and metastases were increased compared to lean controls, but this was not observed in IGF-1 gene deficient mice. In addition, the expression of inflammatory cytokines and cell adhesion molecules was upregulated in obese compared to lean mice, but chronic IGF-1 deficiency was associated with a reduction in these indicators ^[96]. Similar anti-tumour effects of IGF-1 deficiency were observed in other murine studies of colon and pancreatic cancer ^[97, 98]. Hence it is possible that in obesity IGF-1 can affect tumour development both directly, by stimulating tumour growth and indirectly, by creating a microenvironment that is permissive for tumour growth.

This study is the first to clearly demonstrate a link between circulating IGF-1 levels and visceral obesity. While there was no increase in IGF-1 levels in subjects with BMI $\geq 30\text{kg/m}^2$; patients with an elevated VFA had significantly higher circulating IGF-1

levels. BMI is a convenient surrogate measure of obesity but, unlike VFA, it does not take into account quantity and location of adipose tissue. The hypothesis that chronic inflammation in obesity is pro-tumourigenic relates to viscerally located adipose tissue [10, 288]. Indeed, we have recently shown that increasing VFA is a risk factor for oesophageal adenocarcinoma [36]. Research has shown that IGF-1 is crucial for the maturation of adipocytes and both pre-adipocytes and adipocytes express and secrete high amounts of IGF-1 [306]. The fact that VFA, but not BMI, was predictive of higher circulating IGF-1 in patients further supports the hypothesis that adipose tissue influences serum IGF-1 levels. It should also be noted that the median VFA in the adenocarcinoma patients in this study was 168.7cm², compared to 60.0cm² in the SCC patients (p<0.001), confirming that both visceral adiposity and high circulating IGF-1 are strongly associated with oesophageal adenocarcinoma. The observation that patients with the metabolic syndrome had higher serum IGF-1 also supports the hypothesis that the deranged metabolism observed in visceral obesity may drive the increased circulating levels of IGF-1 [292].

This current study has also identified a number of novel findings regarding differential expression of the IGF-1 receptor and interaction with IGF-1 in oesophageal adenocarcinoma compared with oesophageal SCC. At the gene and protein level, SCC cell lines expressed higher levels of the IGF-1R, yet when treated with exogenous IGF-1, little or no effect was observed on cell proliferation. In contrast, adenocarcinoma cell lines expressed lower levels of IGF-1R, but demonstrated significant increases in cell proliferation when treated with low concentrations of IGF-1. Research has shown IGF-1 to stimulate the proliferation of pancreatic [307], colon [308] and breast [309] adenocarcinoma cell lines, with blockade of the IGF-1R leading to a down regulation in growth [310-312]. An important role for IGF-1 in oesophageal adenocarcinoma progression is further strengthened by the observation that patients with adenocarcinoma had significantly higher circulating levels of IGF-1 compared to those with SCC. Studies in a number of other malignancies, including prostate [91], breast [90, 93] and colon cancer [92] have also demonstrated higher IGF-1 levels to be associated with an increased risk of adenocarcinoma development.

In oesophageal SCC, studies have both supported ^[313, 314] and opposed ^[315] the hypothesis that IGF-1 promotes cell proliferation, with research suggesting that IGF-2, not IGF-1, is more important in SCC survival and proliferation ^[316]. Interestingly, in the current study only the SCC cell lines demonstrated co-expression of IGF-2 and IGF-1R, which has been shown to be associated with a more aggressive form of oesophageal SCC and shorter patient survival^[293]. This important role for IGF-2 in oesophageal SCC, but not adenocarcinoma, may provide an explanation for the higher IGF-1R expression in the SCC cell lines. IGF-1 has a fourfold increased affinity for the IGF-1R compared with IGF-2 ^[88], thus the increased demand for IGF-2 in order to continue proliferation may necessitate the SCC cells to up-regulate IGF-1R to compensate for the lower affinity.

Notable variations in IGF-1R expression was observed in oesophageal sections by immunohistochemical analysis, with the highest expression of the receptor observed in the basal squamous epithelial layer. Expression of IGF-1R by the tumour suggests that IGF-1 may be an important growth/survival factor. IGF-1R was further analysed with respect to tumour stage, lymph node involvement and patient survival. No significant association was found, suggesting that the role of the IGF-1 axis in oesophageal tumour progression may be key factor in local tumour growth and development. Tumour-associated macrophages (TAMs) are a significant component of inflammatory infiltrate in neoplastic tissues, the largest accumulation of which occurs at the invasive edge of the tumour. Tissue resident and tumour-associated macrophages have been shown to secrete the growth factor IGF-1, promoting survival, proliferation and invasion of cancer cells ^[299, 301, 302]. In this study, numbers of CD68⁺ cells in tumour sections were assessed by immunohistochemistry to determine macrophage infiltration. Increased CD68⁺ macrophage infiltration was observed in the stroma surrounding the tumour, and was further increased at the invasive tumour edge. This supports our hypothesis that the paracrine secretion of IGF-1 supports oesophageal adenocarcinoma progression.

Oesophageal cancer remains an aggressive malignancy, of which there is an increasing prevalence. In the current study the variations in IGF-1R expression within oesophageal tumours suggests an important role in oesophageal tumour growth whilst

increased circulating levels of IGF-1 suggests a differential role in oesophageal adenocarcinoma compared to SCC. In addition, the association between VFA and high circulating IGF-1 provides mechanistic evidence to substantiate the epidemiological based doctrine that obesity increases the risk of oesophageal adenocarcinoma. Large prospective validation studies are required, but these data identify the IGF-1 axis as a key factor and potential therapeutic target in oesophageal cancer, particularly obesity associated oesophageal adenocarcinoma.

Chapter 5 Investigating the Influence of Visceral Obesity on the Postoperative Inflammatory Response

5.1 INTRODUCTION

Visceral obesity and the associated metabolic syndrome are emerging as key factors that drive malignancy ^[254]. The association is particularly strong in gastrointestinal cancers. Research has identified visceral obesity and the metabolic syndrome as independent risk factors for oesophageal adenocarcinoma ^[36] and colorectal cancer ^[37] as well as their respective premalignant lesions, Barrett's oesophagus ^[125] and colorectal adenomas ^[38, 39].

The curative treatment for gastrointestinal cancer is resection, with chemo-radiotherapy considered if the tumour is advanced ^[199]. As the incidence of oesophageal and colorectal cancer continue to increase in society ^[5], it is inevitable that an increasing number of resective surgeries will be performed on visceraally obese, dysmetabolic patients. Whilst it is now an accepted doctrine that obesity has fuelled the cancer being excised, it remains to be elucidated what impact the presence of visceral obesity and the metabolic syndrome will have on the postoperative inflammatory response and recovery.

Visceral obesity and the metabolic syndrome give rise to abnormal cardiovascular ^[146-148] and pulmonary function ^[134-136] and this may manifest itself in postoperative morbidity. Whilst studies examining the specific effects of visceral obesity and/or the metabolic syndrome on postoperative recovery are limited, they do suggest that these pathological conditions increase the risk of post-operative atrial arrhythmia^[188, 194], respiratory complications ^[189, 317] and infections^[182, 184]. The insulin resistant, hyperglycaemic state observed in the metabolic syndrome can also increase the risk of postoperative complications ^[318-320]. The stress response to surgery induces insulin resistance with subsequent hyperglycaemia, the magnitude of which reflects the invasiveness of the surgery ^[321] and may be influenced by pre-operative insulin resistance. Hyperglycaemia may contribute to morbidity by causing intracellular and extracellular dehydration, inducing electrolyte abnormalities and depressing immune function ^[320, 322] and control of hyperglycaemia leads to improved patient outcomes ^[318, 323].

Central to the etiology of visceral obesity and the metabolic syndrome is chronic systemic inflammation ^[288] and this may critically impact on the normal inflammatory

response following surgery. A gastrointestinal resection is a major operative procedure and may be considered a controlled trauma^[199]. The immune and metabolic response to the surgery initiates an “acute phase” response, to achieve the three primary goals of stimulating antimicrobial functions, reducing tissue damage and promoting wound healing^[225]. This is mediated by an increase in IL-6 in the immediate postoperative phase and is characterised by an increased synthesis of acute phase proteins such as C-reactive protein (CRP). This increased production of acute phase reactants is facilitated by a decrease in hepatic albumin synthesis, leading to an increased CRP:albumin ratio. In response to this initial acute pro-inflammatory environment that arises following surgery, the body attempts to launch an anti-inflammatory response to restore homeostasis. The increased levels of cortisol that occur following surgery, as a result of stimulation by adrenocorticotrophic hormone and in response to rising IL-6, are believed to be responsible for this^[224]. If this increased stimulation of the Th2 response persists, the imbalance gives rise to an immunosuppressive state, also referred to as the catabolic anti-inflammatory response, which can lead to increased morbidity and mortality^[234].

The pro-inflammatory state following surgery in obese patients has been described in a limited number of studies. In a study of elective surgical cases it was found that, compared to non-obese patients, severely obese patients demonstrated higher levels of plasma IL-6 postoperatively and a greater fold change from baseline^[324]. A study in coronary artery bypass patients reported that the combination of elevated waist circumference and elevated pre-operative CRP and IL-6 levels was associated with postoperative atrial fibrillation^[194]. In another study by Tanaka *et al.* oesophagectomy patients with high visceral fat area (VFA $\geq 100\text{cm}^2$) were found to have higher maximal postoperative CRP levels and a longer duration of systemic inflammatory response syndrome than those with low VFA^[325]. The findings of these studies suggest that the systemic inflammation observed in obesity affects postoperative outcomes.

Whilst emerging evidence has linked the systemic inflammation and dysmetabolism associated with visceral obesity with altered postoperative outcomes, the assessment of inflammatory response has been limited to crude, readily available measures such as CRP. A comprehensive assessment of the postoperative inflammatory response in

obese patients, in terms of pro- and anti-inflammatory cytokine production and immune cell function, is warranted to elucidate what effect visceral obesity has on postoperative inflammation and recovery following major surgery.

5.1.1 Specific Aims

This study had two arms, retrospective and prospective.

The specific aim of the retrospective arm was to determine if visceral obesity and dysmetabolism are associated with an altered acute phase response, as measured by CRP and CRP:albumin, and incidence of complications following gastrointestinal resection.

The aim of the prospective arm was to determine the influence of visceral obesity and dysmetabolism on the postoperative inflammatory response. In a cohort of upper gastrointestinal cancer patients the specific aim was to:

- Investigate the impact of obesity on expression of inflammatory genes in specific immune cell populations pre and postoperatively.
- Measure the circulating levels of inflammatory cytokines in obese and non-obese patients pre and postoperatively.
- Assess the relationship between obesity and postoperative complications and postoperative clinical parameters as measured by respiratory rate, heart rate, temperature and blood pressure.

5.2 RESULTS

5.2.1 Patient Characteristics of Retrospective Cohort

Patient characteristics are detailed in Table 5.1 below. Anthropometric data was collected as described in 2.7.1. Visceral obesity was determined using the cut-offs $\geq 80\text{cm}^2$ for females and $\geq 160\text{cm}^2$ for males and metabolic syndrome was determined using the IDF criteria as detailed in section 2.7.4. The cohort consisted of 54 oesophagectomy patients and 63 colectomy patients. Oesophageal resection ($p < 0.003$) and neoadjuvant chemotherapy ($p < 0.001$) were more common amongst males. A postoperative complication was documented in 47 patients.

Table 5.1 Patient characteristics of cohort for retrospective analysis of postoperative inflammation

Patient Characteristics (n=117)	Males (n=81)	Females (n=36)	p value
Age (y), mean (range)	65.5 (45-88)	67.4 (42-94)	0.396
<i>Operation Type</i>			
Oesophagectomy, n (%)	45 (55.6%)	9 (25%)	0.003
Colectomy, n (%)	36 (44.4%)	27 (75%)	
Neoadjuvant CRT, n (%)	51 (63%)	8 (22.2%)	<0.001
Visceral Fat Area, mean (range)	179.7 (13.1-397.1)	97.2 (6.0-261.0)	<0.001
Viscerally Obese, n (%)	44 (54.3%)	19 (52.8%)	1.0
*Elevated waist circumference, n (%)	47 (61%)	21 (61.8%)	1.0
#BMI $\geq 30\text{kg/m}^2$, n (%)	13 (17.3%)	4 (12.1%)	0.578
^Metabolic Syndrome, n (%)	32 (42.1%)	15 (42.9%)	1.0
Postoperative complication, n (%)	35 (43.2%)	12 (33.3%)	0.414

CRT, chemoradiotherapy; BMI, Body mass index; * Waist circumference available on 77 males and 34 females; # BMI available on 75 males and 32 females; ^Metabolic Syndrome diagnosis available on 76 males and 35 females

5.2.2 Incidence of postoperative complications in retrospective cohort

In terms of postoperative complications, there was no difference in overall incidence, or incidence of specific complications between the obese and non-obese patients or between those with or without the metabolic syndrome (Table 5.2).

Table 5.2 Incidence of postoperative complications in retrospective cohort, separated by obesity and metabolic syndrome status.

Complication Type	Viscerally Obese			Metabolic Syndrome*		
	Non-Obese (n=54)	Obese (n=63)	<i>p</i>	No MetSyn (n=64)	MetSyn (n=47)	<i>p</i>
Mortality, n (%)	0 (0%)	1 (1.6%)	0.398	1 (1.6%)	0 (0%)	0.470
Any post-op complication, n (%)	22 (40.7%)	25 (39.7%)	0.907	28 (43.8%)	17 (36.2%)	0.422
Small bowel obstruction, n (%)	2 (3.7%)	2 (3.2%)	0.983	2 (3.1%)	2 (4.3%)	0.712
Anastomotic Leak, n (%)	1 (1.9%)	4 (6.3%)	0.161	2 (3.1%)	3 (6.4%)	0.377
Abdominal Collection, n (%)	0 (0%)	1 (1.6%)	0.310	1 (1.6%)	0 (0%)	0.401
Chylothorax, n (%)	1 (1.9%)	0 (0%)	0.320	1 (1.6%)	0 (0%)	0.401
Pneumothorax, n (%)	3 (5.6%)	1 (1.6%)	0.317	2 (3.1%)	2 (4.3%)	0.712
Pleural Effusion, n (%)	3 (5.6%)	4 (6.3%)	0.673	5 (7.8%)	2 (4.3%)	0.483
Atelectasis, n (%)	1 (1.9%)	3 (4.8%)	0.297	3 (4.7%)	1 (2.1%)	0.503
Organ Failure, n (%)	4 (7.4%)	3 (4.8%)	0.716	4 (6.3%)	2 (4.3%)	0.689
Arrhythmia, n (%)	1 (1.9%)	3 (4.8%)	0.297	2 (3.1%)	2 (4.3%)	0.712
Wound Infection, n (%)	2 (3.7%)	5 (7.9%)	0.239	3 (4.7%)	3 (6.4%)	0.665
Pneumonia, n (%)	6 (11.1%)	5 (7.9%)	0.777	6 (9.4%)	4 (8.5%)	0.940
Infectious complication, n (%)	13 (24%)	12 (19%)	0.815	13 (20.3%)	10 (21.3%)	0.830
Sepsis, n (%)	2 (3.7%)	2 (3.2%)	0.983	3 (4.7%)	1 (2.1%)	0.503

*MetSyn status undetermined on 6 patients as fasting lipids and glucose not reported on laboratory database

5.2.3 Postoperative expression of CRP and CRP:albumin in patients with no complications

CRP and albumin levels documented on the laboratory database were gathered pre-operatively and on days 1, 3, 7 and 14 postoperatively. In patients who experienced a normal recovery from surgery, with no recorded postoperative complications, significantly higher CRP levels were observed on day 1 postoperatively in obese

compared to non-obese patients ($p<0.01$). In addition, patients who were MetSyn positive had significantly higher CRP levels on day 1 postoperatively ($p<0.05$). No significant difference was observed in CRP:albumin ratio between obese and non-obese patients and between MetSyn positive and MetSyn negative patients postoperatively at any timepoint (Figure 5.1).

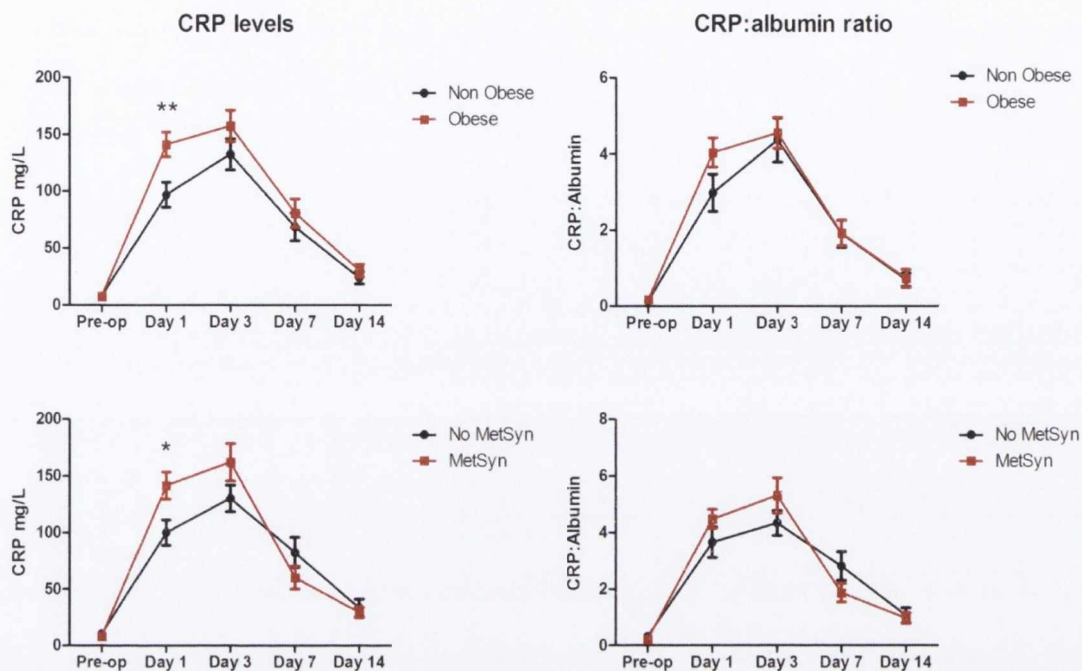


Figure 5.1 Changes in CRP and CRP:albumin ratio postoperatively in patients with no postoperative complications

Changes in CRP and CRP:albumin ratio were assessed pre and postoperatively in patients who experienced no postoperative complications. CRP and CRP:albumin ratio were expressed as mean total level on each day. Differences were assessed between obese and non-obese patients and between metabolic syndrome (MetSyn) positive and MetSyn negative patients using Mann Whitney U. Data are presented as mean $\pm$ SEM, * $p<0.05$; ** $p<0.01$.

5.2.4 Postoperative expression of CRP and CRP:albumin in patients who experienced a postoperative complication

A different pattern of acute inflammation was observed in patients who experienced a postoperative complication (Figure 5.2). No significant difference was observed in CRP or CRP:albumin between obese and non-obese patients pre-operatively or on day 1 and day 3 postoperatively. However, a significantly higher CRP level was observed in obese patients on day 7 postoperatively ($p<0.05$) and there was a trend towards significantly higher CRP:albumin ratio on day 7 postoperatively in the obese patients ($p=0.06$). Similar levels of CRP and CRP:albumin were observed in both groups on day 14 postoperatively (Figure 5.2). Data was also analysed with respect to MetSyn status. CRP levels were significantly higher in the MetSyn positive patients on days 7 and 14 postoperatively ($p<0.05$) and CRP:albumin ratio was significantly higher in the MetSyn positive patients on day 7 ($p<0.05$) and day 14 ($p<0.01$) postoperatively (Figure 5.2).

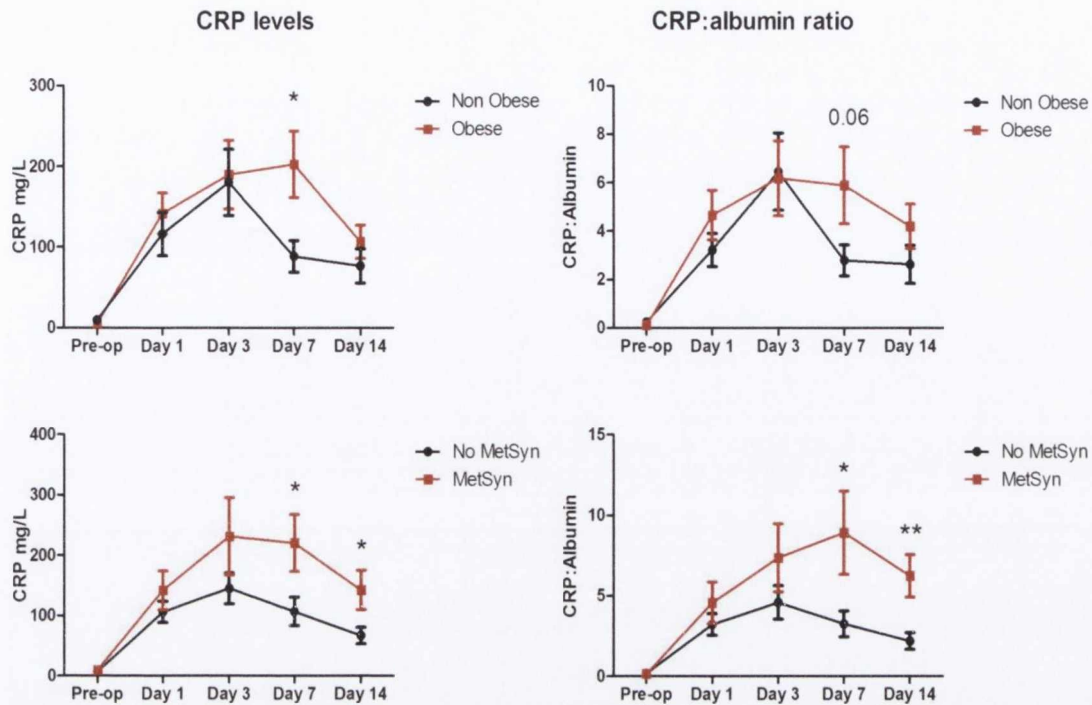


Figure 5.2 Changes in CRP and CRP:albumin ratio postoperatively in patients with postoperative complications

Changes in CRP and CRP:albumin ratio were assessed pre and postoperatively in patients who experienced a postoperative complication. CRP and CRP:albumin ratio were expressed as mean total level on each day. Differences were assessed between obese and non-obese patients and between metabolic syndrome (MetSyn) positive and MetSyn negative patients using Mann Whitney U. Data are presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$.

5.2.5 Prospective investigation into postoperative inflammatory response following upper gastrointestinal resection

In order to further assess the effect of visceral obesity and the metabolic syndrome on the inflammatory response following major resection, a cohort of 42 oesophagectomy and gastrectomy patients were prospectively recruited as detailed in section 2.8. Blood samples were collected preoperatively and on day 1, 3, 7 and 14 postoperatively as described in section 2.10.1. Characteristics of the patient cohort are detailed in Table 5.3 below. The cohort consisted of 29 males and 13 females. The females were significantly older ($p=0.026$) and included adenocarcinoma and squamous cell carcinoma cases whereas all of the male cohort were diagnosed with adenocarcinoma ($p=0.0003$). A postoperative complication occurred in 23 patients.

Table 5.3 Patient characteristics of prospectively recruited cohort

Patient Characteristics	Male (n=29)	Female (n=13)	<i>p</i>
Age, mean (range)	62.4 (29.5-86.3)	72.0 (61.6-78.1)	0.026
*Visceral Fat Area, mean (range)	136.3 (14.7-372.7)	75.7 (9.17-213.3)	0.029
*Visceral Obesity, n (%)	11 (40.7%)	6 (46.2%)	1.0
Elevated Waist Circumference, n (%)	16 (55.2%)	5 (38.5%)	0.506
[#] BMI ≥30kg/m ² #, n (%)	8 (30.8%)	2 (18.2%)	0.453
[^] Metabolic Syndrome [^] , n (%)	9 (32.1%)	5 (41.7%)	0.720
<i>Tumour Type, n (%)</i>			
Oesophageal SCC	0 (0%)	6 (46.2%)	0.0003
Oesophageal Adenocarcinoma	29 (100%)	7 (53.8%)	
<i>Operation Type, n (%)</i>			
Oesophagectomy	18 (63.1%)	10 (76.9%)	0.485
Gastrectomy	11 (37.9%)	3 (23.1%)	
Neoadjuvant CRT, n (%)	5 (17.2%)	2 (15.4%)	1.0
Postoperative Complication, n (%)	14 (48.3%)	9 (69.2%)	0.317

CRT, chemoradiotherapy; BMI, Body mass index; SCC, squamous cell carcinoma, * Visceral fat area available on 27 males and 13 females; # BMI available on 26 males and 12 females; ^Metabolic Syndrome diagnosis available on 27 males and 12 females.

5.2.6 Alterations in inflammatory gene expression in peripheral blood mononuclear cells (PBMC) in obese and non-obese patients.

To detect changes at the gene level between obese and non-obese patients postoperatively, a 370 gene inflammatory array (Appendix I) was ran using PBMC isolated pre-operatively and on day 1 and day 7 postoperatively (section 2.12). The test cohort consisted of 3 obese and 3 non-obese male patients who had undergone oesophagectomy for adenocarcinoma, had no neoadjuvant chemotherapy and no postoperative complications. The array revealed that several genes were altered in obese compared to non-obese patients preoperatively and postoperatively (Table 5.4). Preoperatively, 13 genes were down regulated and 3 genes were upregulated in the obese patients compared to non-obese patients. The chemokine gene CCL3L1 demonstrated an 87.7 fold decrease ($p=0.02$), whilst inhibin β -A (INHBA), azurocidin-1 (AZU1) and oxidized low-density lipoprotein receptor-1 (OLR1) were all >12 fold upregulated. On day 1 postoperatively, 5 genes were downregulated in the obese patients, with the greatest downregulation observed in fibronectin-1 (FN1) (5.6 fold decrease, $p=0.02$). On day 7 postoperatively, 6 genes were downregulated in the obese patients compared to the non-obese and 21 were upregulated. A >4 fold downregulation was observed in interferon- κ (IFNK) ($p<0.02$), whilst monoacylglycerol lipase (MGLL) demonstrated the greatest fold increase (5.7 fold increase, $p=0.01$).

Table 5.4 Significantly altered inflammatory genes in obese compared to non-obese patients preoperatively and on day 1 and day 7 postoperatively. Red indicates up-regulation and blue indicates down-regulation.

Preoperatively			Day 1 postoperatively			Day 7 postoperatively		
Gene	Fold change	<i>p</i>	Gene	Fold change	<i>p</i>	Gene	Fold change	<i>p</i>
CCL3L1	-87.7	0.02	FN1	-5.6	0.02	SPACA3	-5.0	0.00
GFRA2	-4.5	0.09	LEPR	-3.2	0.10	IFNK	-4.3	0.02
CCL8	-3.7	0.09	CXCL16	-2.8	0.06	TNFSF11	-4.0	0.09
CCR10	-3.7	0.10	IL19	-2.1	0.01	FN1	-2.7	0.05
CMTM2	-2.7	0.09	KITLG	-2.0	0.06	SRGAP1	-2.6	0.00
CCL4L1	-2.6	0.01				CCR2	-2.1	0.00
C3	-2.6	0.05				CCL5	2.0	0.01
IL7	-2.5	0.02				GAPDH	2.0	0.03
SIVA1	-2.4	0.09				CMTM1	2.0	0.06
CCL28	-2.2	0.08				CXCL11	2.1	0.09
CCL4	-2.1	0.05				EPOR	2.1	0.07
IL2RA	-2.0	0.02				PRDX5	2.1	0.03
CD4	-2.0	0.02				IL3	2.1	0.06
INHBA	12.8	0.01				NCR3	2.3	0.01
AZU1	14.5	0.09				CCR6	2.3	0.09
OLR1	37.6	0.08				LTA	2.4	0.06
						EDA	2.4	0.03
						TLR10	2.5	0.07
						S100A8	2.5	0.07
						FLT3LG	2.6	0.04
						TYMP	3.0	0.04
						LTB	3.1	0.09
						MDK	3.9	0.10
						MPL	4.5	0.04
						CCL23	4.6	0.07
						PPBP	4.7	0.06
						MGLL	5.7	0.01

5.2.7 Validation of significantly altered genes of interest by qPCR.

Given the magnitude of difference between the obese and non-obese patients the genes CCL3L1, Interferon-kappa (IFNK), Inhibin β -A (INHBA) and MGLL were selected for validation in a separate cohort of 5 obese and 5 non obese patients at all timepoints by qPCR (as detailed in section 2.14). IFNK, a member of the type 1 interferon family and is involved in host defence against viral infection ^[326], was undetectable by qPCR in obese and non-obese patients at all timepoints. CCL3L1 is a ligand of the CCR5 receptor, a chemokine receptor expressed by T cells and macrophages that is involved in regulating the migration of activated and effector T cells ^[327]. Expression of CCL3L1 was lower in the obese patients preoperatively and demonstrated a marked decrease on day 1 postoperatively. Levels increased thereafter and returned to preoperative levels by day 14. No significant difference was observed between obese and non-obese CCL3L1 gene expression at any timepoint. Whilst the test cohort revealed a significant upregulation of INHBA in obese patients preoperatively (12.8 fold increase, $p=0.01$), this was not observed in the validation cohort. A greater fold change was observed in the non-obese patients on day 3, day 7 and day 14 compared to the obese but these differences were not significant. MGLL is involved in controlling the endocannabinoid system and blockade of MGLL has been associated with reduction in inflammation ^[328, 329]. No significant difference in MGLL gene expression was observed between obese and non-obese patients; however a greater fold change was demonstrated in the obese cohort on day 7 postoperatively as was observed in the test cohort (Figure 5.3).

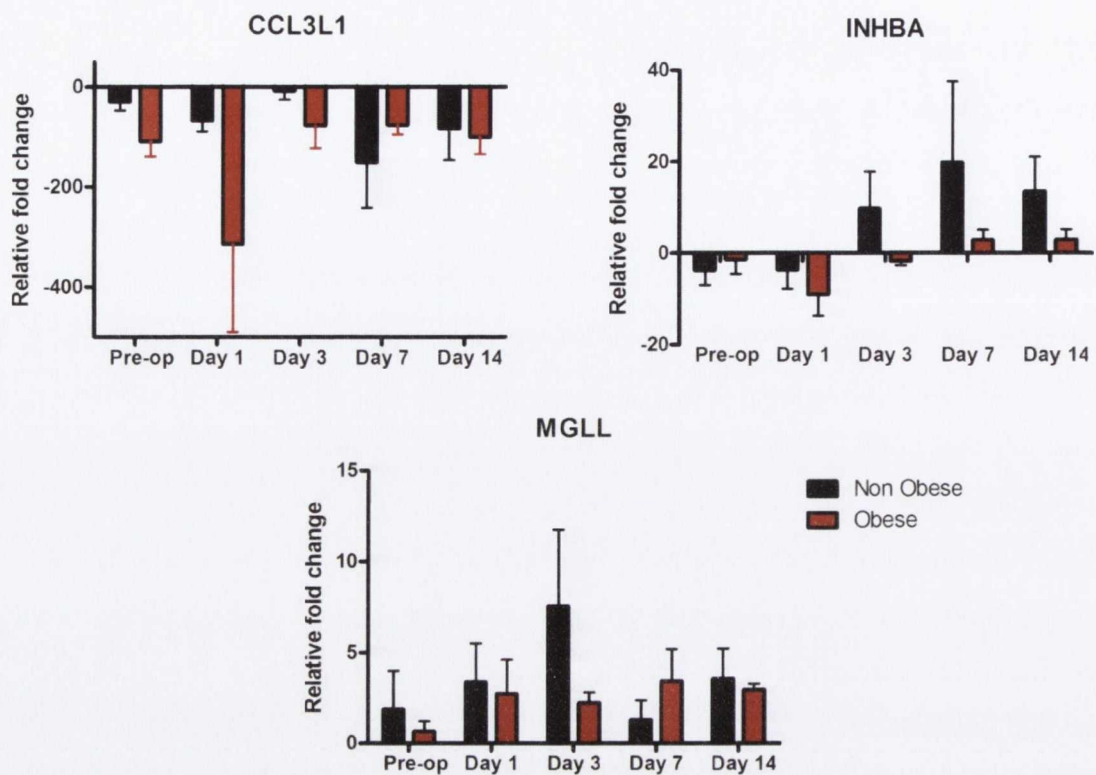


Figure 5.3 Validation of gene expression of CCL3L1, INHBA and MGLL in PBMC from obese and non-obese patients pre and postoperatively

The expression of CCL3L1, INHBA and MGLL was validated by qPCR in a cohort of 5 obese and 5 non-obese patients using PBMC isolated preoperatively and on days 1, 3, 7 and 14 postoperatively. All samples were assayed in triplicate. Data was analysed using the $2^{-\Delta\Delta CT}$ method with 18S serving as an endogenous control and data is expressed as relative fold change. Differences in gene expression between obese and non-obese patients were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM.

5.2.8 Investigation of TNFSF-14, LT β and LT β R gene expression in obese and non-obese patients by qPCR.

Tumour necrosis factor super family-14 (TNFSF14), lymphotoxin- β (LT β) and LT β receptor (LT β R) were also selected for validation in a cohort of 9 obese and 9 non-obese patients by qPCR as described in section 2.14. This is an emerging triad of inflammatory mediators in obesity ^[330-332]. LT β R is abundant on immune cells with TNFSF14 and lymphotoxin its only recognised ligands ^[333]. Binding of TNFSF14 and LT β to LT β R leads to activation of NF- κ B pathways ^[334] and inhibition of LT β R signalling has been shown to be effective in numerous inflammatory and autoimmune disorders ^[333].

Levels of TNFSF14 increased postoperatively in both the obese and non-obese patients (6 and 4-fold respectively) and declined thereafter. Conversely, levels of LT β decreased in both cohorts postoperatively. Levels subsequently rose, to above pre-operative levels in the non-obese cohort only. In the case of LT β R, a sharper increase in levels postoperatively was observed in the non-obese patients on day 1 postoperatively. Levels declined in both cohorts thereafter, returning to preoperative levels by day 14 (Figure 5.4).

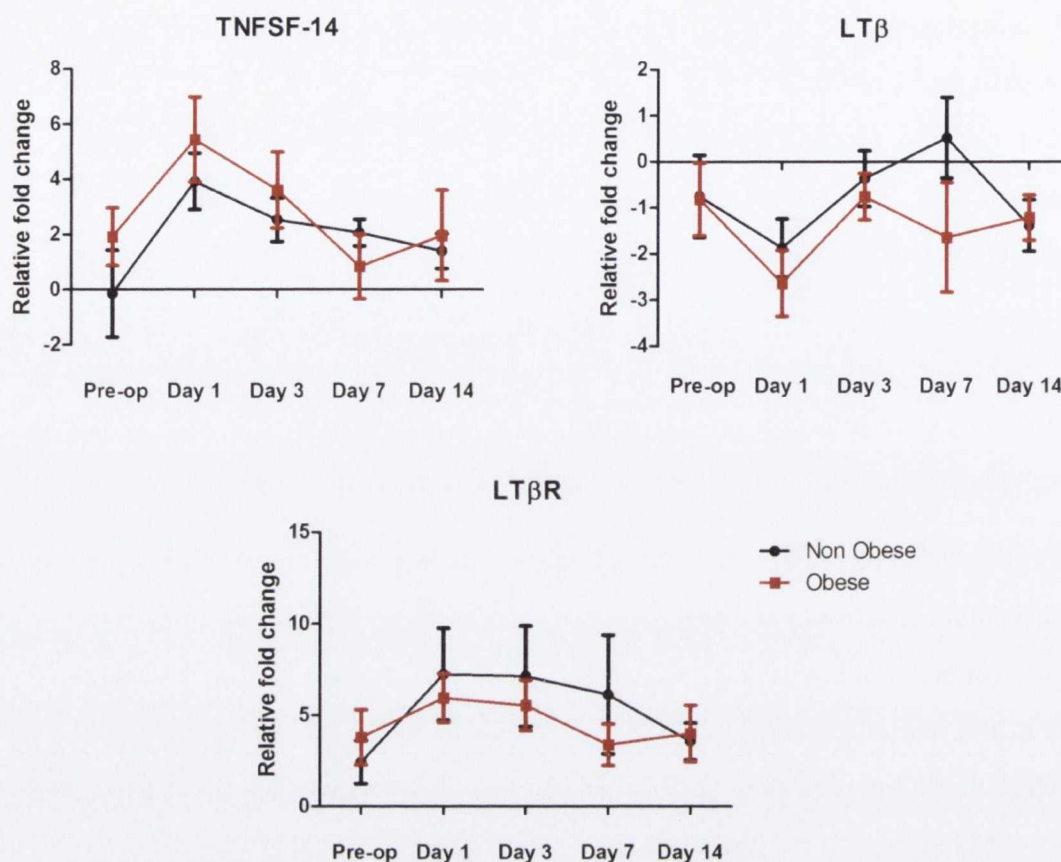


Figure 5.4 Gene expression of TNFSF14, LTβ and LTβR in PBMC from obese and non-obese patients pre and postoperatively

The expression of TNFSF14, LTβ and LTβR was assessed by qPCR in a cohort of 9 obese and 9 non-obese patients using PBMC isolated preoperatively and on days 1, 3, 7 and 14 postoperatively. All samples were assayed in triplicate. Data was analysed using the $2^{-\Delta\Delta CT}$ method with 18S serving as an endogenous control and data is expressed as relative fold change. Differences in gene expression between obese and non-obese patients were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM.

5.2.9 Expression of neutrophil activation markers in obese and non-obese patients pre and postoperatively

Neutrophils are considered the first line of defence in the acute inflammatory response and their function may be altered in obesity and the metabolic syndrome. To investigate neutrophil function further in this cohort, neutrophils were isolated from whole blood collected preoperatively and on days 1, 3, 7 and 14 postoperatively as described in section 2.10.3. The purity of neutrophils isolated was determined by flow cytometry as described in section 2.10.5 (Figure 5.5).

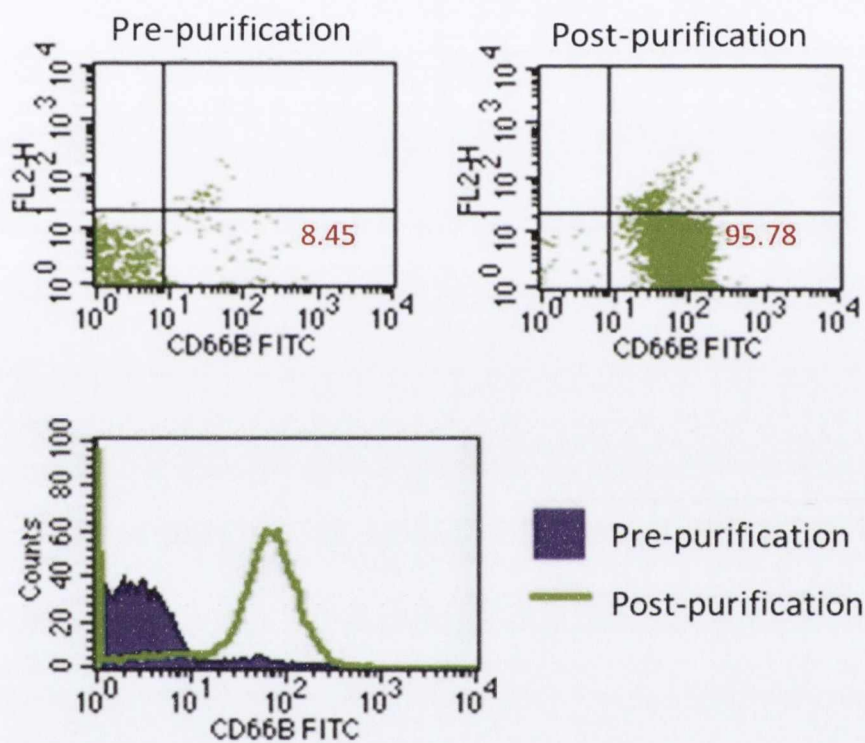


Figure 5.5 Assessment of neutrophil purity by flow cytometry following isolation.

Neutrophils were isolated from whole blood using a dextran sedimentation technique. The purity of isolated neutrophils was assessed by flow cytometry, using CD66b as a positive marker for neutrophils. The flow cytometry dot plots and histogram illustrate purity of neutrophil population (lower right quadrant) pre and post purification. Numbers in lower right quadrant represent % of CD66b positive cells gated on live cell population.

Myeloperoxidase (MPO) is an enzyme released from the neutrophil's azurophilic granules which mediates the cytotoxic killing of pathogens, thus it is a marker of activation ^[335]. CD11b and CD62L are involved in the adherence of neutrophils to endothelial cells, the first step in extravasation ^[336]. The expression of MPO, CD62L, CD11b by neutrophils was measured by qPCR (section 2.15) in samples collected from 3 obese and 3 non-obese patients preoperatively and on day 1, 3, 7 and 14 postoperatively. Similar levels of CD62L were observed in obese and non-obese patients, with levels in both cohorts dropping in the initial postoperative period before rising again to levels approximately 2-fold greater than preoperative levels. Similarly, levels of CD11b rose steadily in the postoperative period in both obese and non-obese patients. In the case of MPO, fluctuating levels were observed postoperatively but no significant difference was observed between the obese and non-obese patients (Figure 5.6).

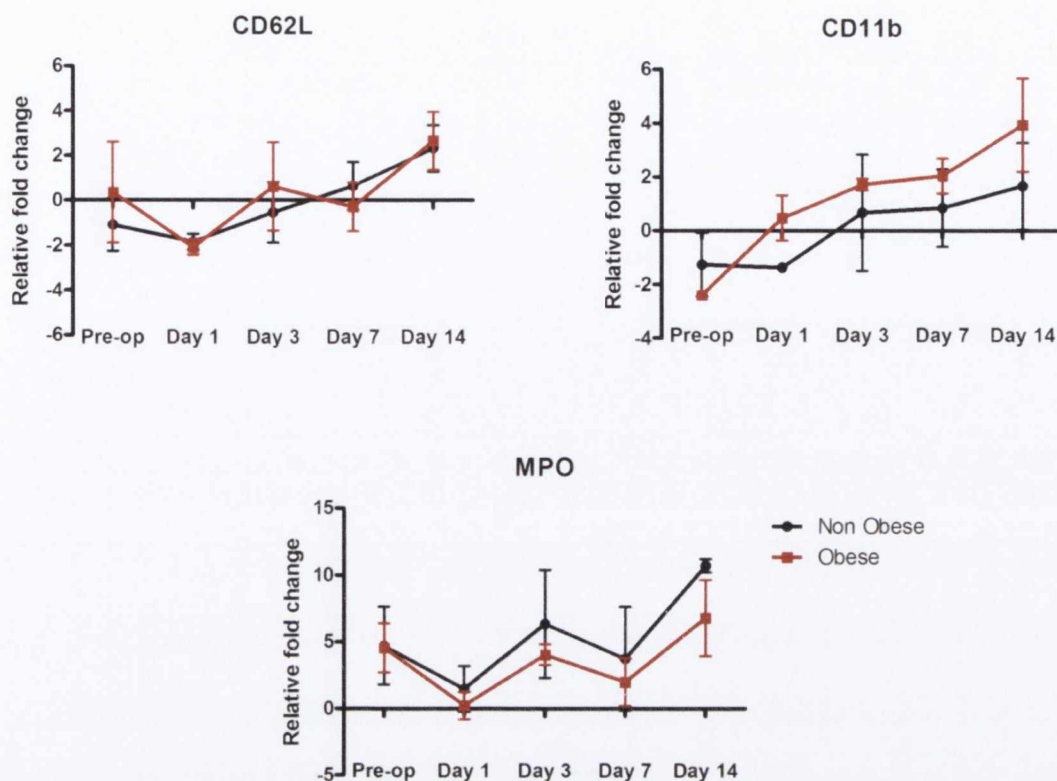


Figure 5.6 Gene expression of CD62L, CD11b and MPO by neutrophils from obese and non-obese patients pre and postoperatively

The gene expression of CD62L, CD11b and MPO was assessed by qPCR in a cohort of 3 obese and 3 non-obese patients using neutrophils isolated preoperatively and on days 1, 3, 7 and 14 postoperatively. All samples were assayed in triplicate. Data was analysed using the $2^{-\Delta\Delta CT}$ method with 18S serving as an endogenous control and data is expressed as relative fold change. Differences in gene expression between obese and non-obese patients were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM.

5.2.10 NF-κβ p65 in non-obese and obese patients in the postoperative period

Central to the regulation of many genes involved in immune and inflammatory responses is the transcription factor NF-κβ, which itself is activated by inflammatory cytokines ^[337]. NF-κβ p65 subunit was assessed in 3 obese and 4 non-obese oesophagectomy patients from nuclear extracts collected at baseline and on days 1, 3, 7 and 14 postoperatively (section 2.10.3 and 2.15). No significant difference was observed in NF-κβ p65 between non-obese and obese patients at any time-point. Interestingly, a significant drop in NF-κβ p65 was observed between baseline and day 1 ($p<0.05$), with a subsequent rise on day 3 ($p<0.05$) in non-obese patients. No significant drop was observed in obese patients (Figure 5.7).

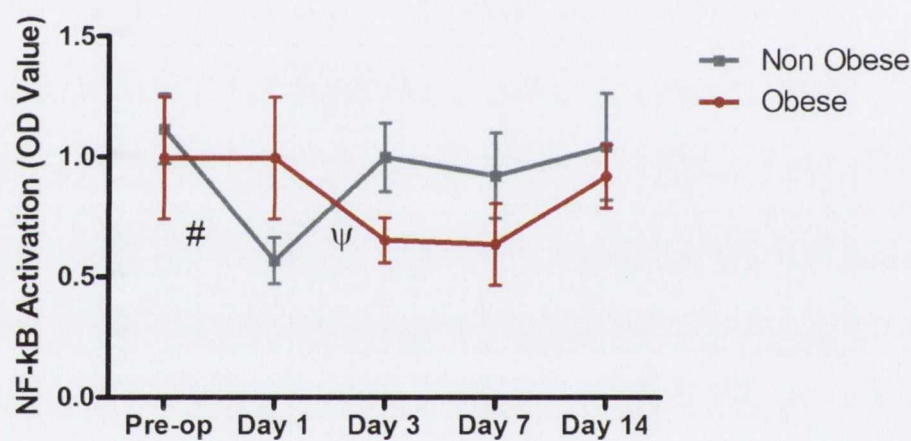


Figure 5.7 NF-κβ p65 in non-obese and obese patients pre and postoperatively

NF-κβ p65 sub-unit was measured semi-quantitatively by ELISA in nuclear extracts isolated from PBMC, collected preoperatively and on day 1, 3, 7 and 14 postoperatively from obese ($n=3$) and non-obese ($n=4$) patients. All samples were assayed in triplicate. Differences in expression between time-points and between obese and non-obese patients was assessed by ANOVA and Mann Whitney U test respectively. Data are presented as mean ± SEM; # $p<0.05$ between pre-op and day 1 in non-obese patients; ψ $p<0.05$ between day 1 and day 3 in non-obese patients.

5.2.11 Circulating levels of inflammatory mediators in obese and non-obese patients postoperatively.

Changes in the postoperative production of well-defined inflammatory cytokines in patients were assessed at the protein level by ELISA as described in section 2.16. A panel of both pro- and anti-inflammatory cytokines was investigated: IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17, IFN- γ , TNF- α and TGF- β . Results were analysed by both obesity and metabolic syndrome status.

Altered patterns of cytokine expression were observed in obese patients compared to non-obese patients pre- and postoperatively. In terms of innate inflammatory response (Figure 5.8), in all patients the levels of IL-6 increased sharply on day 1 postoperatively as previously reported. However the increase was greater in the obese cohort and levels remained higher in the postoperative period. In the case of TNF- α , the same levels were observed in obese and non-obese patients at baseline, however significantly higher levels were observed in the obese patients on day 1 postoperatively ($p < 0.05$). A divergent pattern of expression was observed postoperatively with levels increasing in the obese patients on day 1 postoperatively before declining, whereas non-obese patients demonstrated a fall in TNF- α initially postoperatively, with levels rising on day 7. Significantly higher levels of IL-12p70 were observed in obese preoperatively and on postoperative day 1, day 7 and day 14 ($p < 0.05$). The fluctuation in IL-12p70 levels postoperatively (initially declining sharply before rising again on day 7) was in contrast to the non-obese patients who demonstrated little change in IL-12p70 levels. Fluctuations were also observed in IL-8 levels in obese patients postoperatively, with a sharp decline in levels observed between day 3 and day 7.

In the adaptive inflammatory response (Figure 5.9), IFN- γ levels rose on day 1 in the obese patients, which was in contrast to the decrease in levels postoperatively observed in the non-obese patients. Conversely, higher levels of IL-17 were observed in the non-obese patients at all timepoints, with no detected expression in the obese patients on day 1, 3 or 7 postoperatively.

In the case of the anti-inflammatory response (Figure 5.10), higher levels of IL-4 were observed in the obese patients preoperatively, with levels sharply declining thereafter,

whereas negligible levels of IL-4 were detected in the non-obese patients. IL-10 levels did not change significantly in either cohort postoperatively. TGF- β levels were significantly higher in the obese patients pre-operatively ($p<0.01$) and on day 1 ($p<0.01$) and day 7 postoperatively ($p<0.001$).

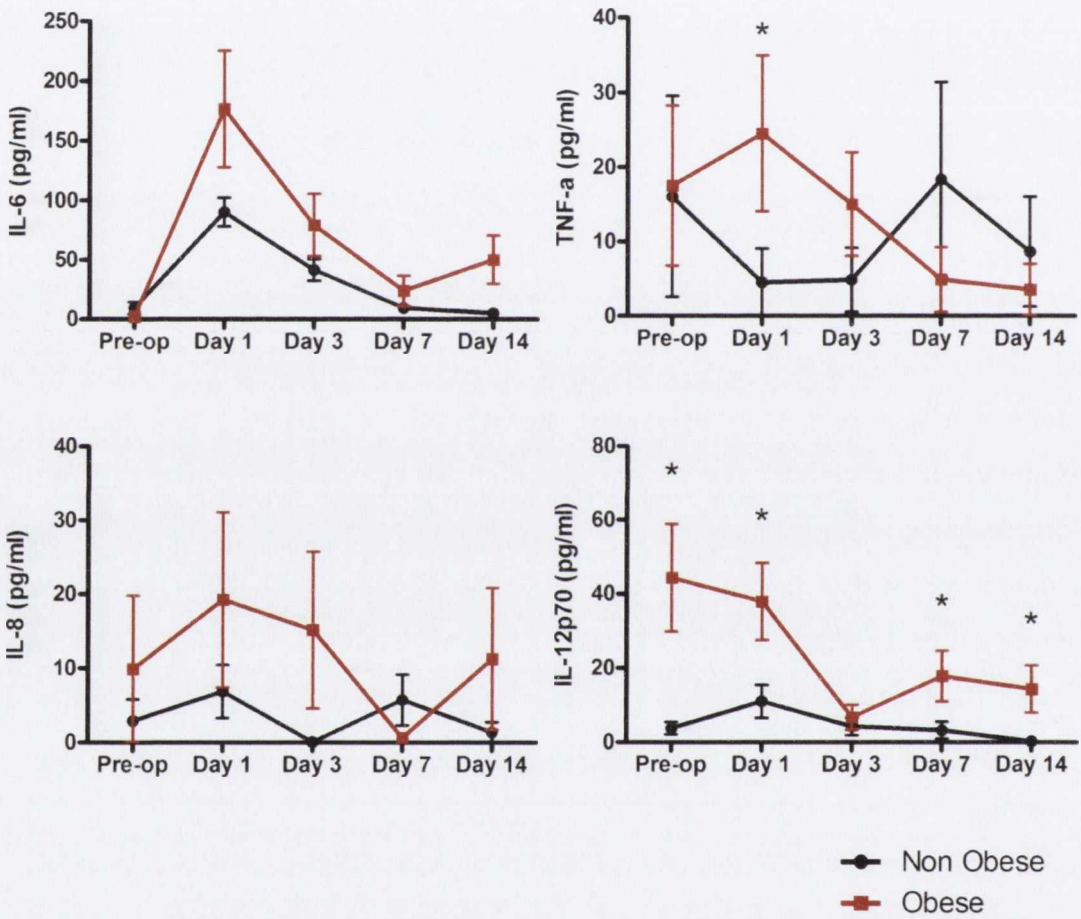


Figure 5.8 Altered innate inflammatory response in obese patients postoperatively.

The expression of IL-6, TNF- α , IL-8 and IL-12p70 was assessed in 16 obese and 15 non-obese patients preoperatively and on days 1, 3, 7 and 14 postoperatively by ELISA. All samples were assayed in triplicate. Differences between expression level in obese and non-obese patients was assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM; * $p<0.05$

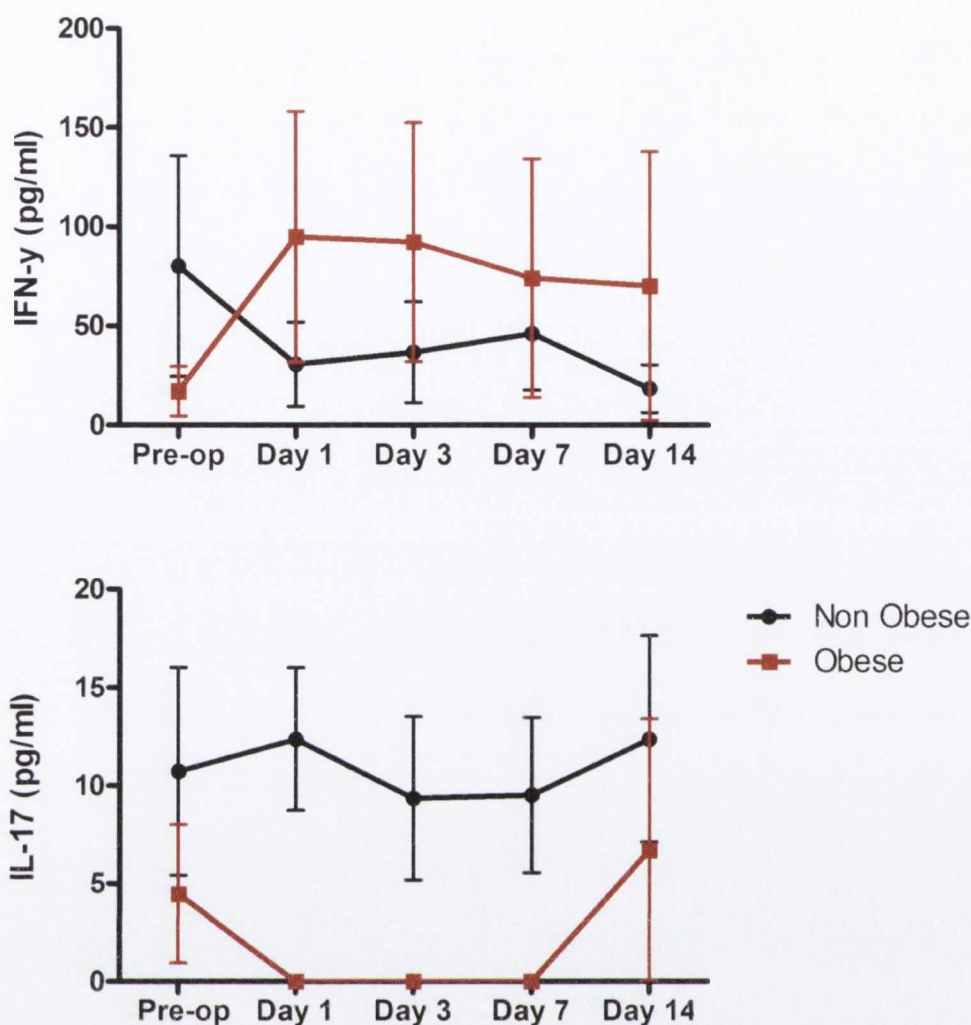


Figure 5.9 Altered adaptive inflammatory response in obese and non-obese patients postoperatively

The expression of IFN- γ and IL-17 was assessed in obese and non-obese patients preoperatively and on days 1, 3, 7 and 14 postoperatively by ELISA (n=16 obese and 15 non-obese for IFN- γ ; n=5 obese and 7 non-obese for IL-17). All samples were assayed in triplicate. Differences between expression level in obese and non-obese patients was assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM.

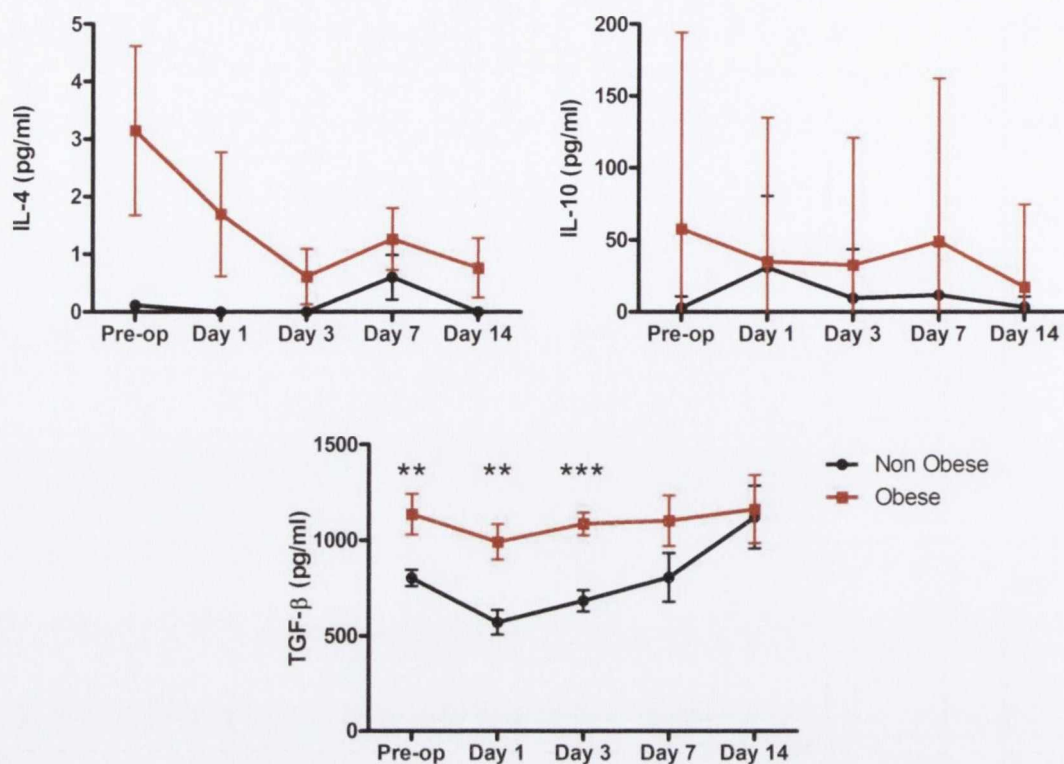


Figure 5.10 Alterations in anti-inflammatory response in obese and non-obese patients postoperatively

The expression of IL-4, IL-10 and TGF- β was assessed in obese and non-obese patients preoperatively and on days 1, 3, 7 and 14 postoperatively by ELISA (IL-4 and TGF- β , n=5 obese and 7 non-obese; IL-10, n=16 obese and 15 non-obese). All samples were assayed in triplicate. Differences between expression level in obese and non-obese patients was assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM; **p<0.01, ***p<0.001

5.2.12 Circulating levels of inflammatory mediators in MetSyn positive and MetSyn negative patients postoperatively.

When the cohort was separated based on MetSyn status, a different pattern of cytokine expression was observed between MetSyn positive and MetSyn negative patients. When the innate inflammatory response was assessed (Figure 5.11), similar levels of IL-6 were observed in both cohorts on day 1 postoperatively but there was higher levels of IL-6 in MetSyn positive patients on day 3 postoperatively. IL-12p70 levels were higher in the MetSyn positive patients preoperatively and on day 1 postoperatively. Levels fell thereafter before rising again in the MetSyn positive patients on day 14 postoperatively ($p=0.07$). TNF- α levels were lower in MetSyn patients pre- and postoperatively. A sharp increase in IL-8 levels was observed on day 1 postoperatively in the MetSyn positive cohort, with levels declining thereafter, and falling below the MetSyn negative patients on day 7.

In terms of adaptive inflammatory response (Figure 5.12), IL-17 levels were lower in MetSyn patients pre- and postoperatively. Interestingly, opposing levels of IFN- γ were observed in patients with or without MetSyn, with MetSyn negative patients demonstrating a decrease in levels postoperatively, with levels rising again on day 7. Conversely, MetSyn positive patients demonstrated an increase in expression postoperatively, with levels declining between day 3 and day 7.

In the anti-inflammatory response (Figure 5.13), no IL-4 expression was demonstrated in the MetSyn positive patients preoperatively or on days 1 and 3 postoperatively. In both cohorts, IL-4 levels peaked on day 7. IL-10 levels did not change significantly in either cohort postoperatively. In the case of TGF- β , levels were significantly higher in the MetSyn positive patients preoperatively and on day 1 and day 3 postoperatively ($p<0.05$).

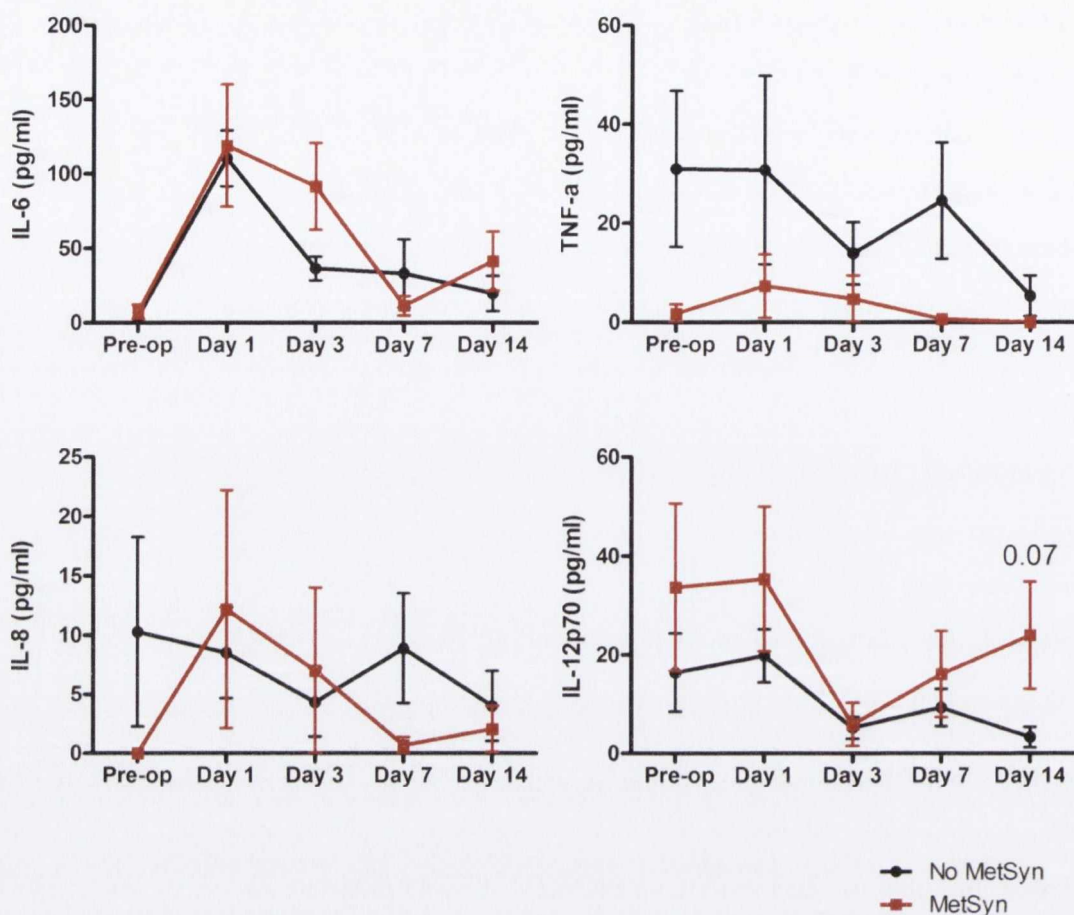


Figure 5.11 Alterations in innate inflammatory response postoperatively in MetSyn positive and MetSyn negative patients

The expression of IL-6, TNF- α , IL-8 and IL-12p70 was assessed in 13 MetSyn positive and 19 MetSyn negative patients preoperatively and on days 1, 3, 7 and 14 postoperatively by ELISA. All samples were assayed in triplicate. Differences between expression level in MetSyn positive and MetSyn negative patients was assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM

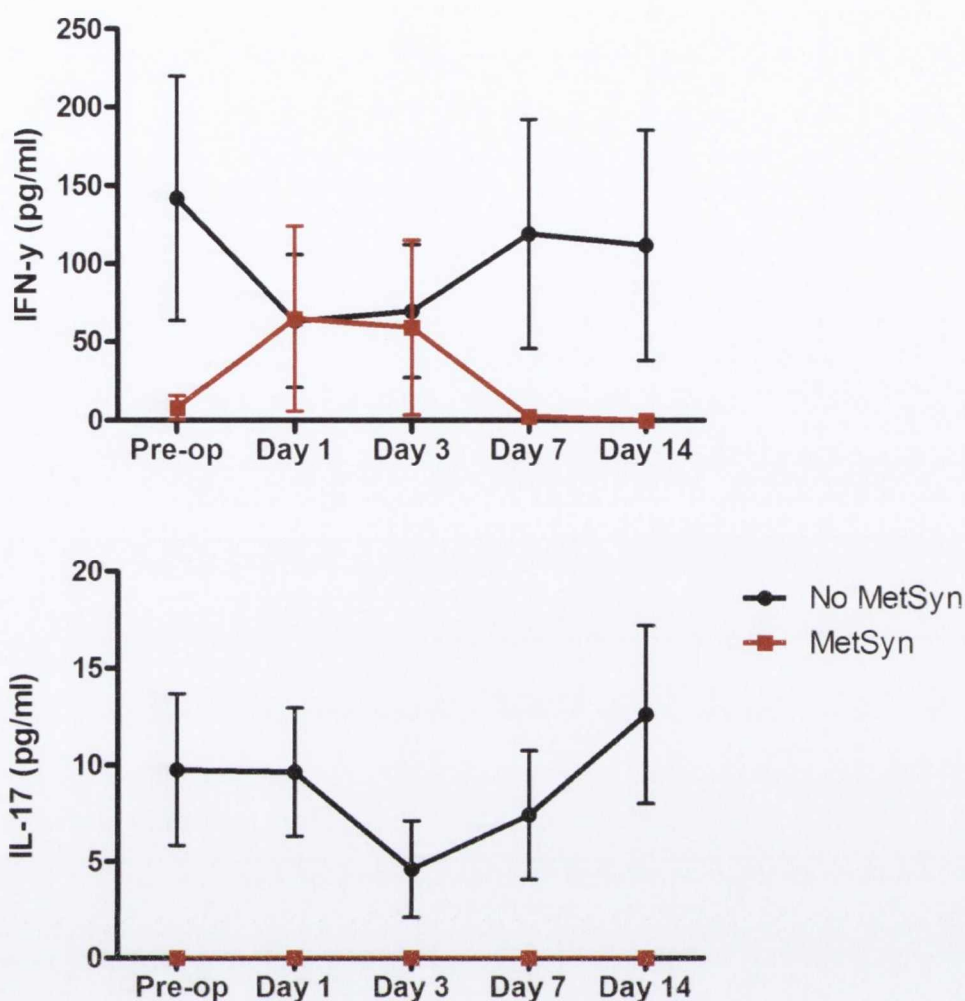


Figure 5.12 Alterations in adaptive inflammatory response postoperatively in MetSyn positive and MetSyn negative patients

The expression of IFN- γ and IL-17 was assessed in MetSyn positive and MetSyn negative patients preoperatively and on days 1, 3, 7 and 14 postoperatively by ELISA (n=12 MetSyn positive and 18 MetSyn negative for IFN- γ ; n=2 MetSyn positive and 10 MetSyn negative for IL-17). All samples were assayed in triplicate. Differences between expression level in MetSyn positive and MetSyn negative patients were assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM

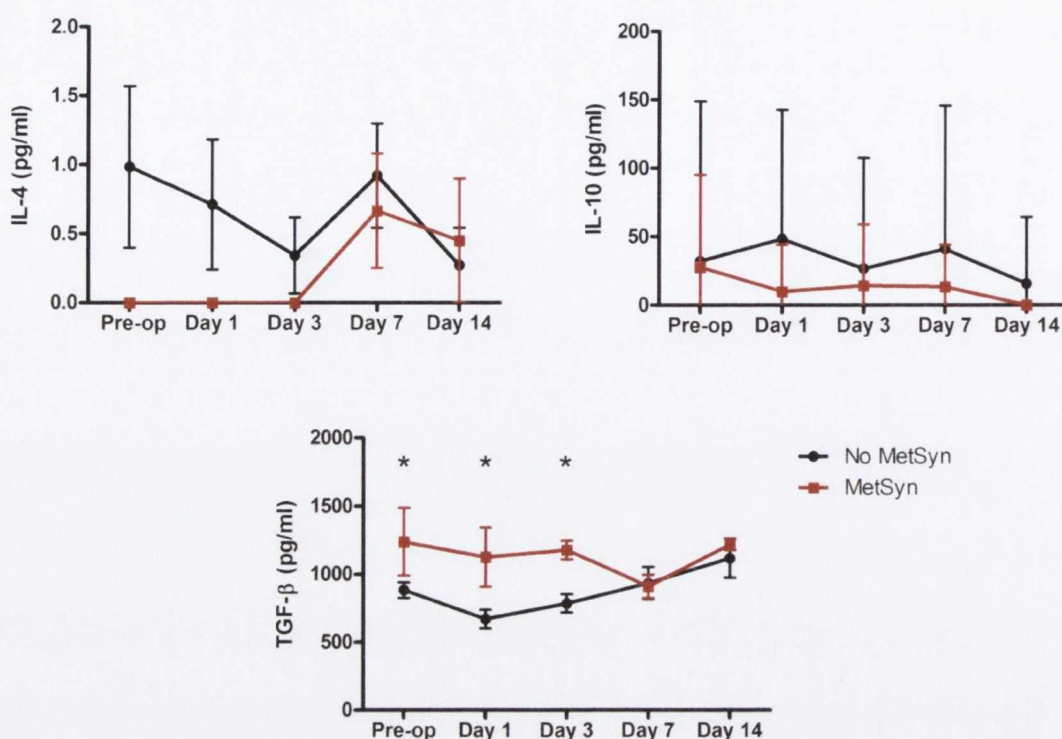


Figure 5.13 Alterations in anti-inflammatory response postoperatively in MetSyn positive and MetSyn negative patients

The expression of IL-4, IL-10 and TGF- β was assessed in MetSyn positive and MetSyn negative patients preoperatively and on days 1, 3, 7 and 14 postoperatively by ELISA (n=2 MetSyn positive and 10 MetSyn negative for IL-4 and TGF- β ; n=12 MetSyn positive and 18 MetSyn negative for IL-10). All samples were assayed in triplicate. Differences between expression level in MetSyn positive and MetSyn negative patients were assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM, *p<0.05

5.2.13 Influence of hyperglycaemia on circulating levels of inflammatory mediators postoperatively.

To assess the possible impact of glycaemic control on inflammatory response, the cohort was separated into patients who were normoglycaemic and patients who were hyperglycaemic preoperatively (Figure 5.14). Higher levels of IL-6 were observed in the hyperglycaemic patients in the initial postoperative period. Similarly, higher levels of IL-8 were observed in the hyperglycaemic patients at all time-points. Conversely, no difference was observed in TNF- α and IL-12p70 levels between the cohorts. In terms of adaptive inflammation, higher levels of IFN- γ were observed in the hyperglycaemic patients at all timepoints. In contrast, higher levels of IL-17 were observed in the normoglycaemic patients on day 1 postoperatively. The anti-inflammatory response was also altered in the hyperglycaemic patients, with higher levels of IL-4 observed preoperatively and on day 1 and day 3 postoperatively. Similarly, significantly higher levels of TGF- β were observed in the hyperglycaemic patients on day 1 postoperatively. No difference was observed in IL-10 levels between the two groups.

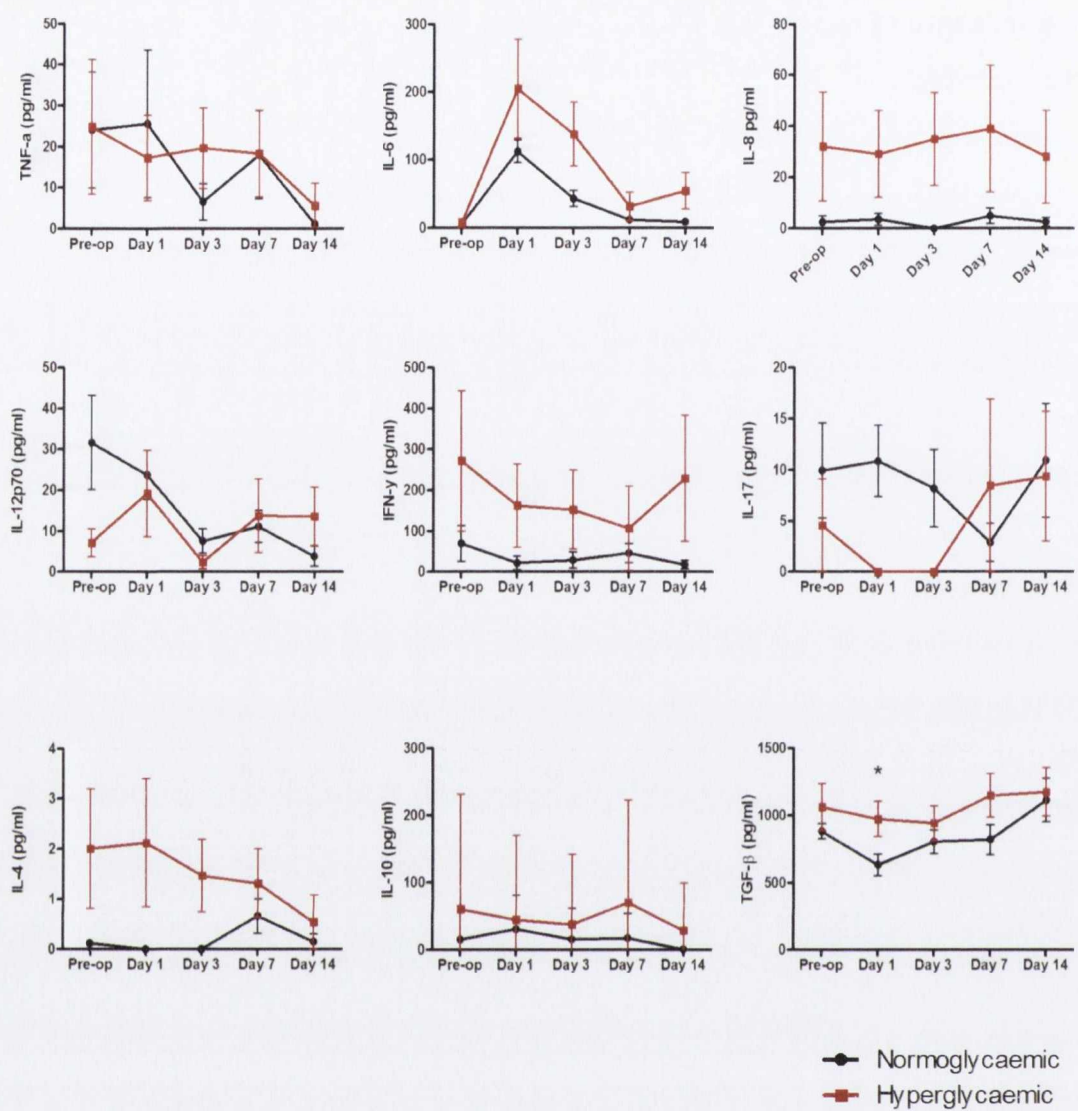


Figure 5.14 Alterations in postoperative inflammatory response in hyperglycaemic and normoglycaemic patients

The expression of a panel of pro- and anti-inflammatory cytokines was assessed in patients who were dichotomised based on preoperative glycaemic status. Levels were assessed preoperatively and on days 1, 3, 7 and 14 postoperatively. All samples were assayed in triplicate. Differences between expression level in hyperglycaemic and normoglycaemic patients were assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM, * $p < 0.05$

5.2.14 Postoperative changes in clinical parameters.

Heart rate, temperature, respiratory rate, systolic and diastolic blood pressure were routinely measured and recorded by nursing staff using standard clinical procedures perioperatively. The data pertaining to these clinical parameters was collected preoperatively and on days 1, 3, 7 and 14 postoperatively in a subset of 13 obese and 11 non-obese patients (Figure 5.15). A higher maximal body temperature was observed in the obese compared to the non-obese patients on day 1 postoperatively (37.6°C compared to 37.1°C, $p=0.07$). Respiratory rate was increased in both cohorts on day 1 postoperatively, as expected. Heart rate also increased postoperatively, with an increased heart rate in obese patients on day 3 postoperatively. Patterns of systolic and diastolic blood pressure changed postoperatively, with systolic rising and diastolic falling leading to an increase in systolic:diastolic ratio. Obese patients demonstrated a lower systolic:diastolic ratio on day 1 postoperatively ($p=0.056$) and a drop in systolic blood pressure on day 7 ($p<0.05$).

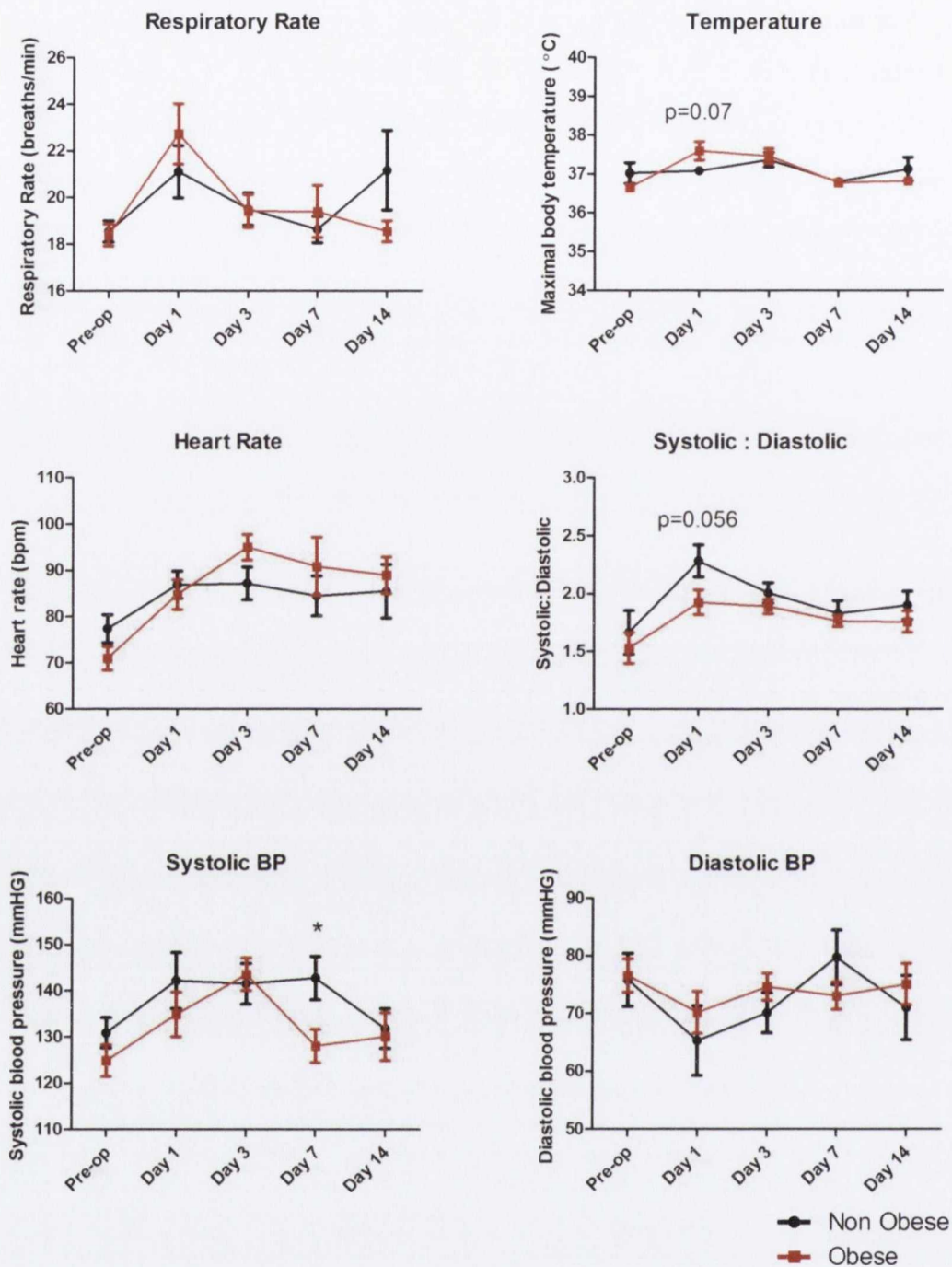


Figure 5.15 Changes in clinical parameters postoperatively in obese and non-obese patients

Data on the clinical parameters of heart rate, respiratory rate, temperature, systolic and diastolic blood pressure were collected preoperatively and on days 1, 3, 7 and 14 postoperatively in 13 obese and 11 non-obese patients. Changes between the obese and non-obese patients were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM, $*p<0.05$.

5.2.15 Incidence of postoperative complications in prospectively recruited cohort

The incidence of postoperative complications was assessed in the prospectively recruited cohort using data recorded in the Upper GI Database. Data was analysed with respect to obesity and MetSyn status. A postoperative complication was reported in 23 patients. There was no difference in overall incidence or incidence of specific complications between obese and non-obese patients or between those with or without the MetSyn (Table 5.5).

Table 5.5 Incidence of postoperative complications in the prospectively recruited upper GI cancer cohort (2010-2011).

Complication Type	Viscerally obese			Metabolic Syndrome*		
	Non-Obese (n=25)	Obese (n=17)	<i>p</i>	No MetSyn (n=26)	MetSyn (n=14)	<i>p</i>
Mortality, n (%)	0 (0%)	1 (5.8%)	0.220	0 (0%)	1 (7.1%)	0.168
Any post-op complication, n (%)	15 (60%)	8 (47%)	0.408	12 (46.1%)	9 (6.4%)	0.273
Anastomotic Leak, n (%)	2 (8%)	0 (0%)	0.506	0 (0%)	2 (14.3%)	0.117
Pneumothorax, n (%)	4 (16%)	2 (1.2%)	0.700	2 (7.7%)	3 (21.4%)	0.210
Pleural Effusion, n (%)	3 (12%)	1 (5.8%)	0.507	1 (3.8%)	3 (21.4%)	0.077
Respiratory Failure, n (%)	2 (8%)	1 (5.8%)	0.794	2 (7.7%)	1 (7.1%)	0.950
Arrhythmia, n (%)	5 (20%)	2 (1.2%)	0.482	2 (7.7%)	3 (21.4%)	0.210
Wound Infection, n (%)	0 (0%)	1 (5.8%)	0.220	1 (3.8%)	0 (0%)	0.457
Pneumonia, n (%)	2 (8%)	1 (5.8%)	0.794	2 (7.7%)	1 (7.1%)	0.950
Infectious complication, n (%)	3 (12%)	3 (17.6%)	0.608	4 (15.4%)	2 (14.3%)	0.926
Sepsis, n (%)	2 (8%)	1 (5.8%)	0.794	2 (7.7%)	1 (7.1%)	0.950

*MetSyn status undetermined on 2 patients as fasting lipids and glucose not reported on laboratory database

5.3 DISCUSSION

In this study we examine, for the first time, the impact of visceral obesity and the metabolic syndrome on the postoperative inflammatory response. The inflammatory response is characterised at the gene and protein level using an array of inflammatory markers.

The retrospective section of this work generated interesting findings with respect to the differential influence of obesity and the metabolic syndrome on the inflammatory response in patients with or without postoperative complications. In patients who experienced a normal postoperative recovery, CRP and CRP:albumin was increased on day 1 postoperatively in the obese patients, suggesting that following surgery a heightened acute inflammatory response is experienced by obese patients. However when the patients who experienced postoperative complications were analysed, increased CRP and CRP:albumin was observed in the obese and dysmetabolic patients on day 7 and day 14 postoperatively. Given these findings it may be speculated that the inflammatory response to a postoperative complication is more extreme in obese patients and those with the MetSyn. Whilst no difference was observed in the incidence of postoperative complications between obese and non-obese patients or between those with or without the metabolic syndrome, the finding that the inflammatory response is exaggerated in these patients might suggest a more exacerbated presentation of complications. The findings of this study are comparable to those reported by Tanaka *et al.* where it was observed that viscerally obese oesophagectomy patients demonstrated higher maximal CRP postoperatively and a prolonged systemic inflammatory response ^[325]. In addition, Donatelli *et al* reported higher postoperative levels of CRP in patients with the metabolic syndrome ^[338].

CRP and CRP:albumin are recognized as markers of inflammation and are routinely measured in surgical patients making them valuable for retrospective studies. However, inflammation is a complex process and a single marker, examined in isolation, does not provide sufficient information. In the prospective arm of this research the aim was to identify additional markers that are altered in obese patients postoperatively. There is a paucity of research investigating the inflammatory response in obese patients or those with the MetSyn, thus this study provides an important and valuable insight into this area.

The 370 gene inflammatory array revealed a number of different genes that were up or downregulated in obese compared to non-obese patients on day 1 and day 7 postoperatively. CCL3L1 was downregulated postoperatively with non-significantly lower levels in obese patients pre-operatively and in the immediate postoperative period. CCL3L1 is a ligand of the CCR5 receptor, a chemokine receptor expressed by T cells and macrophages that is involved in regulating the migration of activated and effector T cells ^[327]. The function of CCL3L1 has been described mainly in the setting of enhanced susceptibility to HIV infection ^[339] and its role in postoperative inflammation is unknown. Pre-operatively, INHBA levels were significantly higher in the obese patients (12.8 fold increase, $p < 0.01$) as determined by the array. However postoperatively, levels were lower in the obese patients at all timepoints. INHBA encodes for Activin A, a member of the TGF- β superfamily and which has been shown to play an important role in adipose tissue deposition and the metabolic derangements observed in obesity ^[340, 341], however its role in the postoperative response is unclear. MGLL is involved in the hydrolysis of intracellular triglyceride and is also responsible for controlling 2-arachidonoylglycerol levels, a key component of the endocannabinoid system. Blockade of MGLL has been associated with reduction in inflammation in murine models ^[328, 329]. The gene expression of MGLL rose acutely in both cohorts postoperatively, with non-significantly higher levels observed in the non-obese patients on day 3 and higher levels in the obese patients on day 7 postoperatively. The implication of these altered levels postoperatively and between obese and non-obese patients remains to be elucidated. The fact that the significant differences observed in the test cohort were not witnessed in the validation cohort may reflect the heterogeneity of the validation cohort. Whilst the patients used in the test cohort were matched in terms of gender and operation type and all were chemoradiotherapy naive and experienced a normal postoperative recovery, there was variation in these parameters in the validation cohort which likely affected gene expression levels. Validation in a much larger cohort would be required.

TNFSF14, LT β and LT β R were also selected for investigation in obese and non-obese patients postoperatively. This triad has emerged recently as potentially important in mediating obesity-associated inflammation ^[330-332]. LT β R is abundant on stromal cells, dendritic cells and macrophages, with TNFSF14 (also known as lymphotoxin-related

inducible protein, or LIGHT) and lymphotoxin its only recognised ligands^[333]. TNFSF14 is produced by both activated and resting T cells, B cells and monocytes^[342], whilst LT β is produced by T cells and B cells as well as activated natural killer cells^[343]. Binding of TNFSF14 and LT β to LT β R leads to activation of NF- κ B pathways^[334]. LT β R ligation also up-regulates T cell adhesion to endothelial cells and expression of CXCL12^[334]. Research has also identified a role for LT β R signalling in dendritic cell function^[333]. Inhibiting LT β R signalling has been shown to be effective in numerous inflammatory and autoimmune disorders^[333] and has been employed as an anti-inflammatory, anti-cancer therapy in some experimental models^[344]. In obesity, TNFSF14 plays an important role in adipose tissue inflammatory responses through macrophage/T cell infiltration and activating the cells to release inflammatory cytokines^[332]. *In vitro* studies have demonstrated that TNFSF14 elicits the release of inflammatory cytokines/chemokines such as TNF, IL-6 and MCP-1 from adipocytes, adipose tissue-derived stromal vascular cells and macrophages. It was also shown TNFSF14 induces chemotactic activity of macrophages and T cell migration^[332]. In the current study, postoperative gene levels of TNFSF14 did not appear to be influenced by obesity, with a sharp increase immediately postoperatively observed in both cohorts suggesting TNFSF14 levels are important in the acute phase response. TNFSF14 levels peaked on day 1, with levels falling sharply in the obese patients, falling below the non-obese on day 7. Activation of human endothelial cells by platelet derived TNFSF14 results in pro-inflammatory and pro-thrombotic changes^[345] but further research is needed to elucidate its role postoperatively including assessment at the protein level. Converse to TNFSF14, levels of LT β declined sharply in patients initially postoperatively, before subsequently increasing. A similar pattern of expression was observed in the obese compared to the non-obese patients with the exception of day 7 postoperatively where levels were lower in the obese patients. The drop in LT β levels postoperatively is contrasted against an increased expression of LT β R, which increased from baseline to day 1 postoperatively before gradually returning to preoperative levels in both the obese and non-obese patients. These results suggest that whilst TNFSF14, LT β and LT β R have been implicated in obesity-associated inflammation and clearly are altered postoperatively, a differential postoperative response does not appear to occur in obese patients. However, assessment of expression of these markers was made at the

gene level. Future work at the protein level may reveal a greater understanding of the role of these inflammatory mediators in the postoperative response and if this is affected by obesity.

Neutrophils are the foot-soldiers of the innate immune system and are major effectors of the acute inflammatory response ^[346]. Surgery and anaesthesia can lead to decreased neutrophil chemotaxis ^[347] which has been linked with increased risk of complications and sepsis ^[348]. The influence of obesity on neutrophil function is an under-studied area. A study by Nijhuis *et al.* found that severely obese individuals demonstrated increased levels of neutrophil activation, as determined by MPO levels and CD66b expression ^[349]. Other studies have reported alterations in the expression of neutrophil adhesion markers ^[350]. In addition, hyperglycaemia is associated with impaired neutrophil function ^[351, 352] and reduced bactericidal capacity has been reported in obesity ^[353]. In this study, the expression of CD62L decreased in both obese and non-obese patients on day 1 postoperatively, which is consistent with a transient drop in chemotaxis as CD62L mediates neutrophil rolling, the first step for extravasation ^[354]. Levels increased thereafter, with 2 fold higher levels on day 14 compared to preoperative levels. MPO gene expression varied in the postoperative phase but no difference was observed between the obese and non-obese cohorts. In the case of CD11b, levels increased steadily in both cohorts postoperatively. These findings suggest that, at a gene level, obesity status does not impact on neutrophil function postoperatively. However, neutrophils have a very short half life ^[355] thus measuring alterations in activation using intra-operatively collected blood samples may shed more light on the possible impact of obesity on acute neutrophil activity.

Key to the regulation of many genes involved in immune and inflammatory responses is the transcription factor, NF- κ B. Usually NF- κ B is bound to I κ B, a cytosolic inhibitory protein that keeps NF- κ B inactive. When activated, the NF- κ B -I κ B complex is phosphorylated, and the complex becomes dissociated. Once dissociated, I κ B is degraded rapidly, and in parallel, there is an accumulation of NF- κ B in the nucleus. This results in the initiation of the transcription of a vast array of inflammatory genes ^[356]. NF- κ B can be rapidly activated by a number of factors including cytokines, adhesion molecules, endotoxins, and ischemia ^[337]. Considering this, it may be anticipated that

NF- κ B levels would rise following the trauma of surgery. However in the current study, both non-obese and obese patients demonstrated a drop in NF- κ B p65 from baseline to day 1. A physiological reason for this acute decrease in NF- κ B p65 postoperatively is unclear. One possible mechanism may relate to transient endothelial cell activation postoperatively. I κ B- α transcript is transiently up-regulated in endothelial cells responding to cytokines, such as TNF α . This acute increased expression of I κ B- α may subsequently decrease cytoplasmic NF- κ B activation^[357]. Furthermore, the decrease in NF- κ B p65 postoperatively was more significant in the non-obese cohort ($p < 0.05$), suggesting altered NF- κ B p65 activity in obese and non-obese patients postoperatively and warrants further investigation.

In the panel of inflammatory cytokines, it was evident that obesity and metabolic syndrome status influenced postoperative inflammatory response. IL-6 is produced in the immediate postoperative phase and correlates with the magnitude of surgery and may indicate increased sepsis risk^[358]. Levels of IL-6 were higher in the obese and metabolic syndrome positive patients on day 1 and day 3 postoperatively respectively. In addition IL-12, a key mediator of the Th1 pro-inflammatory response^[359] was significantly higher in obese patients preoperatively and on days 1, 7 and 14 postoperatively ($p < 0.05$). Conversely, higher levels of the IL-17, responsible for potentiating inflammatory responses^[360], were observed in the non-obese and in the MetSyn negative patients pre and postoperatively. Interestingly, whilst some pro-inflammatory cytokines demonstrated increased levels pre and postoperatively in the obese and metabolic syndrome positive patients, the levels of the classically anti-inflammatory TGF- β were also significantly higher in these patients preoperatively and on days 1 and 3 postoperatively ($p < 0.05$). These higher levels of TGF- β may be acting as a counterfoil to the increased levels of pro-inflammatory mediators and heightened systemic inflammation observed in the immediate postoperative phase in obese dysmetabolic patients. Alternatively, TGF- β may be acting in a pro-thrombotic role in the obese patients^[361]. Of interest, in the case of a number of cytokines, expression levels varied depending on whether patients were classified by obesity or MetSyn status. Levels of TNF- α were higher in obese patients in the immediate postoperative phase, but when classified by MetSyn status, lower levels of TNF- α were observed in MetSyn positive patients at all time-points. A similar change in expression pattern was

observed in IL-4, whereby higher levels were observed in the obese patients preoperatively, but lower levels were observed in the MetSyn positive patients. Furthermore, the magnitude of increase in IFN- γ levels observed in obese patients postoperatively was reduced when patients were classified by MetSyn status and the increased IL-8 levels observed postoperatively between obese and non-obese patients was not replicated when classified by MetSyn status. These findings suggest that adipose tissue excess, in the absence of dysmetabolism, has a different effect on the levels of inflammatory mediators. Whilst this has been observed in non-surgical cohorts ^[326, 362], this is the first study to report such a phenomenon with regards to the postoperative immune response. It is possible that the presence of hyperglycaemia, a characteristic of the MetSyn, also alters inflammatory response. Postoperative hyperglycaemia is a stress response and arises as a result of an acute increase in hepatic glucose production coupled with uptake resistance in peripheral tissues. It is associated with increased postoperative morbidity and mortality and has been shown to be more prevalent in MetSyn positive patients ^[338]. To investigate this further, the cohort was separated based on glycaemic status preoperatively. Again, a different pattern of expression was observed between the two cohorts. IL-6 levels were higher on day 3 and day 14 postoperatively. In addition a marked change was observed in IFN- γ and IL-8 production. Hyperglycaemic patients demonstrated higher levels of IFN- γ preoperatively and postoperatively. Similarly, higher levels of IL-8 were observed preoperatively and postoperatively in the hyperglycaemic patients. These findings again suggest that the potential impact of visceral obesity on postoperative inflammation is multifactorial, with the associated elements of dysmetabolism all exerting different effects on the inflammatory response.

The significance of these changes in inflammatory response is not clear cut. No difference was observed in the overall incidence of complications or the incidence of specific complications between obese and non-obese patients or between MetSyn positive and MetSyn negative patients. However, differences were observed in the clinical parameters of these patients. The obese patients had higher temperature and heart rate postoperatively. These observations suggest that the obese dysmetabolic patients experience a heightened inflammatory response, as was reflected in the CRP and cytokine data.

The results of this study have generated interesting, if not complex results regarding the impact of obesity and the MetSyn on postoperative inflammatory response. No difference was observed in either the retrospective or prospective cohort in terms of postoperative complication incidence. However, using the global marker of acute inflammation, CRP, it was apparent that obese, dysmetabolic patients experienced a heightened inflammatory response postoperatively, and an exacerbated inflammatory response to a complication. The differences in heart rate and temperature postoperatively in the obese patients also supports the hypothesis that obese patients experience a greater acute inflammatory response postoperatively. At the gene level, the array identified a number of inflammatory genes altered by obesity in the postoperative phase, but validation of these genes in separate cohorts yielded different results, most likely due to the heterogeneity of the patients. At a circulating cytokine level, differences were observed in the production of pro- and anti-inflammatory cytokines, but the pattern of expression was influenced differently depending on whether the patients were classed by obesity, MetSyn or glycaemic status. Taken together, these observations suggest that obesity and dysmetabolism most definitely influence the postoperative inflammatory response however further work is warranted to elucidate the exact mechanisms at play.

Chapter 6 The impact of an eicosapentaenoic acid enriched enteral feed on the postoperative inflammatory response and recovery in oesophagectomy patients

6.1 INTRODUCTION

A major surgical procedure is associated with a stress response, characterised by multiple inflammatory and metabolic changes ^[224]. In the immediate phase following the start of a surgical procedure, adrenocorticotrophic hormone (ACTH) is stimulated by afferent neuronal impulses from the site of injury leading to an increase in cortisol levels ^[363]. Cortisol in turn stimulates protein catabolism and gluconeogenesis from amino acids in the liver ^[224, 364]. Alongside this altered endocrine response, there is a stimulation of immune system. The release of TNF- α and IL-1 β from macrophages in the injured tissue stimulates the production of IL-6, which subsequently launches the acute phase response characterised by an increase in acute phase reactants such as C-reactive protein (CRP) ^[365]. This acute phase response also stimulates muscle catabolism to facilitate the production of acute phase reactants, whilst the increase in IL-6 further stimulates ACTH activity ^[224]. This initial hyper-inflammatory response is followed by an excessive compensatory anti-inflammatory response ^[234]. This stress response to surgery results in marked weight loss and protein catabolism in surgical patients. In turn, this loss of muscle mass results in muscle weakness and fatigue and can impair recovery ^[366, 367].

An oesophagectomy is an example of a major surgical procedure which elicits an inflammatory catabolic response. It is an operation that is associated with significant risks, with nearly half of all patients experiencing a postoperative complication ^[200]. Furthermore, the physical impact of the surgery has a profound and long term impact on health related quality of life and functional capacity ^[201, 202]. Oesophageal resections are becoming more frequent as it is an obesity-associated malignancy that is increasing in incidence, with levels in Ireland doubling in the last two decades ^[5]. Thus there is an impetus to identify strategies to optimise the postoperative outcomes in this patient cohort.

Immunonutrition is the modulation of the activity of the immune system by interventions with specific nutrients and it has been employed in critically ill patients and in the postoperative setting ^[368]. Omega-3 polyunsaturated fatty acids (PUFA), i.e., eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) represent promising immunomodulating agents and their effects have been investigated in

oesophagectomy patients ^[237]. The immunomodulating properties of omega-3 PUFA arise from their displacement of omega-6 PUFA, i.e., arachidonic acid (AA). Omega-3 and omega-6 PUFA are resident in phospholipid membranes and upon activation, e.g., in response to trauma, they are metabolised into eicosanoids, powerful mediators of inflammation. Metabolism of AA gives rise to the series-2 prostaglandins (PGE₂), prostacyclins (PGI₂), thromboxanes (TXA₂) and the series-4 leukotrienes (LTA₄, LTB₄), and lipoxins (LXA₄, LXB₄) whereas metabolism of EPA gives rise to series-3 prostaglandins (PGE₃), prostacyclins (PGI₃), thromboxanes (TXA₃) and series-5 leukotrienes (LTA₅, LTB₅) and lipoxins (LXA₅, LXB₅). The metabolites of AA are considered more potent mediators of inflammation than those of EPA ^[212, 213]. In addition, EPA gives rise to the production of resolvin E1, a compound involved in promoting the resolution of inflammation ^[217].

In healthy populations, supplementation with EPA can reduce circulating levels of pro-inflammatory cytokines ^[236]. In addition, in the study of cancer cachexia, EPA supplementation was shown to promote weight stabilisation in cancer patients with cachexia by attenuation of the acute phase response via suppressed IL-6 production ^[221-223]. Thus supplementation with EPA represents a potential therapeutic aid to control the postoperative inflammatory and catabolic response in oesophagectomy patients. A previous double-blind randomised controlled trial carried out by this research group examined the effects of an EPA supplemented enteral feed versus a standard enteral feed on outcomes in 53 oesophagectomy patients. Feed was provided for 5 days pre-operatively and 21 days post-operatively, with those in the treatment arm receiving 2.2g/day of EPA. The results demonstrated that supplementation with EPA was associated with a modest attenuation of inflammation and a reduced maximal body temperature. In addition, patients who received the EPA enriched enteral feed preserved their lean body mass compared to the standard feed group who experienced an average loss of 1.9kg of fat free mass ^[237].

Whilst there is evidence that EPA supplementation attenuates inflammation and catabolism postoperatively in oesophagectomy patients, its impact on specific functional capacity and quality of life remains to be determined. In addition, a more detailed investigation into the impact of EPA supplementation on the function of

specific immune cells and eicosanoid metabolism is warranted. Thus a double blind randomized controlled trial was commenced at this centre in oesophagectomy patients to investigate the long term effects of an enteral nutritional supplement enriched with EPA on lean body mass, physical activity, quality of life and the catabolic/inflammatory response. The results of EPA supplementation on the catabolic inflammatory response for a cohort of trial patients are presented herein.

6.1.1 Specific Aims

The specific aims of this aspect of this study were to determine the impact of perioperative EPA supplementation on:

- The incidence of postoperative complications
- The gene expression of inflammatory mediators and markers of activation in distinct immune cell populations
- The levels of circulating inflammatory cytokines and acute phase reactants
- The production of lipid mediators of inflammation
- Postoperative weight loss and changes in fat mass and fat free mass

6.2 RESULTS

6.2.1 Recruitment and discontinuation of patients in the clinical trial

Recruitment for this study took place between January 2011 and December 2012 (section 2.9). The pattern of patient recruitment and discontinuation is illustrated in Figure 6.1 below. Initially, 109 patients were recruited; 27 pre-multimodal therapy, 49 post-multimodal therapy and 33 surgery only patients. A total of 12 patients were discontinued prior to surgery for a variety of reasons, including disease progression (n=2), receipt of total parenteral nutrition (TPN) (n=3), no longer deemed suitable for surgery (n=4), benign tumour diagnosis (n=1) and at the request of the patient (n=2). Surgical resection was carried out on 97 patients. Postoperatively, 18 patients were discontinued; 5 had major postoperative complications, 6 had no jejunostomy sited at time of surgery and 7 had feeding withheld or tube dislodgements. The remaining 79 patients were deemed to be the study cohort. 72 patients continued feeding for 1 month postoperatively, with 41 patients within the cohort attending the 6 month follow-up appointment (Figure 6.1).

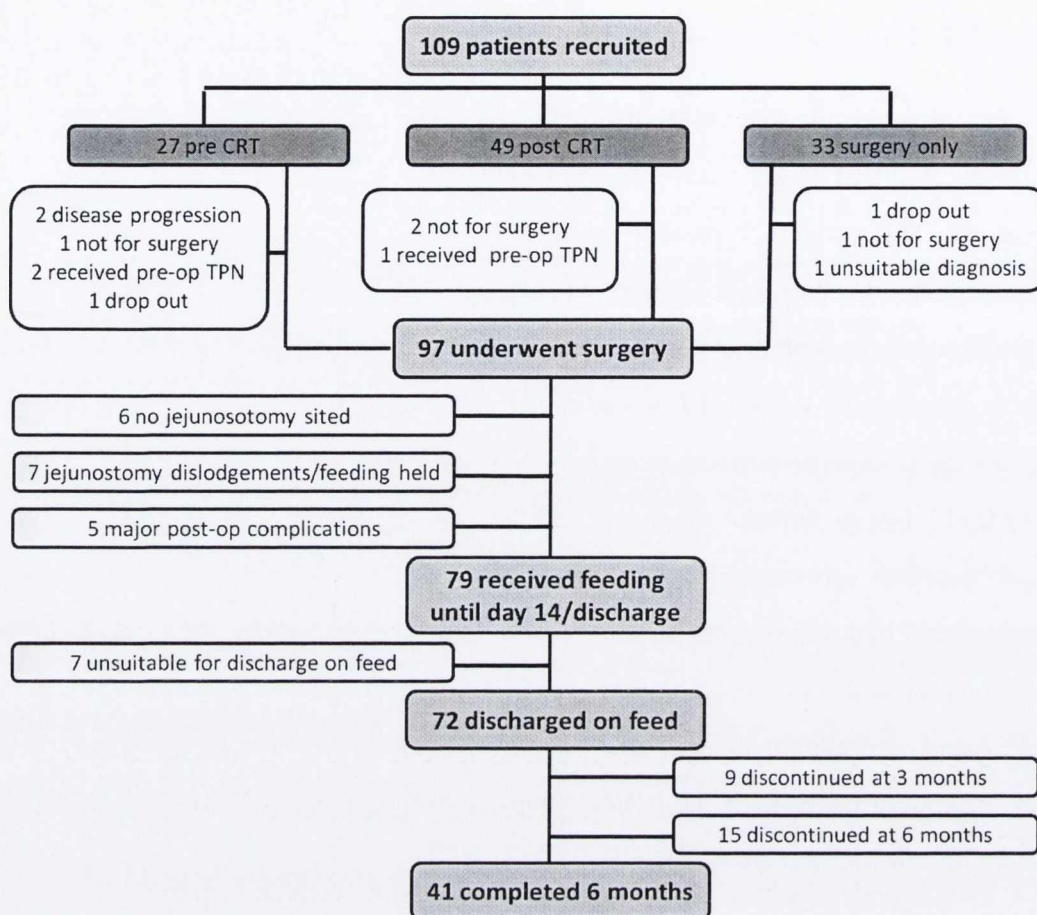


Figure 6.1 Flow chart of patient recruitment and discontinuation to clinical trial

A total of 109 patients were recruited initially with 97 undergoing resection. 79 patients received enteral feeding until discharge, at which point 7 were deemed unsuitable for home enteral feeding. Of the 72 patients discharged on enteral feeding, 41 completed the 6 month follow-up. CRT, chemoradiotherapy; TPN, total parenteral nutrition.

6.2.2 Characteristics of the study cohort

The study cohort ultimately consisted of 79 patients. Pre-operatively patients were randomised to receive either EPA enriched nutritional feed or the iso-caloric standard feed (section 2.9.3 and 2.9.4). The characteristics of the patients in the two arms are listed below in Table 6.1. The standard arm contained 38 patients versus 41 in the EPA arm. There was no significant difference between the groups in terms of age, gender, anthropometry, histological tumour type and operation type.

Table 6.1 Characteristics of the study cohort.

	Standard Feed n=38	EPA Enriched n=41	p
Males, n (%)	31 (81.6%)	38 (92.7)	0.183
Age (y), mean (range)	61.7 (42-78)	60.6 (37-76)	0.698
<i>Tumour Type</i>			
Oesophageal Adenocarcinoma, n (%)	32 (84.2%)	35 (85.4%)	1.0
Oesophageal SCC, n (%)	6 (15.8%)	6 (14.6%)	
Neoadjuvant Chemoradiotherapy, n (%)	24 (63.2%)	33 (80.5%)	0.131
BMI $\geq$ 30kg/m ² , n (%)	22 (57.9%)	31 (75.6%)	0.150
*High Waist Circumference (cm), n (%)	18 (56.3%)	21 (60.0%)	0.808
^Visceral Fat Area (cm ²), mean (range)	149.8 (4.3-354.5)	164.1 (4.7-353.1)	0.457
^Viscerally Obese, n (%)	18 (48.6%)	20 (51.3%)	1.0
#Metabolic Syndrome, n (%)	12 (40.0%)	14 (42.4%)	1.0
Percentage Body Fat (%), mean (range)	25.63 (7.9-36.1)	25.89 (9.7-41.9)	0.818
Fat Mass (kg), mean (range)	19.9 (4.7-36.1)	21.8 (4.5-40.3)	0.349
Fat Free Mass (kg), mean (range)	57.13 (35.0-73.3)	39.8 (76.1-60.1)	0.179
<i>Operation Type</i>			
Transhiatal, n (%)	8 (21.1%)	4 (9.8%)	
2-Stage Oesophagectomy, n (%)	27 (71.1%)	29 (70.7%)	0.169
3-Stage Oesophagectomy, n (%)	3 (7.8%)	8 (19.5%)	

*Waist circumference available on 32 patients in standard arm and 35 patients in EPA arm; ^ Visceral fat area measurement available on 37 patients in standard arm and 39 patients in EPA arm; #Metabolic syndrome status available on 30 patients in standard arm and 33 patients in EPA arm

6.2.3 Incidence of postoperative complications

There was no postoperative mortality in the study cohort. In terms of morbidity, the overall incidence of postoperative complications was significantly higher in the patients receiving the standard feed compared to patients receiving the EPA enriched feed (76.3% versus 46.3%, $p=0.006$). Regarding the type of complication, there was a significantly higher overall incidence of respiratory complications in the standard arm compared to the EPA enriched arm (55.3% versus 22%, $p=0.002$). There was also a higher incidence of respiratory tract infections in the standard arm compared to the EPA enriched arm (36.8% versus 17.1%, $p=0.047$). There was no difference in incidence of wound complications or arrhythmias between the two groups.

Table 6.2 Incidence of postoperative complications in patients receiving standard feed versus EPA enriched feed

Complication Type	Standard Feed (n=38)	EPA enriched (n=41)	<i>p</i>
Any Postoperative Complication, n (%)	29 (76.3%)	19 (46.3%)	0.006
Anastomotic Leak, n (%)	2 (5.3%)	0 (0%)	0.137
Delayed Gastric Emptying, n (%)	2 (5.3%)	1 (2.4%)	0.512
Respiratory Complication, n (%)	21 (55.3%)	9 (22%)	0.002
Respiratory Failure, n (%)	3 (7.9%)	0 (0%)	0.067
Pleural Effusion, n (%)	5 (13.2%)	3 (7.3%)	0.390
Pneumothorax, n (%)	2 (5.3%)	2 (4.9%)	0.938
Respiratory Tract Infection, n (%)	14 (36.8%)	7 (17.1%)	0.047
Infectious Complication, n (%)	16 (42.1%)	10 (24.4%)	0.094
Wound Complication, n (%)	4 (10.5%)	2 (4.9%)	0.344
Wound Infection, n (%)	2 (5.3%)	1 (2.4%)	0.512
Wound Dehiscence, n (%)	2 (5.3%)	1 (2.4%)	0.512
Arrhythmia, n (%)	12 (31.6%)	7 (17.7%)	0.132

EPA, eicosapentaenoic acid.

6.2.4 Alterations in inflammatory gene expression in peripheral blood mononuclear cells (PBMC) following oesophagectomy.

To determine the changes in inflammatory gene levels following surgery, thus identifying targets for EPA supplementation, a test cohort of non-trial patients was utilised. This cohort consisted of 6 male oesophageal adenocarcinoma patients who received standard enteral nutrition postoperatively. Blood samples were collected pre and postoperatively (section 2.8) and peripheral blood mononuclear (PBMC) were isolated (section 2.10.2). To assess the changes in inflammatory response postoperatively, a 370 inflammatory gene array was ran using the RNA extracted from PBMC isolated pre-operatively and on day 1 and day 7 postoperatively (n=6) (section 2.12). A number of inflammatory genes were altered postoperatively. The genes that were >2 fold up or down regulated (p<0.05) on day 1 and day 7 compared to preoperative levels are listed in Table 6.3 below. The greatest down regulation was observed in CCL4, with >11 fold down regulation on day 1 postoperatively. S100A12, S100A8 and IL-10 were all up-regulated on day 1 and day 7 compared to preoperative levels. These significantly altered genes serves as targets to assess the effect of EPA supplementation on the postoperative inflammatory response.

Table 6.3 Alterations in expression of inflammatory genes in PBMC postoperatively.

Red indicates up-regulation and blue indicates down-regulation.

Day 1 versus pre-op			Day 7 versus pre-op	
Gene	Fold change	<i>p</i>	Fold change	<i>p</i>
CCL4	-11.27	0.009	-2.46	0.020
IL5RA	-8.21	0.004	-2.05	0.037
CCL4L1	-5.49	0.006	-3.48	0.021
IL7	-4.62	0.023	-3.55	0.040
IL2RB	-4.16	0.001	-2.53	0.000
IL24	-3.00	0.026	-2.13	0.003
CCL27	-2.78	0.031	-2.58	0.035
S100A8	4.82	0.001	2.99	0.045
IL10	9.19	0.013	2.35	0.015
S100A12	10.99	0.000	3.58	0.014

6.2.5 Impact of EPA supplementation on inflammatory gene expression in PBMC postoperatively

To determine the impact of EPA supplementation on postoperative inflammatory gene expression, the expression of CCL4 and IL-10, targets identified from the inflammatory array, was assessed. Gene expression was measured by qPCR (section 2.14) using PBMC isolated pre-operatively and on days 1, 3, 7, 14 and at 1 month, 3 month and 6 months in 5 standard arm and 7 EPA enriched arm patients (Figure 6.2).

CCL4, also known as macrophage inflammatory protein-1 β (MIP-1 β), is secreted by a variety of immune cells and is a powerful monocyte and lymphocyte chemo-attractant [369]. In the standard arm patients, levels of CCL4 decreased postoperatively, validating the findings of the array. Conversely, in the patients receiving the EPA enriched enteral feed, levels of CCL4 increased postoperatively. At all time-points, CCL4 was higher in the EPA enriched arm patients, with significantly higher levels on day 7 ($p<0.05$), day 14 ($p<0.01$) and a trend towards significantly higher levels at 1 month ($p=0.06$).

IL-10 levels increased postoperatively in the standard arm patients, reflecting the findings of the inflammatory array. Levels of IL-10 also decreased postoperatively in the EPA enriched arm patients. However, the level of IL-10 was lower in the EPA enriched arm at all time-points, with significantly lower levels observed on day 3 postoperatively ($p<0.05$).

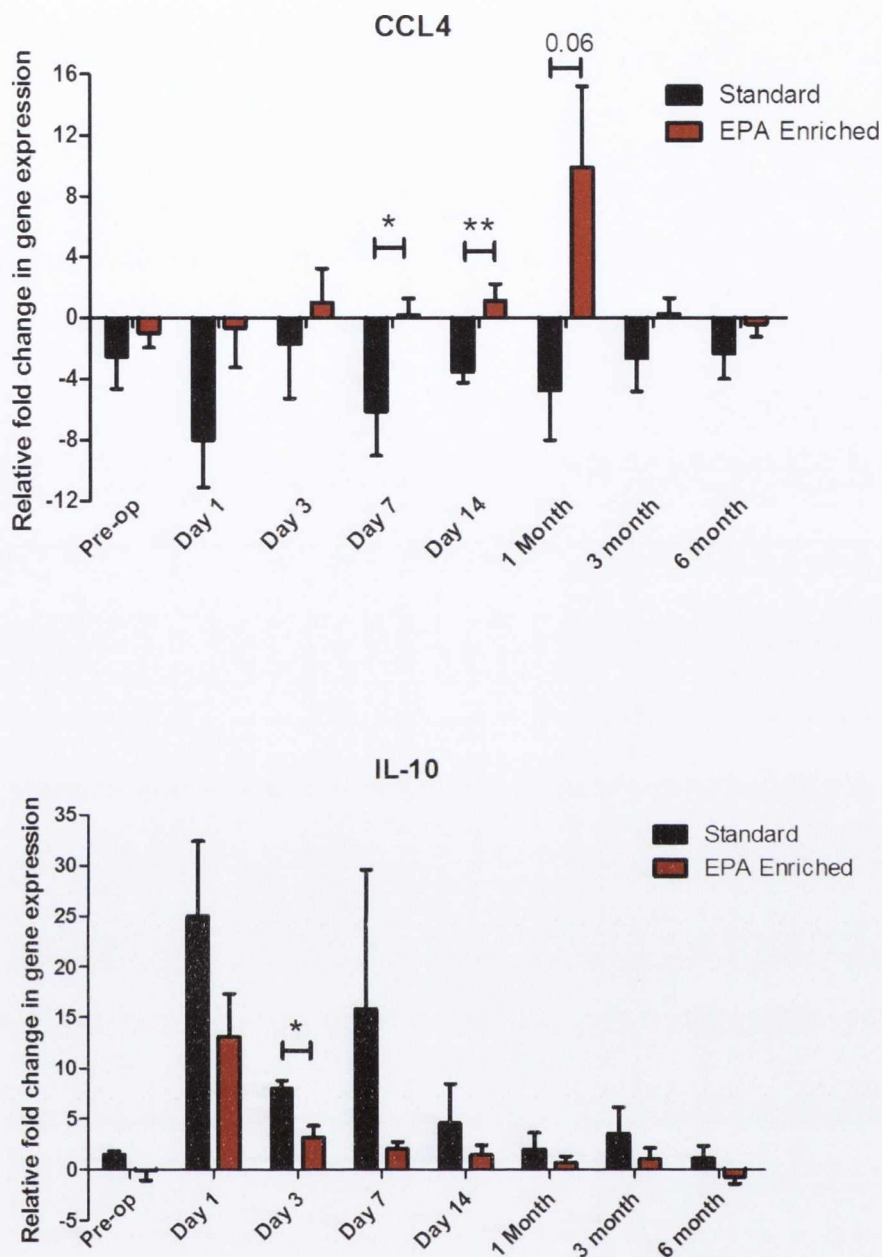


Figure 6.2 Changes in gene expression of CCL4 and IL-10 in PMBC postoperatively in patients receiving EPA enriched versus standard enteral feeding.

The expression of CCL4 and IL-10 in PBMC isolated pre- and postoperatively was assessed in 5 patients receiving standard enteral feeding versus 7 patients receiving EPA enriched enteral feeding. All samples were assayed in triplicate. Data was analysed using the $2^{-\Delta\Delta CT}$ method with 18S serving as an endogenous control and data is expressed as relative fold change. Differences in gene expression between the standard arm and EPA enriched arm were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM, $*=p<0.05$.

6.2.6 Impact of EPA supplementation on the expression of monocyte phenotype indicators pre- and postoperatively

Monocytes were isolated pre- and postoperatively as described in section 2.10.4. The efficacy of monocyte purification was determined by flow cytometry as described in section 2.10.5 (Figure 6.3). The inflammatory M1 and anti-inflammatory M2 phenotype of these circulating peripheral monocytes was determined by assessing the gene expression of the M1 monocyte markers IL-12 and TNF- α and the M2 monocyte markers arginase-1 and IL-10 by qPCR (section 2.16). Expression of these genes was assessed pre-operatively and on days 1, 3, 7 and 14 postoperatively in 3 standard arm patients and 4 EPA enriched arm patients (Figure 6.4).

A negligible change in arginase-1 levels was observed in both groups until day 3 postoperatively, after which both arms increased. The increase was greatest in the standard arm patients, with a near 200 fold increase on day 14, suggesting higher levels of M2 monocytes. IL-10 levels also increased postoperatively, with a sharper increase on day 1 postoperatively observed in the EPA enriched arm. Levels declined in both arms after day 1, returning to pre-operative levels by day 14. Levels of IL-12 decreased sharply postoperatively, with an approximate 3 fold down regulation in expression in the EPA enriched arm and a 6 fold down regulation in the standard arm on day 1 postoperatively, suggesting a drop in M1 monocytes. Levels increased thereafter returning to preoperative levels in both arms. In the case of TNF- α , levels did not vary widely postoperatively, with a gradual decrease observed in both arms in the initial postoperative phase. Levels increased thereafter, with higher than preoperative levels observed in the standard arm on day 14.

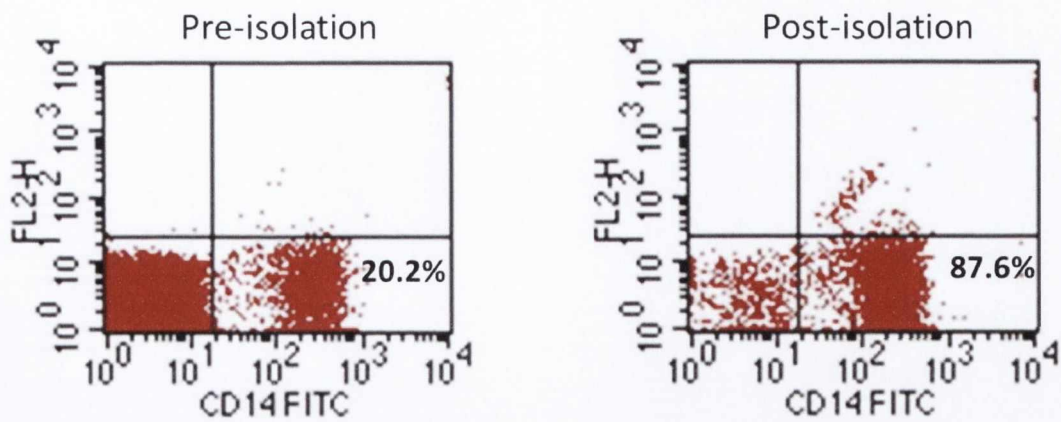


Figure 6.3 Assessment of monocyte purity by flow cytometry following isolation

Monocytes were isolated from peripheral blood mononuclear cell populations using magnetic associated cell sorting. The purity of the technique was assessed by flow cytometry, using CD14 as a positive marker for monocytes. The flow cytometry dot plots illustrate purity of monocyte population (lower right quadrant) pre and post purification. Numbers in lower right quadrant represent % of CD14 positive cells, gated on live cell population.

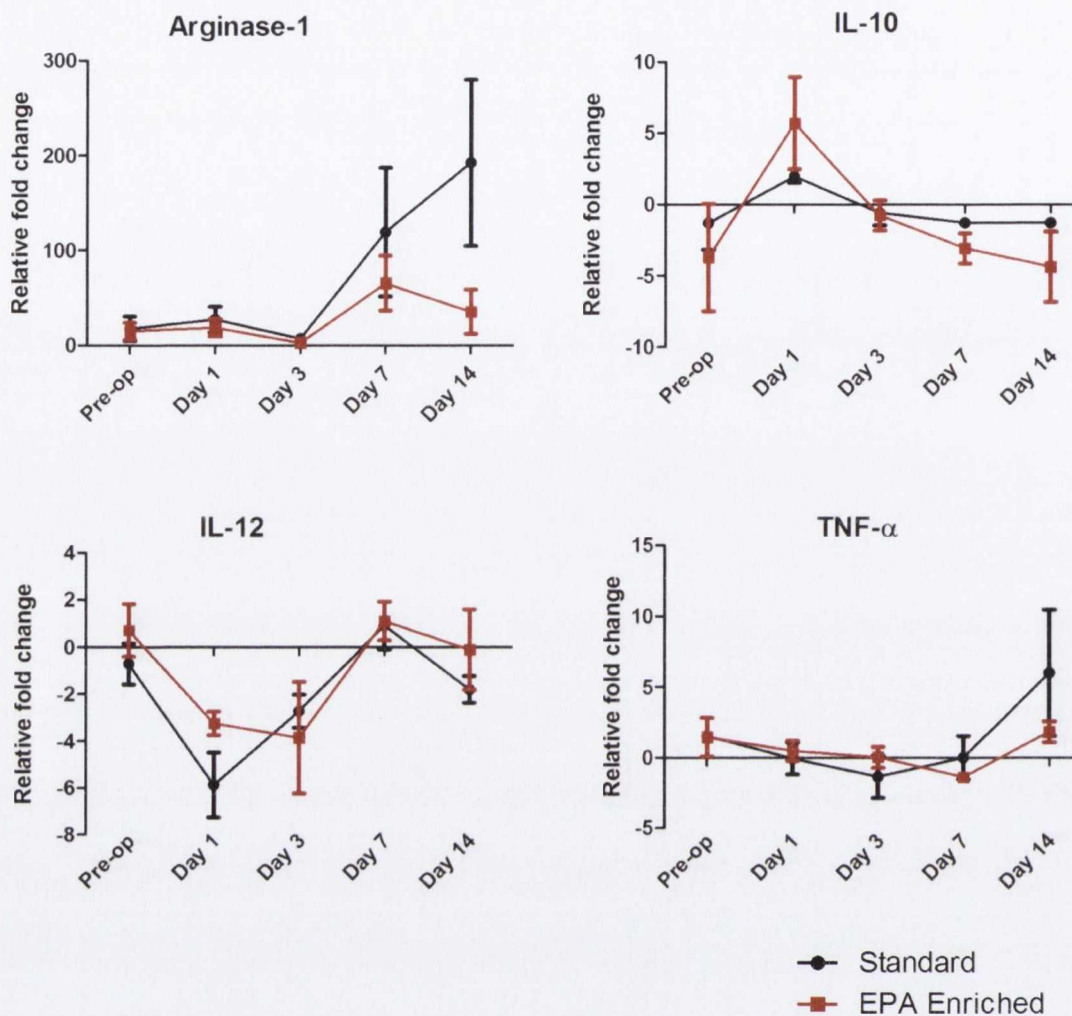


Figure 6.4 Monocyte expression of arginase-1, IL-10, IL-12 and TNF- α postoperatively in patients receiving standard versus EPA enriched enteral feed

The expression of M1 monocyte markers (IL-12 and TNF- α) and M2 monocyte markers (arginase-1 and IL-10) was determined in monocytes isolated pre- and postoperatively in 3 patients receiving standard enteral feed versus 4 patients receiving EPA enriched enteral feed. All samples were assayed in triplicate. Data was analysed using the $2^{-\Delta\Delta CT}$ method with 18S serving as an endogenous control and data is expressed as relative fold change. Differences in gene expression between the standard arm and EPA enriched arm were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM.

6.2.7 Impact of EPA supplementation of the expression of neutrophil activation markers postoperatively

As neutrophils are the first immune cells to respond to tissue damage and infection, neutrophils were isolated from whole blood collected preoperatively and on days 1, 3, 7 and 14 postoperatively as described in section 2.10.3. To determine the effect of EPA supplementation on neutrophil adhesion and activation, the expression of CD62L, CD11b and myeloperoxidase (MPO) by neutrophils was assessed in 5 standard arm and 6 EPA enriched arm patients by qPCR (section 2.15).

CD62L decreased in both arms immediately postoperatively. In the standard arm, levels increased sharply after day 1, whereas a more gradual increase in expression after day 3 was observed in the EPA enriched arm. In the case of CD11b, little change was observed in the expression levels postoperatively in the EPA enriched cohort. However, a sharp increase was observed on day 3 in the standard arm patients. A differential pattern of MPO expression was observed postoperatively in the standard and EPA enriched arms. In both arms, expression of MPO decreased sharply postoperatively. However, after day 1 levels increased, with significantly higher levels observed in the standard arm patients on day 7 postoperatively ($p < 0.05$) (Figure 6.5).

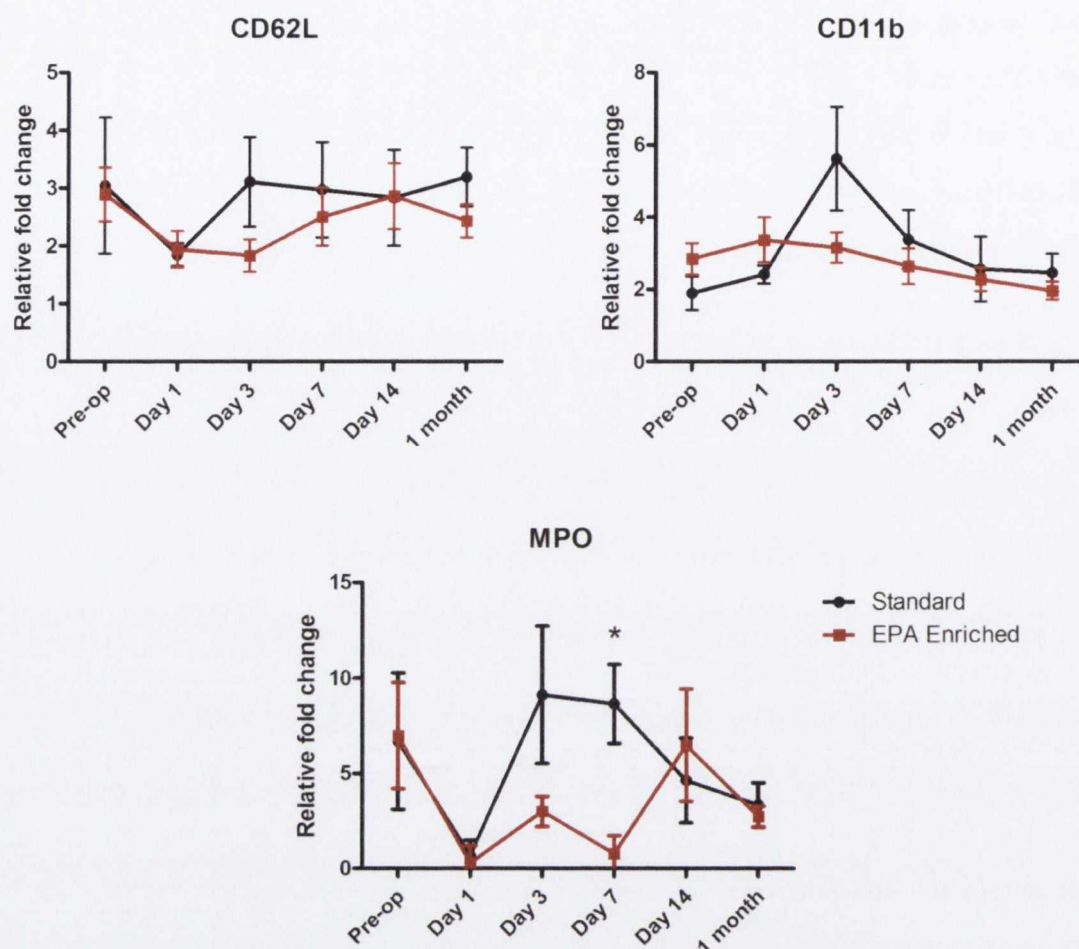


Figure 6.5 Expression of neutrophil activation and adhesion markers postoperatively in patients receiving standard versus EPA enriched enteral feed.

The expression of the neutrophil markers of adhesion and activation CD62L, CD11b and myeloperoxidase (MPO) was determined in neutrophils isolated pre- and postoperatively in 5 patients receiving standard enteral feed versus 6 patients receiving EPA enriched enteral feed. All samples were assayed in triplicate. Data was analysed using the $2^{-\Delta\Delta CT}$ method with 18S serving as an endogenous control and data is expressed as relative fold change. Differences in gene expression between the standard arm and EPA enriched arm were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM; *= $p < 0.05$.

6.2.8 Impact of EPA supplementation on C-reactive protein, albumin and white cell count postoperatively

White cell count (WCC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and albumin were routinely measured pre and postoperatively by the Central Pathology Laboratory in St. James's Hospital. Levels of these inflammatory markers were recorded preoperatively and on days 1, 3, 7 and 14 and 1, 3, and 6 months postoperatively. The pattern of expression of ESR, CRP and CRP:albumin was similar in both arms. Levels rose postoperatively, as expected, peaking on day 3 in the case of CRP and CRP:albumin and day 7 in the case of ESR. Levels declined thereafter to preoperative levels or below. White cell count is a basic indicator of an inflammatory response. As with CRP and ESR, WCC levels increased in both arms immediately postoperatively, peaking on day 1. In the EPA enriched arm, WCC count dropped more quickly following day 1, with significantly lower levels on day 3 compared to the standard arm patients ($p < 0.01$) (Figure 6.6).

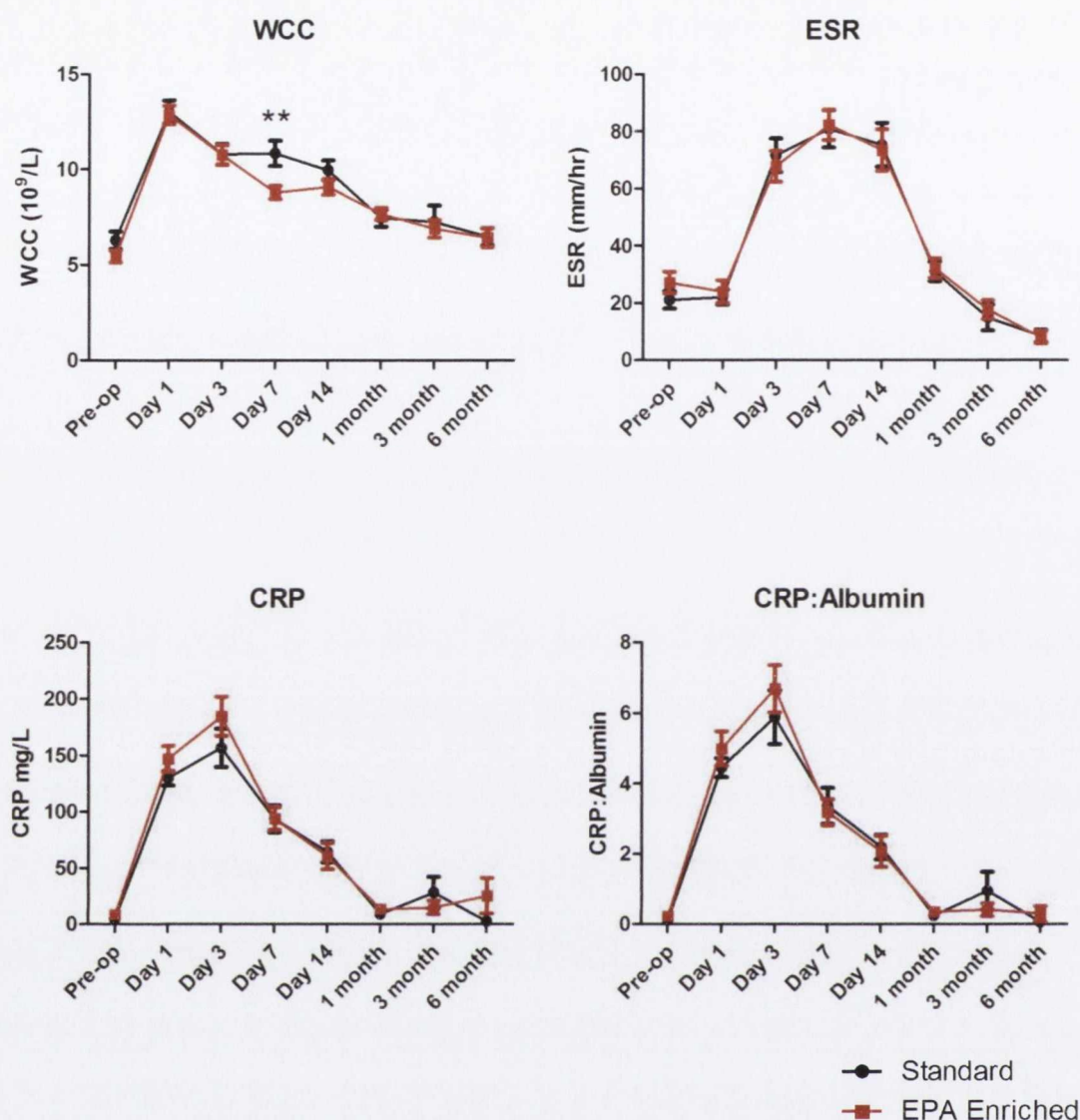


Figure 6.6 The acute inflammatory response postoperatively in patients receiving standard enteral feed versus those receiving EPA enriched feed.

White cell count (WCC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels and CRP:albumin ratio was assessed pre and postoperatively by the Central Pathology Laboratory in St. James's Hospital in patients receiving standard enteral feed versus those receiving EPA enriched enteral feed. Differences in circulating levels of these inflammatory markers between the two arms were assessed by Mann Whitney U. Data are presented as mean $\pm$ SEM; **= $p < 0.01$.

6.2.9 Impact of EPA supplementation on circulating levels of a panel of inflammatory mediators postoperatively

Changes in the postoperative production of well-defined inflammatory cytokines in patients were assessed at the protein level by ELISA as described in section 2.16. A panel of both pro- and anti-inflammatory cytokines was investigated: IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17, IFN- γ , TNF- α and TGF- β . Levels were assessed in both groups pre-operatively, on days 1, 3, 7 and 14 postoperatively and at 1, 3 and 6 months.

In the case of pro-inflammatory cytokines (Figure 6.7), no difference was observed in TNF- α levels in either group. Both groups demonstrated a biphasic increase in circulating levels, with an initial increase on day 1 postoperatively and another increase at 1 month. IL-6 levels increased sharply in both groups immediately, with levels declining thereafter. A different pattern of IL-8 expression was observed between the patients receiving standard feed and those receiving EPA enriched feed, with those in the EPA arm demonstrating higher levels postoperatively. In the standard arm patients, circulating levels of IL-12 increased postoperatively, which was in contrast to the patients in the EPA enriched arm, who demonstrated a decrease in circulating levels postoperatively.

The adaptive immune response was characterised by circulating levels of IFN- γ and IL-17 (Figure 6.8). Levels of IFN- γ did not change remarkably in the acute postoperative phase in either arm. However both arms demonstrated an increase in levels at 1 month post discharge. In the case of IL-17, absolute levels were low in both arms, however a slight increase in postoperative levels was observed in the patients receiving EPA enriched feed.

In terms of the anti-inflammatory response (Figure 6.9), EPA supplementation did not alter circulating levels of TGF- β . Circulating levels of IL-4 were low in both arms postoperatively. Higher levels were observed in the EPA enriched arm patients preoperatively, but negligible levels were detected postoperatively. IL-10 levels did not differ greatly between patients in the EPA enriched arm and those in the standard arm with circulating levels declining on discharge in both groups.

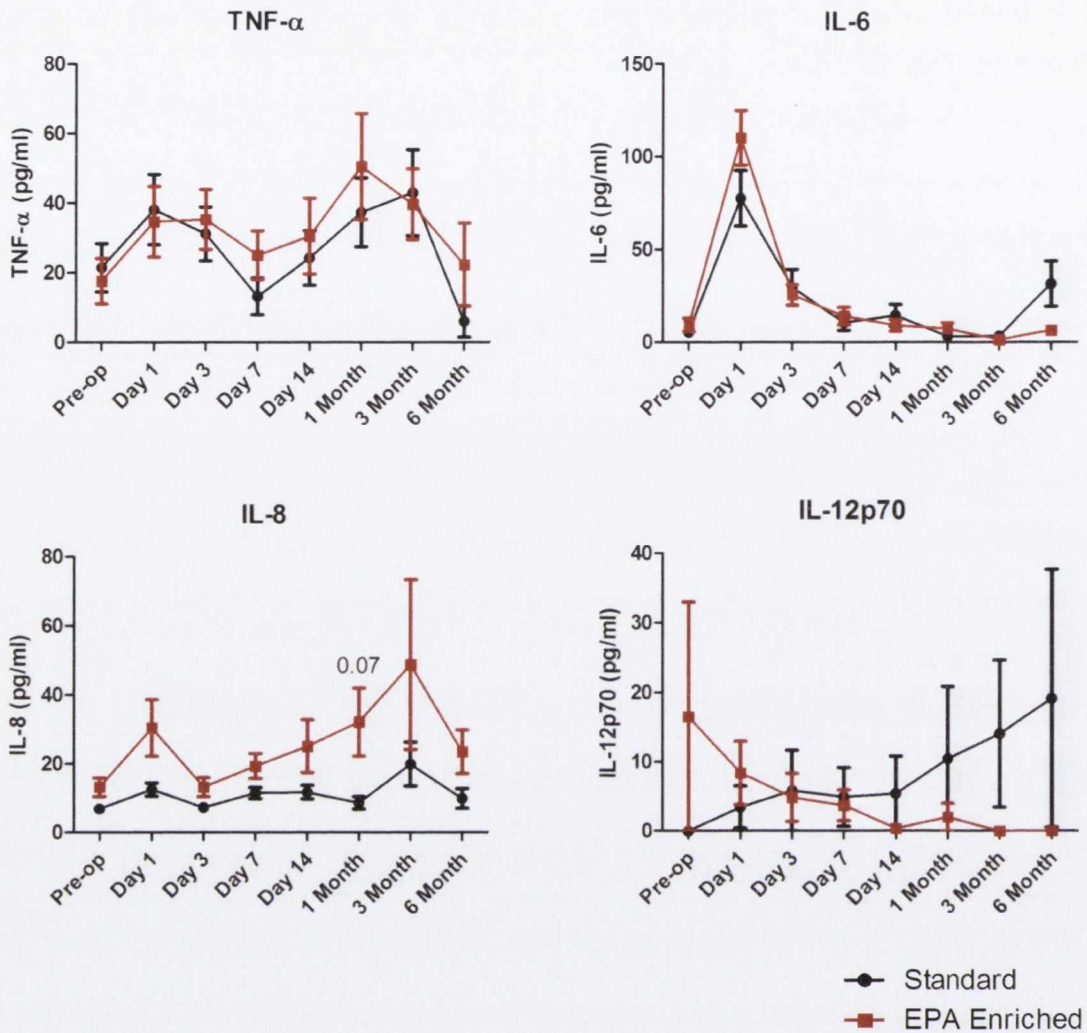


Figure 6.7 Alterations in circulating levels of pro-inflammatory in patients receiving standard versus enteral feed.

The expression of IL-6, TNF- α , IL-8 (n=32 standard enteral feed n=40 EPA enriched enteral feed) and IL-12p70 (n=22 standard enteral feed, n=31 EPA enriched enteral feed) was assessed by ELISA on days 1, 3, 7 and 14 postoperatively and at 1, 3 and 6 months. All samples were assayed in triplicate. Differences between expression levels in patients receiving standard enteral feed versus those receiving EPA enriched enteral feed were assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM

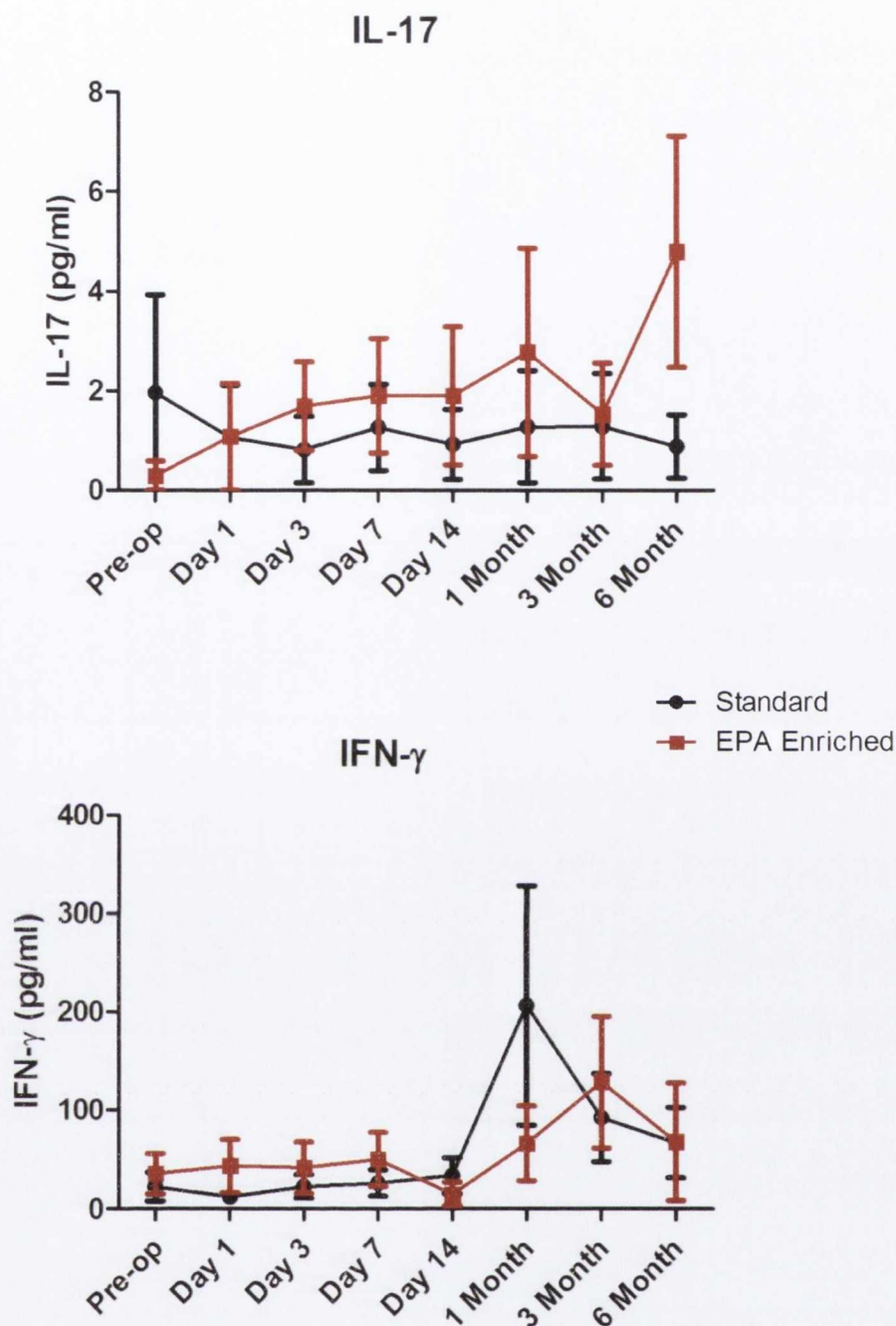


Figure 6.8 Alterations in postoperative circulating levels of adaptive cytokines in patients receiving standard enteral feed versus EPA enriched enteral feed.

The expression of IFN- γ (n=32 standard enteral feed n=40, EPA enriched enteral feed) and IL-17 (n=15 standard enteral feed, n=13 EPA enriched enteral feed) was assessed by ELISA on days 1, 3, 7 and 14 postoperatively and at 1, 3 and 6 months. All samples were assayed in triplicate. Differences between expression levels in patients receiving standard enteral feed versus those receiving EPA enriched enteral feed were assessed using Mann Whitney U. Data are presented as mean $\pm$ SEM.

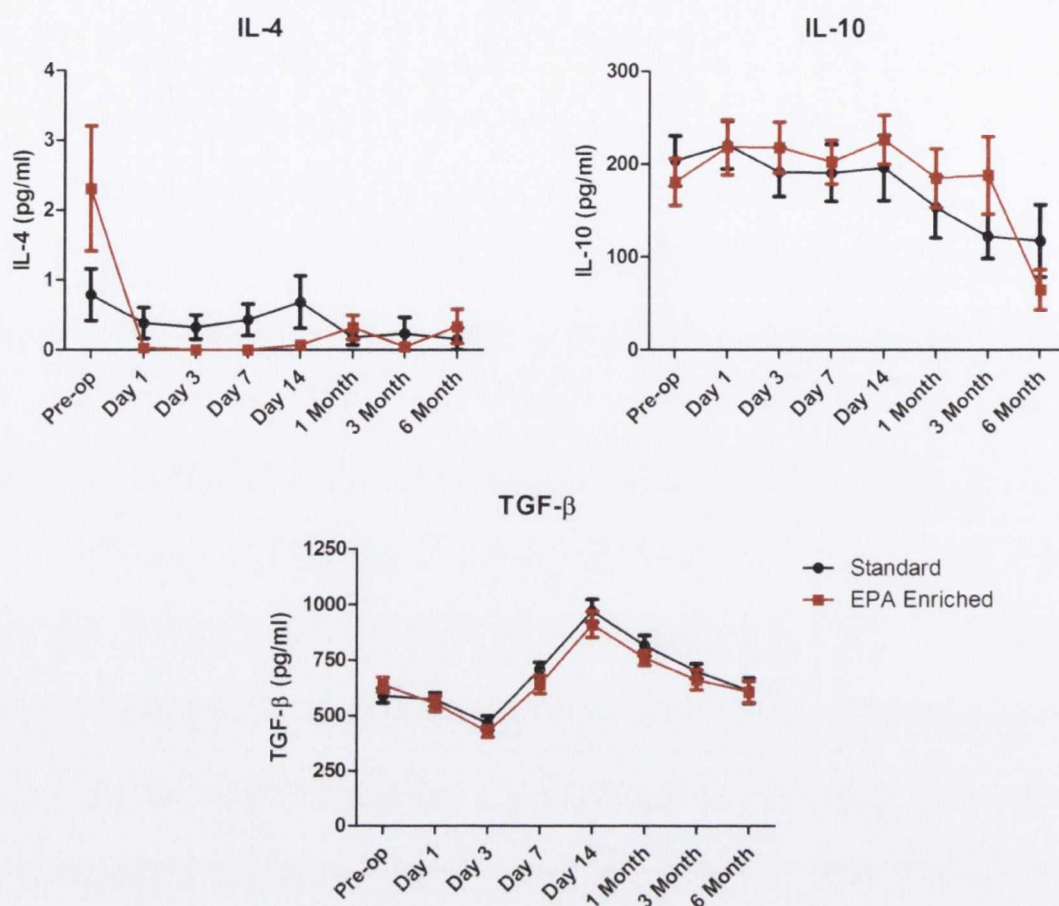


Figure 6.9 Alterations in postoperative circulating levels of anti-inflammatory mediators in patients receiving standard enteral feed versus EPA enteral feed

The expression of IL-4 (n=15 standard enteral feed, n=11 EPA enriched enteral feed) IL-10 (n=22 standard enteral feed, n=31 EPA enriched enteral feed) and TGF-β (n=32 standard enteral feed, n=40 EPA enriched enteral feed) was assessed by ELISA on days 1, 3, 7 and 14 postoperatively and at 1, 3 and 6 months. All samples were assayed in triplicate. Differences between expression levels in patients receiving standard enteral feed versus those receiving EPA enriched enteral feed were assessed using Mann Whitney U. Data are presented as mean ± SEM.

6.2.10 Impact of EPA supplementation on circulating levels of eicosanoids postoperatively

Eicosanoids are powerful mediators of inflammation and their levels of expression may be altered by increased consumption of EPA. Therefore the circulating levels of prostaglandin E_2 (PGE_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) (an indirect measure of prostacyclin I_2), and leukotriene B_4 (LTB $_4$) were assessed by EIA (section 2.17) in 4 patients receiving standard feed and 5 patients receiving EPA enriched feed. Circulating levels were assessed pre-operatively and on day 1, day 7 and day 14 postoperatively.

A marked difference in PGE_2 levels was observed between the two arms. In patients receiving the standard feed, little change was observed in levels postoperatively. Conversely, a sharp increase in levels was observed in the patients receiving the EPA enriched feed, with significantly higher levels on day 7 and day 14 postoperatively ($p < 0.05$). A significant difference between the two arms was also observed in the case of 6-keto-PGF $_{1\alpha}$, with significantly higher levels observed in the EPA enriched arm on day 1 postoperatively ($p < 0.05$). LTB $_4$ levels did not differ significantly between the two arms at any time-point (Figure 6.10).

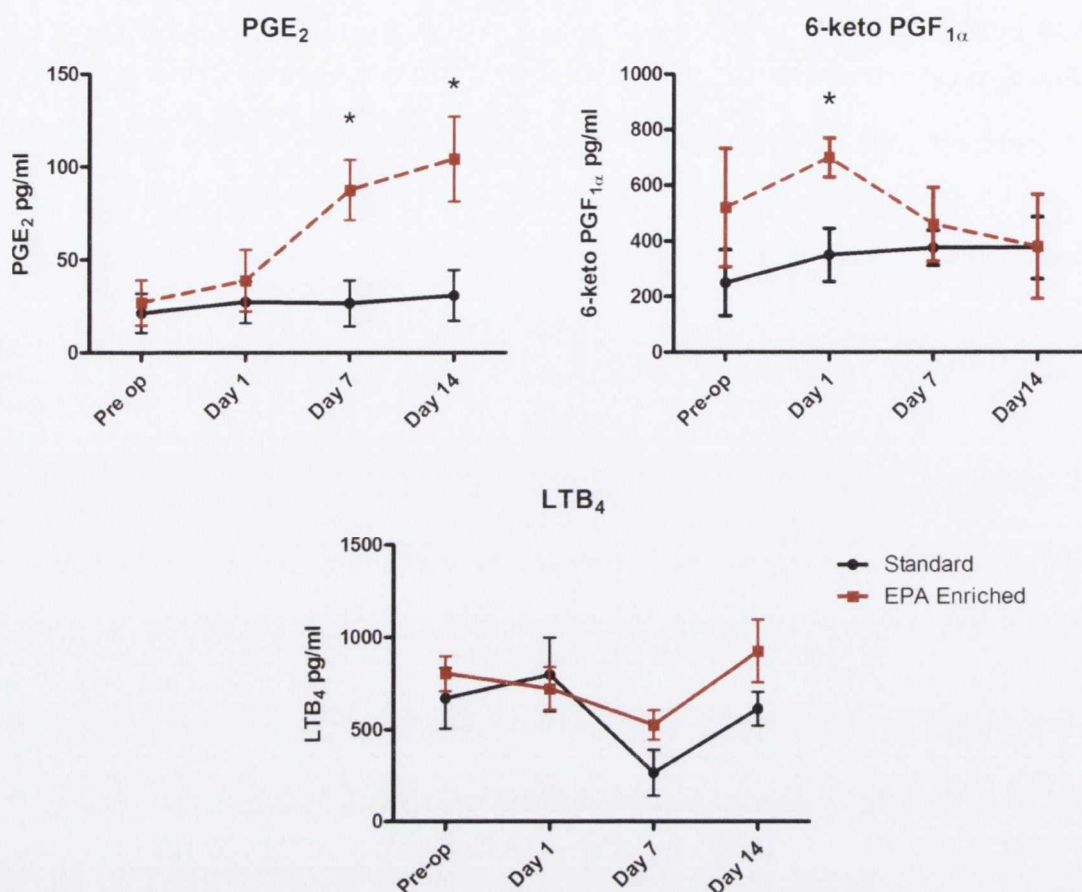


Figure 6.10 Changes in circulating levels of eicosanoids postoperatively in patients receiving standard enteral feed versus EPA enriched enteral feed.

The circulating levels of prostaglandin E₂ (PGE₂), 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}) and leukotriene B₄ (LTB₄) were assessed by EIA in 4 patients receiving standard feed versus 5 patients receiving EPA enriched feed. All samples were assayed in duplicate. Levels were assessed pre-operatively and on days 1, 7 and 14 postoperatively. Differences in the circulating levels between the two arms were assessed using Mann Whitney U. Data is presented as mean ± SEM; *= $p < 0.05$.

6.2.11 Impact of EPA supplementation on muscle and fat mass postoperatively

In all patients, body composition analysis was carried out by the co-research dietitian on the trial using a Tanita BC 418 MA bioelectrical impedance analyzer (Tanita UK Ltd, Middlesex, UK) as described in section 2.9.7. Measurements were taken pre-operatively, at discharge and at 1, 3 and 6 months. Supplementation with EPA is hypothesised to have anabolic effects; therefore changes in weight, fat mass and fat free mass were assessed in both groups (Figure 6.11).

All patients lost weight postoperatively, as expected. However the decline in weight was attenuated in the patients receiving the EPA enriched feed, with significantly higher weights observed at discharge ($p<0.05$) and a trend toward significantly higher levels at 1 month compared with the standard arm patients ($p=0.054$). A greater loss of fat mass and percentage fat was observed in the patients receiving the standard feed but these differences were not significant. In terms of fat free mass, greater losses were observed in the patients receiving standard feed, with significantly lower levels observed at discharge ($p<0.01$) which persisted to the 1 month time-point ($p=0.054$)

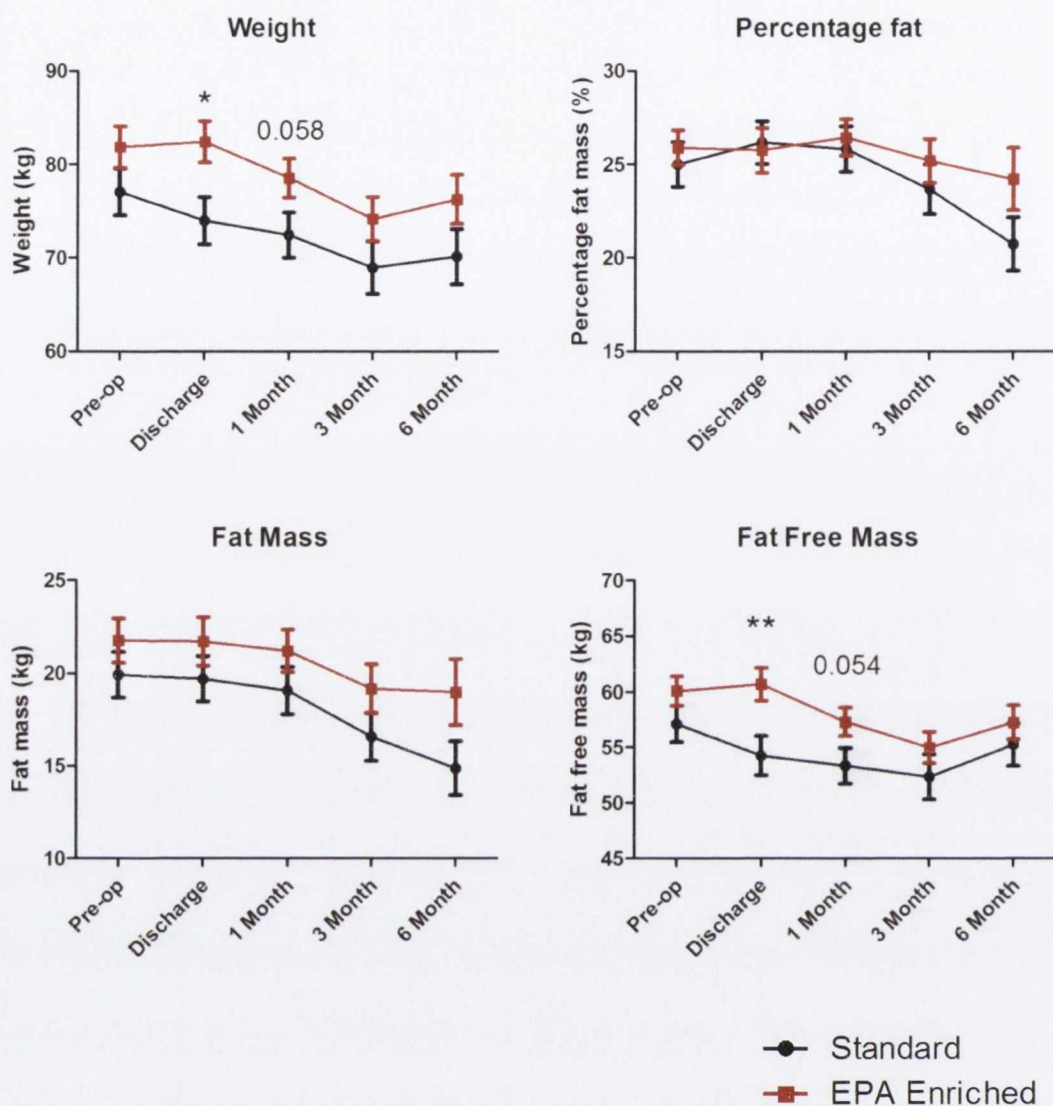


Figure 6.11 Changes in body composition postoperatively in patients receiving standard versus EPA enriched enteral feeding.

Body composition was assessed by bio-electrical impedance pre-operatively, at discharge and at 1, 3 and 6 months in patients receiving standard enteral feed (n=38) versus EPA enriched enteral feed (n=41). Weight, fat mass, percentage fat and fat free mass were determined. Differences in measures between the two arms were assessed by using Mann Whitney U. Data are presented as mean $\pm$ SEM; * p <0.05, ** p <0.01.

6.3 DISCUSSION

In this clinical trial we demonstrate a clear role for the use of EPA supplemented enteral feeding pre and postoperatively in oesophagectomy patients, in terms of reduced incidence of complications and preservation of lean body mass. However this trial also demonstrates the complex interplay between EPA and the immune system and questions the “anti-inflammatory” persona of EPA.

Oesophageal resection represents a major operation with considerable morbidity and mortality ^[200]. In this study we demonstrated that supplementation with EPA reduced the incidence of postoperative complications by 35% compared to standard feed. Furthermore, it was respiratory complications in particular that were reduced in the EPA group. Postoperative respiratory complications are the most common complication following oesophagectomy and are associated with intensive medical treatment and increased length of hospital stay ^[370]. There was a significantly lower incidence of respiratory tract infections in the EPA arm and a reduced number of overall infectious complications. Whilst other studies of EPA supplementation delivered in combination with arginine and RNA have demonstrated a reduced risk of postoperative complications ^[371, 372], to the authors knowledge, this is the first clinical trial to demonstrate a reduction in postoperative complications from perioperative enteral feeding supplementation with EPA alone.

In this clinical trial the mechanisms by which EPA supplementation alters the postoperative inflammatory response in patients is investigated more thoroughly than has been reported to date. Using an expansive gene array and a test cohort of non-trial patients, it was possible to identify inflammatory genes that are significantly altered in patients following oesophagectomy and then determine the impact of EPA supplementation on them. CCL4, which is secreted by a variety of immune cells and is a powerful lymphocyte and monocyte chemo-attractant ^[369], was identified as being significantly down regulated following surgery. However EPA supplementation led to a significant increase in CCL4 expression by PBMC. Conversely, the postoperative increase in levels of anti-inflammatory IL-10 was reduced by supplementation with EPA. This reversal in the expression of pro- and anti-inflammatory genes in PBMC following EPA supplementation is an interesting, and perhaps unexpected finding.

However this pro-inflammatory switch may reflect an attempt to prevent an exaggerated compensatory anti-inflammatory response and immunosuppression.

To further elucidate the impact of EPA supplementation on the activity of immune cells, monocytes and neutrophils were isolated pre- and postoperatively. At the gene level supplementation with EPA did not significantly alter the monocyte expression of IL-10, IL-12 or TNF- α . There was an apparent increased level of arginase-1 expression on day 7 and day 14 in the patients receiving standard feed, although this difference was not significant. Previous research has shown increased arginase-1 activity in peripheral blood mononuclear cells post trauma is associated with decreased plasma arginine levels and may contribute to a state of “arginine malnutrition”. This increase in arginase-1 may be reflective of increased Th2 activity in the standard arm patients [373, 374]. Other studies examining the effect of EPA supplementation in humans have reported a reduction in TNF- α production in isolated macrophages following stimulation [375, 376]. This anti-inflammatory effect of EPA was not witnessed in the postoperative setting in this trial. However previous research on the effect of EPA on monocyte/macrophage inflammatory gene expression has utilised ex-vivo models and may not be representative of the complex postoperative immune environment.

In the case of neutrophils, supplementation with EPA altered expression of the adhesion markers CD62L and CD11b and significantly decreased the expression of the activation marker, MPO. These findings suggest a decrease in neutrophil activation and migration. Studies in cardiac surgery patients have reported that decreased neutrophil activity in the initial postoperative period is associated with a reduced risk of sepsis [377]. A possible explanation would be reduced levels of LTB₄, which is chemotactic and promotes neutrophil activation [378]. However, LTB₄ levels were found to be unchanged by EPA supplementation in this trial, suggesting another mechanism is at play. Neutrophils have a short half-life and are active at the site of injury [355] thus only limited conclusions may be drawn from the observed effects of EPA at the gene level in circulating neutrophils in the days and weeks postoperatively. It would be of interest to investigate the activity of neutrophils in bronchoalveolar lavage as it is associated with respiratory complications [379], which were reduced amongst patients receiving EPA supplemented feed.

Supplementation with EPA is classically considered to have anti-inflammatory or immuno-suppressive effects. However, this study has demonstrated that the impact of EPA supplementation on inflammatory cytokine production is not as clear cut. With the exception of white cell count on day 7 postoperatively, supplementation with EPA did not alter the classic clinical markers of inflammation, i.e., CRP and ESR. In non-surgical groups supplementation with EPA does not affect CRP levels ^[380, 381]. In previous research by this group, CRP levels were not reduced by EPA supplementation ^[237] and in cancer cachexia, supplementation has only been shown to halt rising CRP ^[382]. However, the reduction in white cell count on day 7 in the EPA group does suggest a reduction in systemic inflammation and it may be that CRP is not specific enough a marker to detect the impact of EPA on the immune system. Alternatively it may be that EPA does not act through direct reduction in CRP production, but rather it alters the systemic effects of increased CRP. In a murine model of CRP overexpression and heart failure, EPA supplementation did not affect serum CRP levels but did result in decreased cardiac fibrosis and improved cardiac function ^[383].

The above finding relating to the classic markers used to identify inflammation clinically highlights the importance of examining a panel of inflammatory mediators when assessing immune function. In this trial EPA supplementation did not have a marked effect on the circulating levels of inflammatory cytokines. TNF- α , IL-6, IFN- γ , TGF- β and IL-10 levels were unchanged in the EPA supplemented group. EPA supplementation did appear to negate IL-12 levels, suggesting an anti-inflammatory effect, but this was paralleled by an increased expression of IL-8 and IL-17. To date the impact of EPA supplementation peri-operatively on postoperative cytokine production is unclear. The effect of EPA on markers such as TNF- α , IFN- γ , IL-4, IL-6, IL-8 and IL-10, has been reported to be decreased, increased or unchanged ^[237, 384-386]. In addition, it is difficult to compare the findings of this trial with others due to heterogeneity of study design, i.e. study population, quantity of EPA supplemented and mode of delivery. The gene expression of inflammatory mediators by immune cells and the production of inflammatory cytokines are influenced by a number of factors including transcription factors, microRNA and posttranslational modifications. Furthermore, the Th1, Th2 and Th17 responses all influence and regulate each other ^[359, 360] in a continuous battle to attain homeostasis. Future work delineating the potential role of EPA on immune cell

function postoperatively would benefit from characterisation of transcription factor activity such as NF κ B and real time assessment of activation marker expression on innate and adaptive immune cells.

Whilst no distinct impact on circulating cytokine production was found following EPA supplementation, a clear alteration was observed in eicosanoid production. In the case of PGE₂ and 6-keto-PGF_{1 α} (an indirect measure of prostacyclin), levels increased significantly postoperatively in the EPA group. Prostacyclin is a potent vasodilator and prevents platelet aggregation, inhibiting the effects of TXA₂. It has been utilised in the treatment of peripheral vascular disease and pulmonary hypertension ^[387, 388]. PGE₂ has a number of pro- and anti-inflammatory roles. It has been shown to stimulate IL-6 production ^[215] and induces fever ^[389]. However it is also a powerful inhibitor of TNF- α and IL-1 β production by immune cells ^[390]. In addition it inhibits 5-LOX activity, thus decreasing the production of the 4-series leukotrienes, and it increases lipoxin A₄ production ^[391]. It may be that PGE₂ has an initial pro-inflammatory role post trauma/surgery, followed by an anti-inflammatory role characterised by reduced 5-LOX and increased lipoxin A₄ leading to inflammation resolution.

The up-regulation of PGE₂ and 6-keto-PGF_{1 α} in the EPA group may be counter-intuitive considering the hypothesis that supplementation with EPA reduces the production of the 2-series prostaglandins and prostacyclins. However upregulation of PGE₂ following EPA supplementation has been previously reported. A study by Peck *et al.* in uremic pruritis patients reported that supplementation with omega-3 PUFA was associated with increased PGE₂ and an improvement in symptoms ^[392]. It has been suggested that supplementation with EPA may inhibit the 5-LOX pathway, shifting metabolism through the COX pathway. This results in increased 2-series prostaglandins and prostacyclins but a concurrent decrease in potent LTB₄ ^[393]. However, in the current study, LTB₄ levels were not altered by EPA supplementation suggesting some other mechanism is at play. A limitation of current eicosanoid research is the difficulty in quantifying EPA metabolites such as PGE₃, PGI₃ TXA₃ or LTB₅. Thus investigating the impact of EPA supplementation on eicosanoid production is generally limited to assessing its effects on the downstream metabolites of AA rather than the direct quantification of its metabolites. To address this challenge, a future collaboration will

use nuclear magnetic resonance technology (NMR) to characterise the entire metabolome of a cohort of patients receiving standard versus EPA enriched feed. Two dimensional NMR spectroscopy will allow for the quantification and comparison of all EPA and AA metabolites in the plasma of patients thus allowing for a more comprehensive assessment of the direct impact of EPA supplementation of eicosanoid synthesis. This technique will also allow for the quantification of resolvin E1 levels in both groups. Resolvin E1 is a downstream metabolite of EPA that plays an important role in the resolution of inflammation ^[217] but has not yet been assessed in human trials of EPA supplementation.

In addition to reducing the incidence of postoperative complications, supplementation with EPA also reduced the degree and rate of weight loss. The increased body weight observed on discharge in the EPA group was attributed to an increased fat free mass. The anabolic effects of EPA were reported in previous research from this group ^[237] and in other studies ^[394, 395]. This retention of fat free mass indicates a reduction in catabolism of skeletal muscle. EPA supplementation may prevent skeletal muscle catabolism via a variety of mechanism including reductions in inflammatory cytokines, alterations in the ubiquitin-proteasome proteolytic pathway, and reducing necrosis and apoptosis in myotubes ^[396]. Postoperative muscle catabolism has been shown to affect strength and recovery and impairs respiratory muscle function ^[367]. Thus the prevention of muscle catabolism by EPA supplementation may account for the reduced incidence of respiratory complications observed in the EPA supplemented arm.

The results of this clinical trial demonstrate a clear benefit of EPA supplementation postoperatively in terms of preserved muscle mass and reduced incidence of complications. What is not clear is the mechanism of action by which EPA achieves these results. The impact of EPA supplementation on inflammatory gene expression, cytokine production and eicosanoid metabolism was a complex mix of pro- and anti-inflammatory responses. It may be that EPA has altered roles at different stages postoperatively, influencing the pro-inflammatory and compensatory anti-inflammatory responses. In addition to a more detailed investigation into the impact on the inflammatory response, the impact on functional capacity and quality of life in

patients postoperatively needs to be determined before peri-operative EPA supplementation becomes the standard of care in oesophagectomy patients.

Chapter 7 General Discussion

Oesophageal cancer remains an aggressive malignancy that is increasing in incidence in the Western world ^[2-4]. Thus there is an impetus to understand the mechanisms driving the malignant progression and strategies to optimise outcomes in patients undergoing treatment. In this research novel mechanisms linking visceral obesity and the IGF axis to oesophageal adenocarcinoma progression, insight into how visceral obesity affects postoperative outcomes and a potential role for immunonutrition to improve outcomes in oesophageal cancer patients is presented.

There is robust epidemiological evidence linking obesity with oesophageal adenocarcinoma ^[11] and other malignancies ^[23]. As research in this area has progressed it has become evident that it is specifically an excess of visceral obesity, with its associated systemic inflammation and dysmetabolism that is pro-tumourigenic ^[254]. An increasing number of studies have emerged in recent years linking excess visceral adiposity with impaired response to therapy or a more aggressive tumour phenotype, using CT-defined visceral fat area measurements ^[126, 259, 269, 273-275]. The limiting factor of this research however is the lack of an appropriate measure to classify visceral obesity in Caucasian males and females. Thus the first step in this research programme was to develop an appropriate threshold for the classification of visceral obesity in gastrointestinal patients that also reflects the gender differences in visceral adipose tissue distribution.

In this research, using metabolic syndrome as a readout of dysmetabolism, it was determined that visceral obesity could be classified as VFA thresholds of $\geq 160\text{cm}^2$ for men and $\geq 80\text{cm}^2$ for females. The most interesting, although not surprising, aspect of this finding is the difference between the male and female thresholds. Gender greatly influences fat distribution, with men storing approximately 20-30% of their total body fat in the visceral compartment, irrespective of their obesity status ^[264]. Conversely, women tend to amass excess adipose tissue peripherally, and do not accumulate significant amounts of visceral fat until a moderate level of obesity is reached ^[263]. Other reports show that men have a higher prevalence of obesity-related metabolic diseases ^[252, 276]. This study identified significantly different mean visceral fat areas ($p < 0.0001$) for men (228.6cm^2) and women (146.8cm^2) with the MetSyn, highlighting profound differences between men and women and the absolute requirement for

gender-specific abdominal fat area cut-offs. Although previous studies have used the gold standard techniques of CT or MRI scanning for the quantification of fat compartments in obesity, and report a VFA $\geq 130 \text{ cm}^2$ was associated with obesity related pathologies, no study heretofore has provided gender specific VFA cut-off values in Caucasians [261, 262, 277, 278].

In addition, this research also confirmed the specific inflammatory nature of visceral adipose tissue as a more robust association was evident between metabolic syndrome and visceral fat than subcutaneous fat [284]. These data strongly support the theory that visceral adipose tissue is pathologically distinct and quantification of this depot is essential for research in obesity and its associated morbidities. Furthermore, this research confirms the validity of using waist circumference as a surrogate marker of visceral obesity with the corresponding waist circumference to VFA values obtained in this study for males and females closely relating to the WHO waist circumference values proposed as an increased risk to health.

The generation of these pathologically relevant thresholds is of paramount importance in the study of obesity associated malignancy, allowing for consistency in visceral obesity classification across the field of research. Having successfully determined an appropriate strategy for the classification of visceral obesity in gastrointestinal cancer patients, the next step was to try to understand the mechanisms by which visceral obesity drives oesophageal malignancy.

A core component of the metabolic syndrome is insulin resistance with resultant hyperinsulinaemia. Alongside alterations in the insulin axis, the insulin-like growth factor axis is also affected. The IGF-1 axis has been implicated in the progression of breast, pancreatic, colorectal and oesophageal cancer [90-93]. Obesity increases levels of bioactive free IGF-1 [94, 95], high levels of which are associated with increased risk of malignancy [90-93]. Thus we investigated the IGF axis as a possible lynchpin between visceral obesity and oesophageal malignancy. This study generated a number of novel, interesting findings. The observation that circulating IGF-1 levels are upregulated in adenocarcinoma compared to oesophageal SCC suggests that obesity and its influence on the IGF-1 pathway may be a critical factor for specifically oesophageal

adenocarcinoma development. Hypothesising that obesity is driving oesophageal adenocarcinoma via alterations in IGF-1, it was of great interest to observe that viscerally obese patients had higher circulating levels of IGF-1. Furthermore the fact that visceral obesity but not BMI was associated with increased IGF-1 confirms the specific role of visceral adipose tissue excess on IGF axis activity. Research to date linking IGF-1 with BMI has been fraught with controversy but this study was the first to associate IGF-1 specifically with an excess of visceral adipose tissue. In addition, the observation that patients with the metabolic syndrome had higher serum IGF-1 also supports the hypothesis that the deranged metabolism observed in visceral obesity may drive the increased circulating levels of IGF-1 ^[292]. High circulating levels of IGF-1 have been associated with many cancers and abrogation of IGF-1 leads to attenuation of tumour growth ^[96]. Thus this excess of IGF-1 observed in visceral obesity is a plausible mechanism by which visceral obesity drives oesophageal adenocarcinoma progression.

To further characterise the role of the IGF axis in oesophageal tumourigenesis, resected tumour specimens were examined for expression of IGF-1R. Whilst this study did not find a significant association between obesity and IGF-1R expression, a subsequent study within this research group utilising a larger sample size did find obesity to be associated with significantly higher tumour expression of IGF-1R ^[397]. In this study, a distinct pattern of expression was observed in oesophageal squamous cell and oesophageal adenocarcinoma sections. Higher IGF-1R expression was observed at the tumour invasive edge compared to the tumour island. As tumours upregulate receptors for growth factors in their microenvironment, higher IGF-1R expression indicates that IGF-1 may be an important growth/survival factor in oesophageal cancer. The fact that IGF-1R was not associated with tumour stage, lymph node involvement or patient survival suggests that the role of the IGF-1 axis in oesophageal tumour progression may be key factor in local tumour growth and development.

Whether the higher circulating of IGF-1 in patients influenced IGF-1R expression was not investigated in this study, thus the systemic effects of IGF-1 can only be postulated. However, the possible role of paracrine sources of IGF-1 was investigated. Tumour-associated macrophages (TAMs) are a significant component of inflammatory infiltrate in neoplastic tissues, the largest accumulation of which occurs at the invasive

edge of the tumour. Tissue resident and tumour-associated macrophages have been shown to secrete IGF-1, promoting survival, proliferation and invasion of cancer cells [299, 301, 302]. In this study, numbers of CD68⁺ cells in tumour sections were assessed by immunohistochemistry to determine macrophage infiltration. Increased CD68⁺ macrophage infiltration was observed in the stroma surrounding the tumour, and was further increased at the invasive tumour edge. This supports our hypothesis that the paracrine secretion of IGF-1 supports oesophageal adenocarcinoma progression.

The findings of this study provide evidence for a clear role of the IGF axis in oesophageal cancer progression. In addition, the differential expression of IGF-1 in viscerally obese and oesophageal adenocarcinoma patients provides mechanistic evidence to substantiate the epidemiological based doctrine that obesity increases the risk of oesophageal adenocarcinoma. Investigation of the potential therapeutic benefits of IGF-1R blockade on oesophageal cancer is a current research focus within this research group.

As oesophageal adenocarcinoma is an obesity driven malignancy, its prevalence in society will continue to increase alongside our increasing prevalence of obesity. Thus the goal of research in oesophageal cancer is not only to understand the mechanisms influencing tumour progression, but also the optimisation of outcomes for patients undergoing treatment. The curative approach to oesophageal cancer is resection [199], a major surgery associated with significant morbidity and mortality [200]. As oesophageal adenocarcinoma is an obesity fuelled cancer, many patients present for resection with a viscerally obese body type. Obesity has historically been considered a risk factor for adverse postoperative outcomes. Visceral obesity gives rise to a state of chronic systemic inflammation and research has indicated that visceral obesity increases the risk of postoperative complications [398]. However no study to date has provided a comprehensive assessment of the inflammatory response of obese and non-obese patients undergoing resection for malignancy.

This study generated a number of interesting findings relating to the impact of visceral obesity and the associated metabolic syndrome on the inflammatory response postoperatively. It was determined that visceral obesity and the metabolic syndrome

were associated with alterations in the acute phase response postoperatively, as reflected by increased production of CRP and a reduction in albumin synthesis. Other studies have also supported this finding that CRP is increased postoperatively in obese and dysmetabolic patients ^[325, 338]. The influence of obesity and the metabolic syndrome on CRP and albumin synthesis was different, depending on whether or not the patient experienced a postoperative complication. In patients who experienced a normal postoperative recovery, visceral obesity and the metabolic syndrome were associated with increase CRP and CRP:albumin on day 1 postoperatively, suggesting a heightened initial acute inflammatory response. In patients who experienced a postoperative complication, visceral obesity and the metabolic syndrome were associated with higher CRP and CRP:albumin at day 7 and day 14 postoperatively, indicating an exacerbation of the inflammatory response. These findings indicate a clear influence of visceral obesity and the metabolic syndrome on the acute phase inflammatory response.

In an attempt to further characterise the inflammatory response postoperatively in obese and non-obese patients, the gene expression of immune cell subsets and circulating levels of inflammatory cytokines were assessed. Significant alterations in inflammatory gene expression postoperatively were observed in the obese compared to the non-obese patients postoperatively. Whilst some of the altered genes in this study were not successfully validated, this broad gene analysis has, for the first time, identified a number of other genes altered in the circulating leukocytes of obese patients postoperatively that require further investigation and validation.

Central to the regulation of many genes involved in inflammatory responses is the transcription factor NF- κ B ^[337]. In this study, there was a different pattern of NF- κ B activation postoperatively in the obese compared to the non-obese patients, with the non-obese patients demonstrating a greater reduction in activation on day 1 postoperatively. This finding has not been reported to date and the clinical significance is unclear. However, this observation does raise interesting questions regarding the impact of visceral obesity on key regulators of the inflammatory response postoperatively.

These alterations in NF- κ B expression may underlie the changes observed in inflammatory cytokine production in obese and dysmetabolic patients. In the panel of inflammatory cytokines, it was evident that obesity and metabolic syndrome status influenced postoperative inflammatory response. IL-6, which is produced in the immediate postoperative phase, correlating with the magnitude of surgery ^[358], was higher in the obese and metabolic syndrome positive patients postoperatively. In addition IL-12, a key mediator of the Th1 pro-inflammatory response ^[359] was significantly higher in obese patients preoperatively and postoperatively. However not all pro-inflammatory cytokines were increased in the obese and dysmetabolic patients as these patients also demonstrated lower levels of IL-17, which potentiates inflammatory responses ^[360]. In addition, higher levels of anti-inflammatory TGF- β were observed in the obese patients. This may be an attempt to counter the pro-inflammatory environment observed in obesity or it may be that this increased TGF- β , is having a negative pro-thrombotic effect ^[361].

Of great interest was the finding that, in the case of a number of cytokines, expression levels varied depending on whether patients were classified by obesity or metabolic syndrome status. These findings suggest that adipose tissue excess, in the absence of dysmetabolism, has a different effect on postoperative immune response. A possible explanation may be the role of hyperglycaemia, which is characteristic of the metabolic syndrome and a factor known to be associated with increased morbidity postoperatively ^[338]. When the cytokine data was analysed with respect to glycaemic status, clear alterations in expression levels were observed between the two cohorts, with higher IL-6, IFN- γ and IL-8 levels in the hyperglycaemic cohort. These findings again suggest that the potential impact of visceral obesity on postoperative inflammation is multifactorial, with the associated elements of dysmetabolism all potentially exerting different effects on the inflammatory response.

The inflammatory response is a finely tuned mechanism and the impact of these alterations in inflammatory cytokine production may be significant. No difference was observed in the overall incidence of complications or the incidence of specific complications between obese and non-obese patients. However, differences were

observed in the clinical parameters of these patients. The obese patients had higher temperatures and heart rates postoperatively, suggesting an underlying systemic inflammatory response ^[399] which is also reflected in the CRP and cytokine data. Whilst a previous study reported that obesity was not associated with systemic inflammatory response syndrome (SIRS), BMI was used to classify obesity as opposed to visceral fat area ^[400]. The observations in this study suggest that obesity and dysmetabolism most definitely influence the postoperative inflammatory response however further work is warranted to elucidate the molecular mechanisms at play before alterations in their clinical management can be recommended.

Irrespective of a patient's anthropomorphic status, an oesophagectomy is a major procedure that elicits a catabolic stress response, which if uncontrolled can give rise to significant morbidity and mortality. In addition, the long term consequences of the surgery can be very debilitating and greatly impact on the patient's quality of life ^[201, 202]. Over the past number of years there has been an emergence of immunonutrition as a strategy to optimise the outcomes in these patients. In this research we examined the impact of peri-operative supplementation with an eicosapentaenoic acid (EPA) enriched enteral feed on the inflammatory response and recovery in oesophagectomy patients.

It was found that supplementation with EPA significantly reduced the incidence of postoperative complications in oesophagectomy patients, specifically respiratory complications. This was a very interesting finding. Firstly, no study to date has demonstrated that enteral feeding supplemented with EPA alone can reduce postoperative complications. Secondly, respiratory complications are the most common post-oesophagectomy complication and they are associated with significant morbidity and delayed recovery.

In addition to reducing the incidence of postoperative complications, supplementation with EPA also reduced the degree and rate of weight loss. Of greater importance, the EPA group retained their fat free mass. This retention of fat free mass indicates a reduction in catabolism of skeletal muscle. Postoperative muscle catabolism has been shown to affect strength and recovery and impairs both skeletal and respiratory muscle function ^[367, 401]. Thus, the prevention of muscle catabolism by EPA

supplementation may account for the reduced incidence of respiratory complications observed in the EPA supplemented arm. Taking these findings together, a strong case can be made for peri-operative supplementation with EPA in this cohort.

This study also provides a more detailed examination of the impact of EPA supplementation on the postoperative inflammatory response than has been reported to date. Again, the use of an expansive gene array allowed for the identification of novel inflammatory targets for EPA supplementation. Using this technique it was found that EPA supplementation dramatically impacted on the expression of CCL4, increasing its production postoperatively where it was initially identified as being reduced. Similarly, the array revealed an up regulation in IL-10 postoperatively and this was reduced by EPA supplementation. This flip in inflammatory gene production has not been reported to date and calls into question the widespread belief that EPA has solely anti-inflammatory properties. It may be that this more pro-inflammatory gene production is an attempt to prevent an extreme compensatory anti-inflammatory response which is associated with increased morbidity. However, these are two genes looked at in isolation and a more widespread validation of other genes identified on the array may provide greater insight into the mechanisms at play.

This study also characterised the effect of peri-operative EPA supplementation on the activity of distinct immune cell populations, monocytes and neutrophils. At a gene level EPA supplementation did not appear to impact on M1/M2 monocyte markers. A more apparent effect was observed in neutrophils, whereby production of myeloperoxidase was significantly reduced by EPA. The relevance of these gene changes must be interpreted with caution as neutrophils have a very short half and are active at the tissue site ^[355]. In light of the finding that EPA reduced respiratory complications, it would be of interest to examine neutrophil activity in the lung postoperatively, utilising bronchoalveolar lavage. In addition, collecting blood samples intraoperatively may shed more light on the impact of EPA on the acute, short lived neutrophils.

With respect to circulating markers of inflammation, EPA supplementation did not reduce CRP or ESR levels but did lead to a reduced white cell count, suggesting an attenuation of systemic inflammation. The mechanism surrounding this proposed

reduction in inflammation is unclear as an examination of inflammatory cytokines did not demonstrate a reduction in pro-inflammatory cytokine production following EPA supplementation. In fact many trials of EPA supplementation have reported conflicting effects on cytokine production. The postoperative course is varied in every patient and the immune response is continuously trying to maintain balance between pro- and anti-inflammatory responses ^[359, 360]. These factors may make it difficult to ascertain the specific effect of EPA on the inflammatory response. Alternatively, it may be that the reduction in morbidity observed following EPA supplementation is not related to alterations in cytokine production.

Whilst no distinct impact on circulating cytokine production was found following EPA supplementation, a clear alteration was observed in eicosanoid production. Interestingly, peri-operative EPA supplementation led to an increase in 2-series prostaglandin and prostacyclin levels. This finding is counter-intuitive given the premise that increased consumption of EPA leads to a reduction in AA metabolites. In the context of the reduced incidence of complications in the EPA supplemented cohort, this unexpected manipulation of the eicosanoid pathway must be beneficial. It may be that PGE₂ has an initial pro-inflammatory role post trauma/surgery, followed by an anti-inflammatory role characterised by reduced 5-LOX and increased lipoxin A₄ leading to inflammation resolution ^[393]. What is required, and what is lacking from studies investigating the impact of EPA supplementation, is an assessment of the production of 3-series prostaglandins and 5-series leukotrienes derived from EPA. This poses difficulty due to the lack of any commercially available high throughput detection techniques. However, attempting to deduce the impact of EPA supplementation by its proposed inhibitory effects on AA metabolism is an ineffective approach. To tackle this problem we have identified a collaborator who will use NMR technology to characterise the entire metabolome of a cohort of patients receiving standard versus EPA enriched feed. This will allow for the quantification and comparison of all EPA and AA metabolites in the plasma of patients thus allowing for a more comprehensive assessment of the direct impact of EPA supplementation on eicosanoid synthesis. In addition, it will be possible to quantify resolvin E1 levels in both groups, which may be playing a key role in promoting recovery in patients receiving EPA ^[217].

The results of this clinical trial demonstrate a clear benefit of EPA supplementation peri-operatively in terms of preserved muscle mass and reduced incidence of complications. However a more detailed investigation into the mechanisms by which EPA achieves these beneficial outcomes is warranted. In addition, it is important to assess whether or not EPA supplementation translates into improved functional status and tangible benefits to the postoperative and long term quality of life in patients.

This body of work has generated a number of interesting findings relating to the pathogenesis of oesophageal cancer and the recovery of oesophagectomy patients. An appropriate threshold for classification of visceral obesity has been determined allowing a more unified approach to the study of visceral obesity and gastrointestinal cancer. In addition, plausible mechanisms by which visceral obesity may increase oesophageal cancer via the IGF-axis were identified. For the first time, the impact of visceral obesity and dysmetabolism of the postoperative inflammatory response and recovery was characterised. Finally, the effectiveness of peri-operative immunonutrition with EPA in reducing complications and preventing catabolism in oesophagectomy patients was demonstrated. Together these findings add to the knowledge base regarding oesophageal cancer, which will hopefully result in improved treatment strategies and outcomes in patients suffering from this aggressive, ever increasing malignancy.

7.1 FUTURE DIRECTIONS

This research has identified a number of areas for future work and has spawned a number of other research projects.

The promising findings relating to the role of the IGF axis in obesity and cancer resulted in a separate 3 year research project which confirmed a strong association between visceral obesity and the IGF axis in oesophageal adenocarcinoma. In addition the potential therapeutic benefits to IGF-1R blockade and interactions with another obesity associated hormone, vascular endothelial growth factor, were investigated. Furthermore, another 4 year research project has commenced which will focus on the role of the STAT3 pathway, a downstream signalling from the IGF-1R, in obesity and oesophageal cancer.

With respect to the postoperative inflammatory response in patients, this is going to be investigated in greater detail in a forthcoming DCCR funded project. Using biobanked serum and plasma samples, the levels of the acute phase reactants fibrinogen, serum amyloid A and cortisol will be assessed in obese and non-obese patients to further characterise the acute inflammatory response postoperatively.

An alteration in the postoperative inflammatory response is just one aspect of the oesophageal cancer patient journey that may be altered by visceral obesity. It is plausible that visceral obesity will impact on the metabolic activity of the tumour and the response to chemoradiotherapy. To investigate this further a project has been designed using a retrospective cohort of 200 patients. The metabolic activity of tumours pre-treatment will be assessed by FDG uptake on PET-CT, the response to chemoradiotherapy will be determined by TRG status and the post treatment/pre-surgical inflammatory state will be assessed using biobanked blood samples and a cytokine array. I was recently awarded a research bursary from IrSPEN to carry out this study.

With respect to the clinical trial investigating EPA, recruitment and follow up is ongoing. The future work in this area is the NMR characterisation of the metabolome in patients and the synthesis of the results presented herein, pertaining to the stress response, with the other primary outcomes of the trial, i.e., the functional status and long term quality of life in oesophagectomy patients.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global Cancer Statistics, 2002. *CA: A Cancer Journal for Clinicians* 2005 **55** 74-108
2. Bollschweiler E, Wolfgarten E, Gutschow C, Holscher AH. Demographic variations in the rising incidence of esophageal adenocarcinoma in white males. *Cancer* 2001 **92** 549-555
3. Bosetti C, Levi F, Ferlay J, Garavello W, Lucchini F, Bertuccio P, *et al.* Trends in oesophageal cancer incidence and mortality in Europe. *International Journal of Cancer* 2008 **122** 1118-1129
4. Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiology Biomarkers & Prevention* 2010 **19** 1893-1907
5. National Cancer Registry of Ireland (NCRI). Updated 2009
6. Vaughan TL, Davis S, Kristal A, Thomas DB. Obesity, alcohol, and tobacco as risk factors for cancers of the esophagus and gastric cardia: adenocarcinoma versus squamous cell carcinoma. *Cancer Epidemiology Biomarkers & Prevention* 1995 **4** 85-92
7. Lagergren J, Bergström R, Lindgren A, Nyrén O. symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *New England Journal of Medicine* 1999 **340** 825-831
8. Jankowski JA, Wright NA, Meltzer SJ, Triadafilopoulos G, Geboes K, Casson AG, *et al.* Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *American Journal of Pathology* 1999 **154** 965-973
9. McCarthy SN, Gibney MJ, Flynn A. Overweight, obesity and physical activity levels in Irish adults: evidence from the North/South Ireland food consumption survey. *Proceedings of the Nutrition Society* 2002 **61** 3-7
10. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nature Reviews Cancer* 2004 **4** 579-591
11. Ryan AM, Rowley SP, Fitzgerald AP, Ravi N, Reynolds JV. Adenocarcinoma of the oesophagus and gastric cardia: male preponderance in association with obesity. *European Journal of Cancer* 2006 **42** 1151-1158
12. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *The Lancet* 2008 **371** 569-578
13. Kubo A, Corley DA. Body mass index and adenocarcinomas of the esophagus or gastric cardia: a systematic review and meta-analysis. *Cancer Epidemiology Biomarkers & Prevention* 2006 **15** 872-878
14. Friedenberg FK, Xanthopoulos M, Foster GD, Richter JE. The association between gastroesophageal reflux disease and obesity. *American Journal of Gastroenterology* 2008 **103** 2111-2122
15. Lagergren J, Bergstrom R, Nyren O. Association between body mass and adenocarcinoma of the esophagus and gastric cardia. *Annals of Internal Medicine* 1999 **130** 883-890
16. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA: A Cancer Journal for Clinicians* 2011 **61** 69-90

17. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, *et al.* Origins and evolution of the Western diet: health implications for the 21st century. *The American Journal of Clinical Nutrition* 2005 **81** 341-354
18. Eaton SB, Konner M, Shostak M. Stone agers in the fast lane: chronic degenerative diseases in evolutionary perspective. *The American Journal of Medicine* 1988 **84** 739-749
19. Irish Universities Nutrition Alliance. National Adult Nutrition Survey. 2011
20. National Longitudinal Study of Children. Growing up in Ireland, overweight and obesity amongst 9-year-olds 2011 **Report 2**
21. Serdula MK, Ivery D, Coates RJ, Freedman DS, Williamson DF, Byers T. Do obese children become obese adults? A Review of the Literature. *Preventive Medicine* 1993 **22** 167-177
22. Klein S, Wadden T, Sugerman HJ. AGA technical review on obesity. *Gastroenterology* 2002 **123** 882-932
23. World Cancer Research Fund, American Institute for Cancer Research. Food, nutrition, physical activity and the prevention of cancer: global perspective. 2007
24. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *New England Journal of Medicine* 2003 **348** 1625-1638
25. Dirx MJM, Zeegers MPA, Dagnelie PC, van den Bogaard T, van den Brandt PA. Energy restriction and the risk of spontaneous mammary tumors in mice: A meta-analysis. *International Journal of Cancer* 2003 **106** 766-770
26. Sjöström L, Gummesson A, Sjöström CD, Narbro K, Peltonen M, Wedel H, *et al.* Effects of bariatric surgery on cancer incidence in obese patients in Sweden (Swedish Obese Subjects Study): a prospective, controlled intervention trial. *The Lancet Oncology* 2009 **10** 653-662
27. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. *World Health Organ Tech Rep Ser* 2000 **894** i-xii, 1-253
28. Vague J. La différenciation sexuelle, facteur déterminant des formes de l'obésité. *Presse Medicale* 1947 **53** 339-340
29. Vague J. The degree of masculine differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease. 1956. *Obesity Research* 1956 **4** 204-212
30. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu C-Y, *et al.* Abdominal visceral and subcutaneous adipose tissue compartments. *Circulation* 2007 **116** 39-48
31. Galic S, Oakhill JS, Steinberg GR. Adipose tissue as an endocrine organ. *Molecular and Cellular Endocrinology* 2010 **316** 129-139
32. Alberti KG, Zimmet P, Shaw J. Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med* 2006 **23** 469 - 480
33. Fujioka S, Matsuzawa Y, Tokunaga K, Tarui S. Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity. *Metabolism* 1987 **36** 54-59
34. Nedungadi TP, Clegg DJ. Sexual dimorphism in body fat distribution and risk for cardiovascular diseases. *Journal of Cardiovascular Translational Research* 2009 **2** 321-327

35. Schapira DV, Clark RA, Wolff PA, Jarrett AR, Kumar NB, Aziz NM. Visceral obesity and breast cancer risk. *Cancer* 1994 **74** 632-639
36. Beddy P, Howard J, McMahon C, Knox M, de Blacam C, Ravi N, *et al.* Association of visceral adiposity with oesophageal and junctional adenocarcinomas. *British Journal of Surgery* **97** 1028-1034
37. Schoen RE, Tangen CM, Kuller LH, Burke GL, Cushman M, Tracy RP, *et al.* Increased blood glucose and insulin, body size, and incident colorectal cancer. *Journal of the National Cancer Institute* 1999 **91** 1147-1154
38. Yamaji T, Iwasaki M, Sasazuki S, Kurahashi N, Mutoh M, Yamamoto S, *et al.* Visceral fat volume and the prevalence of colorectal adenoma. *American Journal of Epidemiology* 2009 **170** 1502-1511
39. Nam SY, Kim BC, Han KS, Ryu KH, Park BJ, Kim HB, *et al.* Abdominal visceral adipose tissue predicts risk of colorectal adenoma in both sexes. *Clinical Gastroenterology and Hepatology* 2010 **8** 443-450.e442
40. Cowey S, Hardy RW. The metabolic syndrome: a high-risk state for cancer? *The American Journal of Pathology* 2006 **169** 1505-1522
41. Giovannucci E. Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. *The American Journal of Clinical Nutrition* 2007 **86** 836S-842S
42. Colangelo LA, Gapstur SM, Gann PH, Dyer AR, Liu K. Colorectal cancer mortality and factors related to the insulin resistance syndrome. *Cancer Epidemiology Biomarkers & Prevention* 2002 **11** 385-391
43. Bowers K, Albanes D, Limburg P, Pietinen P, Taylor PR, Virtamo J, *et al.* A prospective study of anthropometric and clinical measurements associated with insulin resistance syndrome and colorectal cancer in male smokers. *American Journal of Epidemiology* 2006 **164** 652-664
44. Stocks T, Borena W, Strohmaier S, Bjørge T, Manjer J, Engeland A, *et al.* Cohort profile: the metabolic syndrome and cancer project (Me-Can). *International Journal of Epidemiology* 2010 **39** 660-667
45. Stocks T, Lukanova A, Bjørge T, Ulmer H, Manjer J, Almquist M, *et al.* Metabolic factors and the risk of colorectal cancer in 580,000 men and women in the metabolic syndrome and cancer project (Me-Can). *Cancer* 2010 **117** 2398-2407
46. Bjørge T, Stocks T, Lukanova A, Tretli S, Selmer R, Manjer J, *et al.* Metabolic syndrome and endometrial carcinoma. *American Journal of Epidemiology* 2010 **171** 892-902
47. Häggström C, Stocks T, Rapp K, Bjørge T, Lindkvist B, Concin H, *et al.* Metabolic syndrome and risk of bladder cancer: prospective cohort study in the metabolic syndrome and cancer project (Me-Can). *International Journal of Cancer* 2011 **128** 1890-1898
48. Johansen D, Stocks T, Jonsson HK, Lindkvist B, Bjørge T, Concin H, *et al.* Metabolic factors and the risk of pancreatic cancer: a prospective analysis of almost 580,000 men and women in the metabolic syndrome and cancer project. *Cancer Epidemiology Biomarkers & Prevention* 2010 **19** 2307-2317
49. Russo A, Autelitano M, Bisanti L. Metabolic syndrome and cancer risk. *European Journal of Cancer* 2008 **44** 293-297
50. Kang HW, Kim D, Kim HJ, Kim CH, Kim YS, Park MJ, *et al.* Visceral obesity and insulin resistance as risk factors for colorectal adenoma: a cross-sectional, case-control study. *American Journal of Gastroenterology* 2009 **105** 178-187

51. Siegel EM, Ulrich CM, Poole EM, Holmes RS, Jacobsen PB, Shibata D. The effects of obesity and obesity-related conditions on colorectal cancer prognosis. *Cancer Control* 2010 **17** 52-57
52. Healy L, Howard J, Ryan A, Beddy P, Mehigan B, Stephens R, *et al.* Metabolic syndrome and leptin are associated with adverse pathological features in male colorectal cancer patients. *Colorectal Dis* 2012 **14** 157-165
53. Healy LA, Ryan AM, Carroll P, Ennis D, Crowley V, Boyle T, *et al.* Metabolic syndrome, central obesity and insulin resistance are associated with adverse pathological features in postmenopausal breast cancer. *Clinical Oncology (Royal College of Radiologist (Great Britain))* 2012 **22** 281-288
54. Moon H-G, Ju Y-T, Jeong C-Y, Jung E-J, Lee Y-J, Hong S-C, *et al.* Visceral obesity may affect oncologic outcome in patients with colorectal cancer. *Annals of Surgical Oncology* 2008 **15** 1918-1922
55. Shen Z, Wang S, Ye Y, Yin M, Yang X, Jiang K, *et al.* Clinical study on the correlation between metabolic syndrome and colorectal carcinoma. *ANZ Journal of Surgery* 2010 **80** 331 - 336
56. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, *et al.* Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature Medicine* 2011 **17** 1498-1503
57. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, *et al.* Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Research* 2011 **71** 2455-2465
58. Elliott BE, Tam SP, Dexter D, Chen ZQ. Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: effect of estrogen and progesterone. *International Journal of Cancer* 1992 **51** 416-424
59. Elliott BE, Maxwell L, Arnold M, Wei WZ, Miller FR. Expression of epithelial-like markers and class I major histocompatibility antigens by a murine carcinoma growing in the mammary gland and in metastases: orthotopic site effects. *Cancer Research* 1988 **48** 7237-7245
60. Celis JE, Moreira JM, Cabezon T, Gromov P, Friis E, Rank F, *et al.* Identification of extracellular and intracellular signaling components of the mammary adipose tissue and its interstitial fluid in high risk breast cancer patients: toward dissecting the molecular circuitry of epithelial-adipocyte stromal cell interactions. *Molecular & Cellular Proteomics* 2005 **4** 492-522
61. Cook KS, Min HY, Johnson D, Chaplinsky RJ, Flier JS, Hunt CR, *et al.* Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. *Science* 1987 **237** 402-405
62. Flier JS, Cook KS, Usher P, Spiegelman BM. Severely impaired adipsin expression in genetic and acquired obesity. *Science* 1987 **237** 405-408
63. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994 **372** 425-432
64. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to c1q, produced exclusively in adipocytes. *Journal of Biological Chemistry* 1995 **270** 26746-26749
65. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nature Reviews Immunology* 2011 **11** 85-97

66. Harvey AE, Lashinger LM, Hursting SD. The growing challenge of obesity and cancer: an inflammatory issue. *Annals of the New York Academy of Sciences* 2011 **1229** 45-52
67. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocrine Reviews* 2005 **26** 439-451
68. Rose DP, Komninou D, Stephenson GD. Obesity, adipocytokines, and insulin resistance in breast cancer. *Obesity Reviews* 2004 **5** 153-165
69. Cummings DE, Foster KE. Ghrelin-leptin tango in body-weight regulation. *Gastroenterology* 2003 **124** 1532-1535
70. Pär S, Annekatrin L, Carine B, Stefan S, Richard P, Rudolf K, *et al.* Obesity and colon cancer: Does leptin provide a link? *International Journal of Cancer* 2004 **109** 149-152
71. Howard JM, Pidgeon GP, Reynolds JV. Leptin and gastro-intestinal malignancies. *Obesity Reviews* 2010 **11** 863-874
72. Somasundar P, McFadden DW, Hileman SM, Vona-Davis L. Leptin is a growth factor in cancer. *The Journal of Surgical Research* 2004 **116** 337-349
73. Garofalo C, Surmacz E. Leptin and cancer. *Journal of Cellular Physiology* 2006 **207** 12-22
74. Howard JM, Beddy P, Ennis D, Keogan M, Pidgeon GP, Reynolds JV. Associations between leptin and adiponectin receptor upregulation, visceral obesity and tumour stage in oesophageal and junctional adenocarcinoma. *British Journal of Surgery* **97** 1020-1027
75. Bergman RN, Ader M. Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends in Endocrinology and Metabolism* 2000 **11** 351-356
76. Ebeling P, Koivisto V. Non-esterified fatty acids regulate lipid and glucose oxidation and glycogen synthesis in healthy man. *Diabetologia* 1994 **37** 202-209
77. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *New England Journal of Medicine* 2003 **348** 1625-1638
78. Moller DE, Flier JS. Insulin resistance - mechanisms, syndromes, and implications. *New England Journal of Medicine* 1991 **325** 938-948
79. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocrine Reviews* 2000 **21** 697-738
80. Fasshauer M, Paschke R. Regulation of adipocytokines and insulin resistance. *Diabetologia* 2003 **46** 1594-1603
81. Greenberg AS, McDaniel ML. Identifying the links between obesity, insulin resistance and beta-cell function: potential role of adipocyte-derived cytokines in the pathogenesis of type 2 diabetes. *European Journal of Clinical Investigation* 2002 **32 Suppl 3** 24-34
82. Stocks T, Rapp K, Bjorge T, Manjer J, Ulmer H, Selmer R, *et al.* Blood glucose and risk of incident and fatal cancer in the metabolic syndrome and cancer project (Me-Can): analysis of six prospective cohorts. *PLoS Medicine* 2009 **6** e1000201
83. Hsing AW, Gao Y-T, Chua S, Deng J, Stanczyk FZ. Insulin resistance and prostate cancer risk. *Journal of the National Cancer Institute* 2003 **95** 67-71
84. Yang Y-X, Hennessy S, Lewis JD. Insulin therapy and colorectal cancer risk among type 2 diabetes mellitus patients. *Gastroenterology* 2004 **127** 1044-1050

85. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nature Reviews Cancer* 2008 **8** 915-928
86. Osborne CK, Bolan G, Monaco ME, Lippman ME. Hormone responsive human breast cancer in long-term tissue culture: effect of insulin. *Proceedings of the National Academy of Sciences* 1976 **73** 4536-4540
87. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, *et al.* The role of insulin receptors and IGF-I receptors in cancer and other diseases. *Archives Of Physiology And Biochemistry* 2008 **114** 23 - 37
88. Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocrine Reviews* 2007 **28** 20-47
89. Jones JJ, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* 1995 **16** 3-34
90. Nemoto T, Ohashi K, Akashi T, Johnson JD, Hirokawa K. Overexpression of protein tyrosine kinases in human esophageal cancer. *Pathobiology* 1997 **65** 195-203
91. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, *et al.* Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* 1998 **279** 563-566
92. Ma J, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH, *et al.* Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and igf-binding protein-3. *Journal of the National. Cancer Institute.* 1999 **91** 620-625
93. Renehan AG, Frystyk J, Flyvbjerg A. Obesity and cancer risk: the role of the insulin-IGF axis. *Trends in Endocrinology & Metabolism* 2006 **17** 328-336
94. Frystyk J, Vestbo E, Skjaerbaek C, Mogensen CE, Orskov H. Free insulin-like growth factors in human obesity. *Metabolism* 1995 **44** 37-44
95. Nam SY, Lee EJ, Kim KR, Cha BS, Song YD, Lim SK, *et al.* Effect of obesity on total and free insulin-like growth factor (IGF)-1, and their relationship to IGF-binding protein (BP)-1, IGFBP-2, IGFBP-3, insulin, and growth hormone. *International Journal of Obesity & Related Metabolic Disorders* 1997 **21** 355
96. Wu Y, Brodt P, Sun H, Mejia W, Novosyadlyy R, Nunez N, *et al.* Insulin-like growth factor-I regulates the liver microenvironment in obese mice and promotes liver metastasis. *Cancer Research* 2010 **70** 57-67
97. Lashinger LM, Malone LM, McArthur MJ, Goldberg JA, Daniels EA, Pavone A, *et al.* Genetic reduction of insulin-like growth factor-1 mimics the anticancer effects of calorie restriction on cyclooxygenase-2-driven pancreatic neoplasia. *Cancer Prevention Research (Philadelphia PA)* **4** 1030-1040
98. Olivo-Marston SE, Hursting SD, Lavigne J, Perkins SN, Maarouf RS, Yakar S, *et al.* Genetic reduction of circulating insulin-like growth factor-1 inhibits azoxymethane-induced colon tumorigenesis in mice. *Molecular Carcinogenesis* 2009 **48** 1071-1076
99. Alberini CM, Chen DY. Memory enhancement: consolidation, reconsolidation and insulin-like growth factor 2. *Trends in Neurosciences* 2012 **35** 274-283
100. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *The Lancet* 2001 **357** 539-545
101. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002 **420** 860-867

102. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* 2009 **30** 1073-1081
103. Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *Journal of Clinical Endocrinology & Metabolism* 1998 **83** 847-850
104. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. *Journal of the American Medical Association* 1999 **282** 2131-2135
105. Weyer C, Yudkin JS, Stehouwer CD, Schalkwijk CG, Pratley RE, Tataranni PA. Humoral markers of inflammation and endothelial dysfunction in relation to adiposity and in vivo insulin action in Pima Indians. *Atherosclerosis* 2002 **161** 233-242
106. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor- α in human obesity and insulin resistance. *The Journal of Clinical Investigation* 1995 **95** 2409-2415
107. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *The Journal of Clinical Investigation* 1995 **95** 2111-2119
108. Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE, *et al.* Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *International Journal of Obesity (London)* 2006 **30** 1347-1355
109. Schaffler A, Muller-Ladner U, Scholmerich J, Buchler C. Role of adipose tissue as an inflammatory organ in human diseases. *Endocrine Reviews* 2006 **27** 449-467
110. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of Clinical Investigation* 2003 **112** 1821-1830
111. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of Clinical Investigation* 2003 **112** 1796-1808
112. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *Journal of Clinical Endocrinology & Metabolism* 2004 **89** 2548-2556
113. Neels JG, Olefsky JM. Inflamed fat: what starts the fire? *The Journal of Clinical Investigation* 2006 **116** 33-35
114. Curat CA, Miranville A, Sengenès C, Diehl M, Tonus C, Busse R, *et al.* From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 2004 **53** 1285-1292
115. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* 2003 **100** 7265-7270
116. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. *The Journal of Clinical Investigation* 2003 **112** 1785-1788
117. Bassols J, Ortega FJ, Moreno-Navarrete JM, Peral B, Ricart W, Fernandez-Real JM. Study of the proinflammatory role of human differentiated omental adipocytes. *Journal of Cellular Biochemistry* 2009 **107** 1107-1117

118. Morris DL, Singer K, Lumeng CN. Adipose tissue macrophages: phenotypic plasticity and diversity in lean and obese states. *Current Opinion in Clinical Nutrition & Metabolic Care* **14** 341-346
119. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, *et al.* CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nature Medicine* 2009 **15** 914-920
120. Lysaght J, Allott EH, Donohoe CL, Howard JM, Pidgeon GP, Reynolds JV. T lymphocyte activation in visceral adipose tissue of patients with oesophageal adenocarcinoma. *British Journal of Surgery* **98** 964-974
121. Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, *et al.* Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* 2012 **37** 574-587
122. Lynch L, O'Shea D, Winter DC, Geoghegan J, Doherty DG, O'Farrelly C. Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. *European Journal of Immunology* 2009 **39** 1893-1901
123. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Current Opinion in Immunology* 2008 **20** 358-368
124. Lukens John R, Kanneganti T-D. Fat chance: not much against NKT Cells. *Immunity* 2012 **37** 447-449
125. Ryan AM, Healy LA, Power DG, Byrne M, Murphy S, Byrne PJ, *et al.* Barrett esophagus: prevalence of central adiposity, metabolic syndrome, and a proinflammatory state. *Annals of Surgery* 2008 **247** 909-915
126. Allott EH, Lysaght J, Cathcart MC, Donohoe CL, Cummins R, McGarrigle SA, *et al.* MMP9 expression in oesophageal adenocarcinoma is upregulated with visceral obesity and is associated with poor tumour differentiation. *Molecular Carcinogenesis* 2013 **52** 144-154
127. Lysaght J, van der Stok EP, Allott EH, Casey R, Donohoe CL, Howard JM, *et al.* Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue. *Cancer Letters* 2011 **312** 62-72
128. Allott EH, Morine MJ, Lysaght J, McGarrigle SA, Donohoe CL, Reynolds JV, *et al.* Elevated tumor expression of PAI-1 and SNAI2 in obese esophageal adenocarcinoma patients and impact on prognosis. *Clinical and Translational Gastroenterology* 2012 **3** e12
129. Felson DT, Zhang Y, Hannan MT, Naimark A, Weissman B, Aliabadi P, *et al.* Risk factors for incident radiographic knee osteoarthritis in the elderly: the Framingham Study. *Arthritis & Rheumatism* 1997 **40** 728-733
130. Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. *Circulation* 1983 **67** 968-977
131. Carrasco Sanchez FJ, Diaz Alcaide F, Maran Fernajndez Y, Chaparro Moreno I, Pujol de la Llave E. Prevalence of obesity in hospitalized internal medicine patients. *Anales de Medicina Interna* 2002 **19** 453-456
132. Fettes SB, Davidson HIM, Richardson RA, Pennington CR. Nutritional status of elective gastrointestinal surgery patients pre- and post-operatively. *Clinical Nutrition* 2002 **21** 249-254

133. Planas M, Audivert S, Pérez-Portabella C, Burgos R, Puiggrós C, Casanelles JM, *et al.* Nutritional status among adult patients admitted to an university-affiliated hospital in Spain at the time of genoma. *Clinical Nutrition* 2004 **23** 1016-1024
134. Harik-Khan RI, Wise RA, Fleg JL. The effect of gender on the relationship between body fat distribution and lung function. *Journal of Clinical Epidemiology* 2001 **54** 399-406
135. Jenkins SC, Moxham J. The effects of mild obesity on lung function. *Respiratory Medicine* 1991 **85** 309-311
136. Canoy D, Luben R, Welch A, Bingham S, Wareham N, Day N, *et al.* Abdominal obesity and respiratory function in men and women in the EPIC-Norfolk Study, United Kingdom. *American Journal of Epidemiology* 2004 **159** 1140
137. Eichenberger AS, Proietti S, Wicky S, Frascarolo P, Suter M, Spahn DR, *et al.* Morbid obesity and postoperative pulmonary atelectasis: an underestimated problem. *Anesthesia & Analgesia* 2002 **95** 1788-1792
138. Merkow RP, Bilimoria KY, McCarter MD, Bentrem DJ. Effect of body mass index on short-term outcomes after colectomy for cancer. *Journal of the American College of Surgeons* 2009 **208** 53-61
139. Brooks-Brunn JA. Predictors of postoperative pulmonary complications following abdominal surgery. *Chest* 1997 **111** 564-571
140. Hall JC, Tarala RA, Hall JL, Mander J. A multivariate analysis of the risk of pulmonary complications after laparotomy. *Chest* 1991 **99** 923-927
141. Benoist S, Panis Y, Alves A, Valleur P. Impact of obesity on surgical outcomes after colorectal resection. *The American Journal of Surgery* 2000 **179** 275-281
142. Moulton MJ, Creswell LL, Mackey ME, Cox JL, Rosenbloom M. Obesity is not a risk factor for significant adverse outcomes after cardiac surgery. *Circulation* 1996 **94** 1187-92
143. Mullen JT, Davenport DL, Hutter MM, Hosokawa PW, Henderson WG, Khuri SF, *et al.* Impact of body mass index on perioperative outcomes in patients undergoing major intra-abdominal cancer surgery. *Annals of Surgical Oncology* 2008 **15** 2164-2172
144. Healy LA, Ryan AM, Gopinath B, Rowley S, Byrne PJ, Reynolds JV. Impact of obesity on outcomes in the management of localized adenocarcinoma of the esophagus and esophagogastric junction. *Journal of Thoracic & Cardiovascular Surgery* 2007 **134** 1284-1291
145. Zacharias A, Schwann TA, Riordan CJ, Durham SJ, Shah AS, Habib RH. Obesity and risk of new-onset atrial fibrillation after cardiac surgery. *Circulation* 2005 **112** 3247-3255
146. Lauer MS, Anderson KM, Kannel WB, Levy D. The impact of obesity on left ventricular mass and geometry. The Framingham Heart Study. *Journal of the American Medical Association* 1991 **266** 231-236
147. Pritchett AM, Jacobsen SJ, Mahoney DW, Rodeheffer RJ, Bailey KR, Redfield MM. Left atrial volume as an index of left atrial size: a population-based study. *Journal of the American College of Cardiology* 2003 **41** 1036-1043
148. Vaziri SM, Larson MG, Lauer MS, Benjamin EJ, Levy D. Influence of blood pressure on left atrial size : The Framingham Heart Study. *Hypertension* 1995 **25** 1155-1160

149. Iacobellis G, Ribaudo MC, Leto G, Zappaterreno A, Vecchi E, Di Mario U, *et al.* Influence of excess fat on cardiac morphology and function: study in uncomplicated obesity. *Obesity* 2002 **10** 767-773
150. Wang TJ, Parise H, Levy D, D'Agostino RB, Sr., Wolf PA, Vasan RS, *et al.* Obesity and the risk of new-onset atrial fibrillation. *Journal of the American Medical Association* 2004 **292** 2471-2477
151. Kuduvalli M, Grayson AD, Oo AY, Fabri BM, Rashid A. The effect of obesity on mid-term survival following coronary artery bypass surgery. *European Journal of Cardiothoracic Surgery* 2003 **23** 368-373
152. Fasol R, Schindler M, Schumacher B, Schlaudraff K, Hannes W, Seitelberger R, *et al.* The influence of obesity on perioperative morbidity: retrospective study of 502 aortocoronary bypass operations. *The Thoracic and Cardiovascular Surgeon* 1992 **40** 126-129
153. Prasad US, Walker WS, Sang CT, Campanella C, Cameron EW. Influence of obesity on the early and long term results of surgery for coronary artery disease. *European Journal of Cardiothoracic Surgery* 1991 **5** 67-72
154. Wanahita N, Messerli FH, Bangalore S, Gami AS, Somers VK, Steinberg JS. Atrial fibrillation and obesity--results of a meta-analysis. *American Heart Journal* 2008 **155** 310-315
155. Choban PS, Heckler R, Burge JC, Flancbaum L. Increased incidence of nosocomial infections in obese surgical patients. *The American Surgeon* 1995 **61** 1001-1005
156. Canturk Z, Canturk NZ, Cetinarslan B, Utkan NZ, Tarkun I. Nosocomial infections and obesity in surgical patients. *Obesity Research* 2003 **11** 769-775
157. Choban PS, Flancbaum L. The impact of obesity on surgical outcomes: a review. *Journal of the American College of Surgeons* 1997 **185** 593-603
158. Mangram AJ, Horan TC, Pearson ML, Silver LC, Jarvis WR. Committee THICPA. Guideline for Prevention of Surgical Site Infection, 1999. Hospital Infection Control Practices Advisory Committee. *Infection Control and Hospital Epidemiology* 1999 **20** 250-280
159. Graham DJ, Stevenson JT, McHenry CR. The association of intra-abdominal infection and abdominal wound dehiscence. *The Journal of Urology* 1999 **161** 371-371
160. Green JW, Wenzel RP. Postoperative wound infection: a controlled study of the increased duration of hospital stay and direct cost of hospitalization. *Annals of Surgery* 1977 **185** 264-268
161. Karunakar MA, Shah SN, Jerabek S. Body mass index as a predictor of complications after operative treatment of acetabular fractures. *The Journal of Bone and Joint Surgery American Volume* 2005 **87** 1498-1502
162. Namba RS, Paxton L, Fithian DC, Stone ML. Obesity and perioperative morbidity in total hip and total knee arthroplasty patients. *The Journal of Arthroplasty* 2005 **20** 46-50
163. Loop FD, Lytle BW, Cosgrove DM, Mahfood S, McHenry MC, Goormastic M, *et al.* Sternal wound complications after isolated coronary artery bypass grafting: Early and late mortality, morbidity, and cost of care. *Annals of Thoracic Surgery* 1990 **49** 179-187

164. Ridderstolpe L, Gill H, Granfeldt H, Ahlfeldt H, Rutberg H. Superficial and deep sternal wound complications: incidence, risk factors and mortality. *European Journal of Cardiothoracic Surgery* 2001 **20** 1168-1175
165. Lu JC, Grayson AD, Jha P, Srinivasan AK, Fabri BM. Risk factors for sternal wound infection and mid-term survival following coronary artery bypass surgery. *European Journal of Cardiothoracic Surgery* 2003 **23** 943-949
166. Birkmeyer NJO, Charlesworth DC, Hernandez F, Leavitt BJ, Marrin CAS, Morton JR, *et al.* Obesity and risk of adverse outcomes associated with coronary artery bypass surgery. *Circulation* 1998 **97** 1689-1694
167. Crabtree TD, Codd JE, Fraser VJ, Bailey MS, Olsen MA, Damiano RJ, Jr. Multivariate analysis of risk factors for deep and superficial sternal infection after coronary artery bypass grafting at a tertiary care medical center. *Seminars in Thoracic and Cardiovascular Surgery* 2004 **16** 53-61
168. Parisian Mediastinitis Study Group. Risk factors for deep sternal wound infection after sternotomy: a prospective, multicenter study. *Journal of Thoracic and Cardiovascular Surgery* 1996 **111** 1200-1207
169. Greif R, Akca O, Horn E-P, Kurz A, Sessler DI; The Outcomes Research Group. Supplemental perioperative oxygen to reduce the incidence of surgical-wound infection. *New England Journal of Medicine* 2000 **342** 161-167
170. Anaya DA, Dellinger EP. The obese surgical patient: a susceptible host for infection. *Surgical Infections* 2006 **7** 473-480
171. Milano CA, Kesler K, Archibald N, Sexton DJ, Jones RH. Mediastinitis after coronary artery bypass graft surgery : risk factors and long-term survival. *Circulation* 1995 **92** 2245-2251
172. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 1988 **37** 1595-1607
173. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. *The Journal of Clinical Investigation* 2007 **117** 1219-1222
174. Hu FB. Obesity and mortality: watch your waist, not just your weight. *Archives of Internal Medicine* 2007 **167** 875-876
175. Rankinen T, Kim SY, Perusse L, Despres JP, Bouchard C. The prediction of abdominal visceral fat level from body composition and anthropometry: ROC analysis. *International Journal of Obesity* 1999 **23** 801-809
176. Rossner S, Bo WJ, Hiltbrandt E, Hinson W, Karstaedt N, Santago P, *et al.* Adipose tissue determinations in cadavers--a comparison between cross-sectional planimetry and computed tomography. *International Journal of Obesity* 1990 **14** 893-902
177. House M, Fong Y, Arnaoutakis D, Sharma R, Winston C, Protic M, *et al.* Preoperative predictors for complications after pancreaticoduodenectomy: impact of bmi and body fat Distribution. *Journal of Gastrointestinal Surgery* 2008 **12** 270-278
178. Kim TN, Park MS, Lim KI, Yang SJ, Yoo HJ, Kang HJ, *et al.* Skeletal muscle mass to visceral fat area ratio is associated with metabolic syndrome and arterial stiffness: The Korean Sarcopenic Obesity Study (KSOS). *Diabetes Research & Clinical Practice* 2011 **93** 285-291
179. Balentine CJ, Enriquez J, Cruz G, Hodges S, Bansal V, Jo E, *et al.* Obesity does not increase complications following pancreatic surgery. *Journal of Surgical Research* **170** 220-225

180. Tanaka K, Miyashiro I, Yano M, Kishi K, Motoori M, Seki Y, *et al.* Accumulation of excess visceral fat is a risk factor for pancreatic fistula formation after total gastrectomy. *Annals of Surgical Oncology* 2009 **16** 1520-1525
181. Sugisawa N, Tokunaga M, Tanizawa Y, Bando E, Kawamura T, Terashima M. Intra-abdominal infectious complications following gastrectomy in patients with excessive visceral fat. *Gastric Cancer* 2012 **15** 206-212
182. Tokunaga M, Hiki N, Fukunaga T, Ogura T, Miyata S, Yamaguchi T. Effect of individual fat areas on early surgical outcomes after open gastrectomy for gastric cancer. *British Journal of Surgery* 2009 **96** 496-500
183. Ishii Y, Hasegawa H, Nishibori H, Watanabe M, Kitajima M. Impact of visceral obesity on surgical outcome after laparoscopic surgery for rectal cancer. *British Journal of Surgery* 2005 **92** 1261-1262
184. Tsujinaka S, Konishi F, Kawamura Y, Saito M, Tajima N, Tanaka O, *et al.* Visceral obesity predicts surgical outcomes after laparoscopic colectomy for sigmoid colon cancer. *Diseases of the Colon & Rectum* 2008 **51** 1757-1767
185. Tsukada K, Miyazaki T, Kato H, Masuda N, Fukuchi M, Fukai Y, *et al.* Body fat accumulation and postoperative complications after abdominal surgery. *American Surgeon* 2004 **70** 347-351
186. Kobayashi J, Tadokoro N, Watanabe M, Shinomiya M. A novel method of measuring intra-abdominal fat volume using helical computed tomography. *International Journal of Obesity and other Related Metabolic Disorders* 2002 **26** 398-402
187. The Examination Committee of Criteria for 'Obesity Disease' in Japan. Japan Society for the Study of Obesity. New criteria for 'obesity disease' in Japan. *Circulation Journal* 2002 **66** 987-992
188. Echahidi N, Mohty D, Pibarot P, Despres J-P, O'Hara G, Champagne J, *et al.* Obesity and metabolic syndrome are independent risk factors for atrial fibrillation after coronary artery bypass graft surgery. *Circulation* 2007 **116** 1-213-219
189. Echahidi N, Pibarot P, Després J-P, Daigle J-M, Mohty D, Voisine P, *et al.* Metabolic syndrome increases operative mortality in patients undergoing coronary artery bypass grafting surgery. *Journal of the American College of Cardiology* 2007 **50** 843-851
190. Angeloni E, Melina G, Benedetto U, Refice S, Capuano F, Roscitano A, *et al.* Metabolic syndrome affects midterm outcome after coronary artery bypass grafting. *Annals of Thoracic Surgery* 2012 **93** 537-544
191. Hong S, Youn YN, Yoo KJ. Metabolic syndrome as a risk factor for postoperative kidney injury after off-pump coronary artery bypass surgery. *Circulation Journal* 2010 **74** 1121-1126
192. Brackbill ML, Sytsma CS, Sykes K. Perioperative outcomes of coronary artery bypass grafting: effects of metabolic syndrome and patient's sex. *American Journal of Critical Care* 2009 **18** 468-473
193. Grundy SM, Cleeman JJ, Daniels SR, Donato KA, Eckel RH, Franklin BA, *et al.* Diagnosis and management of the metabolic syndrome: An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 2005 **112** 2735-2752
194. Girerd N, Pibarot P, Fournier D, Daleau P, Voisine P, O'Hara G, *et al.* Middle-aged men with increased waist circumference and elevated C-reactive protein

- level are at higher risk for postoperative atrial fibrillation following coronary artery bypass grafting surgery. *European Heart Journal* 2009 **30** 1270-1278
195. Lohsiriwat V, Pongsanguansuk W, Lertakyamanee N, Lohsiriwat D. Impact of metabolic syndrome on the short-term outcomes of colorectal cancer surgery. *Diseases of the Colon & Rectum* **53** 186-191
 196. Memtsoudis SG, Kirksey M, Ma Y, Chiu YL, Mazumdar M, Pumberger M, *et al.* Metabolic syndrome and lumbar spine fusion surgery: epidemiology and perioperative outcomes. *Spine (Philadelphia PA 1976)* 2012 **37** 989-995
 197. Bhayani NH, Hyder O, Frederick W, Schulick RD, Wolfgang CL, Hirose K, *et al.* Effect of metabolic syndrome on perioperative outcomes after liver surgery: A National Surgical Quality Improvement Program (NSQIP) analysis. *Surgery* 2012 **152** 218-226
 198. Dy CJ, Wilkinson JD, Tamariz L, Scully SP. Influence of preoperative cardiovascular risk factor clusters on complications of total joint arthroplasty. *American Journal of Orthopedics (Belle Mead NJ)* 2011 **40** 560-565
 199. Reynolds JV, Donohoe CL, McGillicuddy E, Ravi N, O'Toole D, O'Byrne K, *et al.* Evolving progress in oncologic and operative outcomes for esophageal and junctional cancer: Lessons from the experience of a high-volume center. *The Journal of Thoracic and Cardiovascular Surgery* 2012 **143** 1130-1137
 200. Bailey SH, Bull DA, Harpole DH, Rentz JJ, Neumayer LA, Pappas TN, *et al.* Outcomes after esophagectomy: a ten-year prospective cohort. *The Annals of Thoracic Surgery* 2003 **75** 217-222
 201. Lagergren P, Avery KNL, Hughes R, Barham CP, Alderson D, Falk SJ, *et al.* Health-related quality of life among patients cured by surgery for esophageal cancer. *Cancer* 2007 **110** 686-693
 202. Reynolds JV, McLaughlin R, Moore J, Rowley S, Ravi N, Byrne PJ. Prospective evaluation of quality of life in patients with localized oesophageal cancer treated by multimodality therapy or surgery alone. *British Journal of Surgery* 2006 **93** 1084-1090
 203. Weimann A, Braga M, Harsanyi L, Laviano A, Ljungqvist O, Soeters P, *et al.* ESPEN guidelines on enteral nutrition: surgery including organ transplantation. *Clinical Nutrition* 2006 **25** 224-244
 204. Ryan AM, Rowley SP, Healy LA, Flood PM, Ravi N, Reynolds JV. Post-oesophagectomy early enteral nutrition via a needle catheter jejunostomy: 8-year experience at a specialist unit. *Clinical Nutrition* 2006 **25** 386-393
 205. Baker A, Wooten L-A, Malloy M. Nutritional considerations after gastrectomy and esophagectomy for malignancy. *Current Treatment Options in Oncology* 2011 **12** 85-95
 206. Beier-Holgersen R, Boesby S. Influence of postoperative enteral nutrition on postsurgical infections. *Gut* 1996 **39** 833-835
 207. Bozzetti F, Braga M, Gianotti L, Gavazzi C, Mariani L. Postoperative enteral versus parenteral nutrition in malnourished patients with gastrointestinal cancer: a randomised multicentre trial. *The Lancet* 2001 **358** 1487-1492
 208. Aiko S, Yoshizumi Y, Sugiura Y, Matsuyama T, Naito Y, Matsuzaki J, *et al.* Beneficial effects of immediate enteral nutrition after esophageal cancer surgery. *Surgery Today* 2001 **31** 971-978

209. Kromann N, Green A. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Medica Scandinavica* 1980 **208** 401-406
210. Dyerberg J, Bang HO. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *The Lancet* 1979 **2** 433-435
211. Hirai A, Hamazaki T, Terano T, Nishikawa T, Tamura Y, Kumagai A, *et al.* Eicosapentaenoic acid and platelet function in Tapanese. *The Lancet* 1980 **316** 1132-1133
212. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. *Journal of the American College of Nutrition* 2002 **21** 495-505
213. Calder PC. Omega-3 fatty acids and inflammatory processes. *Nutrients* 2010 **2** 355-374
214. Kalinski P. Regulation of immune responses by prostaglandin E2. *Journal of Immunology* 2012 **188** 21-28
215. Bagga D, Wang L, Farias-Eisner R, Glaspy JA, Reddy ST. Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *Proceedings of the National Academy of Sciences of the United States of America* 2003 **100** 1751-1756
216. Moreno JJ. Differential effects of arachidonic and eicosapentaenoic Acid-derived eicosanoids on polymorphonuclear transmigration across endothelial cell cultures. *The Journal of pharmacology and experimental therapeutics* 2009 **331** 1111-1117
217. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology* 2008 **8** 349-361
218. Arita M, Yoshida M, Hong S, Tjonahen E, Glickman JN, Petasis NA, *et al.* Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. *Proceedings of the National Academy of Sciences of the United States of America* 2005 **102** 7671-7676
219. Wang C, Harris WS, Chung M, Lichtenstein AH, Balk EM, Kupelnick B, *et al.* n-3 Fatty acids from fish or fish-oil supplements, but not α -linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *The American Journal of Clinical Nutrition* 2006 **84** 5-17
220. Lee YH, Bae SC, Song GG. Omega-3 polyunsaturated fatty acids and the treatment of rheumatoid arthritis: a meta-analysis. *Archives of Medical Research* 2012 **43** 356-362
221. Barber MD, Preston T, McMillan DC, Slater C, Ross JA, Fearon KC. Modulation of the liver export protein synthetic response to feeding by an n-3 fatty-acid-enriched nutritional supplement is associated with anabolism in cachectic cancer patients. *Clinical Science (London)* 2004 **106** 359-364
222. Wigmore SJ, Fearon KC, Maingay JP, Ross JA. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. *Clinical Science (London)* 1997 **92** 215-221
223. Barber MD, Ross JA, Voss AC, Tisdale MJ, Fearon KC. The effect of an oral nutritional supplement enriched with fish oil on weight-loss in patients with pancreatic cancer. *British Journal of Cancer* 1999 **81** 80-86

224. Desborough JP. The stress response to trauma and surgery. *British Journal of Anaesthesia* 2000 **85** 109-117
225. Moldawer LL, Copeland EM. Proinflammatory cytokines, nutritional support, and the cachexia syndrome. *Cancer* 1997 **79** 1828-1839
226. Birkhahn RH, Long CL, Fitkin D, Geiger JW, Blakemore WS. Effects of major skeletal trauma on whole body protein turnover in man measured by L-[1,14C]-leucine. *Surgery* 1980 **88** 294-300
227. Birkhahn RH, Long CL, Fitkin D, Jeevanandam M, Blakemore WS. Whole-body protein metabolism due to trauma in man as estimated by L-[15N]alanine. *American Journal of Physiology* 1981 **241** E64-71
228. Arora NS, Rochester DF. Respiratory muscle strength and maximal voluntary ventilation in undernourished patients. *American Review of Respiratory Disease* 1982 **126** 5-8
229. Tellado JM, Garcia-Sabrido JL, Hanley JA, Shizgal HM, Christou NV. Predicting mortality based on body composition analysis. *Annals of Surgery* 1989 **209** 81-87
230. Castaneda C, Charnley JM, Evans WJ, Crim MC. Elderly women accommodate to a low-protein diet with losses of body cell mass, muscle function, and immune response. *American Journal of Clinical Nutrition* 1995 **62** 30-39
231. Blotta MH, DeKruyff RH, Umetsu DT. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. *Journal of Immunology* 1997 **158** 5589-5595
232. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. *Annals of the New York Academy of Sciences* 2004 **1024** 138-146
233. Marik PE, Flemmer M. Immunonutrition in the surgical patient. *Minerva Anestesiologica* 2012 **78** 336-342
234. Kimura F, Shimizu H, Yoshidome H, Ohtsuka M, Miyazaki M. Immunosuppression following surgical and traumatic injury. *Surgery Today* 2010 **40** 793-808
235. Michaeli B, Berger M, Revelly J-P, Tappy L, Chioléro R. Effects of fish oil on the neuro-endocrine responses to an endotoxin challenge in healthy volunteers. *Clinical Nutrition* 2007 **26** 70-77
236. Myhrstad MW, Retterstøl K, Telle-Hansen V, Ottestad I, Halvorsen B, Holven K, et al. Effect of marine n-3 fatty acids on circulating inflammatory markers in healthy subjects and subjects with cardiovascular risk factors. *Inflammation Research* 2011 **60** 309-319
237. Ryan AM, Reynolds JV, Healy L, Byrne M, Moore J, Brannelly N, et al. Enteral nutrition enriched with eicosapentaenoic acid (EPA) preserves lean body mass following esophageal cancer surgery: results of a double-blinded randomized controlled trial. *Annals of Surgery* 2009 **249** 355-363
238. Detre S, Saclani Jotti G, Dowsett M. A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. *Journal of Clinical Pathology* 1995 **48** 876-878
239. Lean MEJ, Han TS, Morrison CE. Waist circumference as a measure for indicating need for weight management. *British Medical Journal* 1995 **311** 158-161

240. Baumgartner RN, Heymsfield SB, Roche AF, Bernardino M. Abdominal composition quantified by computed tomography. *The American Journal of Clinical Nutrition* 1988 **48** 936-945
241. Miller KD, Jones E, Yanovski JA, Shankar R, Feuerstein I, Falloon J. Visceral abdominal-fat accumulation associated with use of indinavir. *The Lancet* 1998 **351** 871-875
242. Belghazi J, El Feghali RN, Moussalem T, Rejdych M, Asmar RG. Validation of four automatic devices for self-measurement of blood pressure according to the International Protocol of the European Society of Hypertension. *Vascular Health & Risk Management* 2007 **3** 389-400
243. Schofield WN. Predicting basal metabolic rate, new standards and review of previous work. *Human Nutrition. Clinical Nutrition* 1985 **39 Suppl 1** 5-41
244. Barak N, Wall-Alonso E, Sitrin MD. Evaluation of stress factors and body weight adjustments currently used to estimate energy expenditure in hospitalized patients. *Journal of Parenteral and Enteral Nutrition* 2002 **26** 231-238
245. Nauseef WM. Isolation of human neutrophils from venous blood. *Methods in Molecular Biology* 2007 **412** 15-20
246. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001 **25** 402-408
247. Bessesen DH. Update on Obesity. *Journal of Clinical Endocrinology & Metabolism* 2008 **93** 2027-2034
248. Donohoe CL, Pidgeon GP, Lysaght J, Reynolds JV. Obesity and gastrointestinal cancer. *British Journal of Surgery* 2010 **97** 628-642
249. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 2005 **54** 2003-2011
250. World Health Organisation. Physical status: the use and interpretation of anthropometry. Report of a WHO expert committee. *WHO Technical Report Series* **854**
251. Okorodudu DO, Jumeau MF, Montori VM, Romero-Corral A, Somers VK, Erwin PJ, *et al.* Diagnostic performance of body mass index to identify obesity as defined by body adiposity: a systematic review and meta-analysis. *International Journal of Obesity* **34** 791-799
252. Oka R, Kobayashi J, Yagi K, Tanii H, Miyamoto S, Asano A, *et al.* Reassessment of the cutoff values of waist circumference and visceral fat area for identifying Japanese subjects at risk for the metabolic syndrome. *Diabetes Research and Clinical Practice* 2008 **79** 474-481
253. Klopfenstein BJ, Kim MS, Krisky CM, Szumowski J, Rooney WD, Purnell JQ. Comparison of 3 T MRI and CT for the measurement of visceral and subcutaneous adipose tissue in humans. *British Journal of Radiology* 2012 **85** e826-830
254. Doyle SL, Donohoe CL, Lysaght J, Reynolds JV. Visceral obesity, metabolic syndrome, insulin resistance and cancer. *Proceedings of the Nutrition Society* 2012 **71** 181-189
255. Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG, *et al.* Visceral and subcutaneous adipose tissue volumes are cross-sectionally

- related to markers of inflammation and oxidative stress: The Framingham Heart Study. *Circulation* 2007 **116** 1234-1241
256. Takahashi H, Takayama T, Yoneda K, Endo H, Iida H, Sugiyama M, *et al.* Association of visceral fat accumulation and plasma adiponectin with rectal dysplastic aberrant crypt foci in a clinical population. *Cancer Science* 2009 **100** 29-32
 257. Ohki T, Tateishi R, Shiina S, Goto E, Sato T, Nakagawa H, *et al.* Visceral fat accumulation is an independent risk factor for hepatocellular carcinoma recurrence after curative treatment in patients with suspected NASH. *Gut* 2009 **58** 839-844
 258. Pouliot M-C, Després J-P, Nadeau A, Moorjani S, Prud'Homme D, Lupien PJ, *et al.* Visceral obesity in men: associations with glucose tolerance, plasma insulin, and lipoprotein levels. *Diabetes* 1992 **41** 826-834
 259. Guiu B, Petit JM, Bonnetain F, Ladoire S, Guiu S, Cercueil J-P, *et al.* Visceral fat area is an independent predictive biomarker of outcome after first-line bevacizumab-based treatment in metastatic colorectal cancer. *Gut* 2010 **59** 341-347
 260. Steffens S, Grünwald V, Ringe KI, Seidel C, Eggers H, Schrader M, *et al.* Does obesity influence the prognosis of metastatic renal cell carcinoma in patients treated with vascular endothelial growth factor-targeted therapy? *The Oncologist* 2011 **16** 1565-1571
 261. Despres JP, Lamarche B. Effects of diet and physical activity on adiposity and body fat distribution: implications for the prevention of cardiovascular disease. *Nutrition Research Reviews* 1993 **6** 137-159
 262. Hunter GR, Snyder SW, Kekes-Szabo T, Nicholson C, Berland L. Intra-abdominal adipose tissue values associated with risk of possessing elevated blood lipids and blood pressure. *Obesity Research* 1994 **2** 563-568
 263. Bjorntorp P. Abdominal obesity and the metabolic syndrome. *Annals of Medicine* 1992 **24** 465-468
 264. Bjorntorp P. Metabolic difference between visceral fat and subcutaneous abdominal fat. *Diabetes & Metabolism* 2000 **26 Suppl 3** 10-12
 265. Flint AJ, Rexrode KM, Hu FB, Glynn RJ, Caspard H, Manson JE, *et al.* Body mass index, waist circumference, and risk of coronary heart disease: A prospective study among men and women. *Obesity Research & Clinical Practice* 2010 **4** e171-e181
 266. Kashiwara H, Lee JS, Kawakubo K, Tamura M, Akabayashi A. Criteria of waist circumference according to computed tomography-measured visceral fat area and the clustering of cardiovascular risk factors. *Circulation Journal* 2009 **73** 1881-1886
 267. Katzmarzyk PT, Bray GA, Greenway FL, Johnson WD, Newton RL, Ravussin E, *et al.* Racial differences in abdominal depot-specific adiposity in white and African American adults. *The American Journal of Clinical Nutrition* 2010 **91** 7-15
 268. Kim JA, Choi CJ, Yum KS. Cut-off values of visceral fat area and waist circumference: diagnostic criteria for abdominal obesity in a Korean population. *Journal of Korean Medical Science* 2006 **21** 1048-1053
 269. Rickles A, Iannuzzi J, Mironov O, Deeb A-P, Sharma A, Fleming F, *et al.* Visceral obesity and colorectal cancer: are we missing the boat with BMI? *Journal of Gastrointestinal Surgery* 2013 **17** 133-143

270. El-Serag HB, Hashmi A, Garcia J, Richardson P, Alsarraj A, Fitzgerald S, *et al.* Visceral abdominal obesity measured by CT scan is associated with an increased risk of Barrett's oesophagus: a case-control study. *Gut* 2013 [Epub ahead of print]
271. International Diabetes Federation. The IDF consensus worldwide definition of the metabolic syndrome. http://www.idf.org/webdata/docs/IDF_Meta_def_final.pdf 2006
272. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, *et al.* Harmonizing the Metabolic Syndrome: A joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 2009 **120** 1640-1645
273. von Hafe P, Pina F, Perez A, Tavares M, Barros H. Visceral fat accumulation as a risk factor for prostate cancer. *Obesity Research* 2004 **12** 1930-1935
274. Doyle SL, Donohoe CL, Finn SP, Howard JM, Lithander FE, Reynolds JV, *et al.* IGF-1 and its receptor in esophageal cancer: association with adenocarcinoma and visceral obesity. *American Journal of Gastroenterology* 2012 **107** 196-204
275. Yamamoto S, Nakagawa T, Matsushita Y, Kusano S, Hayashi T, Irokawa M, *et al.* Visceral fat area and markers of insulin resistance in relation to colorectal neoplasia. *Diabetes Care* 2010 **33** 184-189
276. Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *Journal of the American Medical Association* 2002 **287** 356 - 359
277. Lemieux S, Prud'homme D, Bouchard C, Tremblay A, Despres JP. A single threshold value of waist girth identifies normal-weight and overweight subjects with excess visceral adipose tissue. *American Journal of Clinical Nutrition* 1996 **64** 685-693
278. Ross R, Aru J, Freeman J, Hudson R, Janssen I. Abdominal adiposity and insulin resistance in obese men. *American Journal of Physiology - Endocrinology And Metabolism* 2002 **282** E657-E663
279. Tanaka S, Horimai C, Katsukawa F. Ethnic differences in abdominal visceral fat accumulation between Japanese, African-Americans, and Caucasians: a meta-analysis. *Acta Diabetologica* 2003 **40 Suppl 1** S302-304
280. Brochu M, Starling RD, Tchernof A, Matthews DE, Garcia-Rubi E, Poehlman ET. Visceral adipose tissue is an independent correlate of glucose disposal in older obese postmenopausal women. *Journal of Clinical Endocrinology & Metabolism* 2000 **85** 2378-2384
281. Hu G, Tuomilehto J, Silventoinen K, Barengo Ne, Jousilahti P. Joint effects of physical activity, body mass index, waist circumference and waist-to-hip ratio with the risk of cardiovascular disease among middle-aged Finnish men and women. *European Heart Journal* 2004 **25** 2212-2219
282. Larsson B, Svardstudd K, Welin L, Wilhelmsen L, Bjorntorp P, Tibblin G. Abdominal adipose tissue distribution, obesity, and risk of cardiovascular disease and death: 13 year follow up of participants in the study of men born in 1913. *British Medical Journal* 1984 **288** 1401-1404
283. Pouliot MC, Despres JP, Lemieux S, Moorjani S, Bouchard C, Tremblay A, *et al.* Waist circumference and abdominal sagittal diameter: best simple

- anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women. *American Journal of Cardiology* 1994 **73** 460-468
284. Snijder MB, van Dam RM, Visser M, Seidell JC. What aspects of body fat are particularly hazardous and how do we measure them? *International Journal of Epidemiology* 2006 **35** 83-92
 285. Balentine CJ, Marshall C, Robinson C, Wilks J, Anaya D, Albo D, *et al.* Validating quantitative obesity measurements in colorectal cancer patients. *Journal of Surgical Research* 2010 **164** 18-22
 286. Park J, Euhus DM, Scherer PE. Paracrine and endocrine effects of adipose tissue on cancer development and progression. *Endocrine Reviews* 2011 **32** 550-570
 287. van Kruijsdijk RCM, van der Wall E, Visseren FLJ. Obesity and cancer: the role of dysfunctional adipose tissue. *Cancer Epidemiology Biomarkers & Prevention* 2009 **18** 2569-2578
 288. Despres J-P, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006 **444** 881-887
 289. Vona-Davis L, Howard-McNatt M, Rose DP. Adiposity, type 2 diabetes and the metabolic syndrome in breast cancer. *Obesity Reviews* 2007 **8** 395-408
 290. Iftikhar SY, James PD, Steele RJ, Hardcastle JD, Atkinson M. Length of Barrett's oesophagus: an important factor in the development of dysplasia and adenocarcinoma. *Gut* 1992 **33** 1155-1158
 291. Iravani S, Zhang HQ, Yuan ZQ, Cheng JQ, Karl RC, Jove R, *et al.* Modification of insulin-like growth factor 1 receptor, c-Src, and Bcl-XL protein expression during the progression of Barrett's neoplasia. *Human Pathology* 2003 **34** 975-982
 292. Kaaks R, Lukanova A. Energy balance and cancer: the role of insulin and insulin-like growth factor-I. *Proceedings of the Nutrition Society* 2001 **60** 91-106
 293. Imsumran A, Adachi Y, Yamamoto H, Li R, Wang Y, Min Y, *et al.* Insulin-like growth factor-I receptor as a marker for prognosis and a therapeutic target in human esophageal squamous cell carcinoma. *Carcinogenesis* 2007 **28** 947-956
 294. Piao W, Wang Y, Adachi Y, Yamamoto H, Li R, Imsumran A, *et al.* Insulin-like growth factor-I receptor blockade by a specific tyrosine kinase inhibitor for human gastrointestinal carcinomas. *Molecular Cancer Therapeutics* 2008 **7** 1483-1493
 295. Chen SC, Chou CK, Wong FH, Chang CM, Hu CP. Overexpression of epidermal growth factor and insulin-like growth factor-I receptors and autocrine stimulation in human esophageal carcinoma cells. *Cancer Research* 1991 **51** 1898-1903
 296. Liu YC, Leu CM, Wong FH, Fong WS, Chen SC, Chang C, *et al.* Autocrine stimulation by insulin-like growth factor I is involved in the growth, tumorigenicity and chemoresistance of human esophageal carcinoma cells. *Journal of Biomedical Science* 2002 **9** 665-674
 297. Sohda M, Kato H, Miyazaki T, Nakajima M, Fukuchi M, Manda R, *et al.* The role of insulin-like growth factor 1 and insulin-like growth factor binding protein 3 in human esophageal cancer. *Anticancer Research* 2004 **24** 3029-3034
 298. Yilmaz O, Eroglu A, Dag E, Karaoglanoglu N, Yilmaz A. Serum levels of IGF-I and IGFBP-III and their relation with carcinoembryonic antigen and carbohydrate antigen 19-9 in cases of esophageal cancer. *International Journal of Clinical Practice* 2006 **60** 1604-1608

299. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C. Macrophages and angiogenesis. *Journal of Leukocyte Biology* 1994 **55** 410-422
300. Oberlin D, Fellbaum C, Eppler E. Insulin-like growth factor I messenger RNA and protein are expressed in the human lymph node and distinctly confined to subtypes of macrophages, antigen-presenting cells, lymphocytes and endothelial cells. *Immunology* 2009 **128** 342-350
301. Kodelja V, Muller C, Tenorio S, Schebesch C, Orfanos CE, Goerdts S. Differences in angiogenic potential of classically vs alternatively activated macrophages. *Immunobiology* 1997 **197** 478-493
302. Kopfstein L, Christofori G. Metastasis: cell-autonomous mechanisms versus contributions by the tumor microenvironment. *Cellular and Molecular Life Sciences* 2006 **63** 449-468
303. Argente J, Caballo N, Barrios V, Munoz MT, Pozo J, Chowen JA, *et al.* Multiple endocrine abnormalities of the growth hormone and insulin-like growth factor axis in patients with anorexia nervosa: effect of short- and long-term weight recuperation. *Journal of Clinical Endocrinology & Metabolism* 1997 **82** 2084-2092
304. Fowke JH, Matthews CE, Yu H, Cai Q, Cohen S, Buchowski MS, *et al.* Racial differences in the association between body mass index and serum IGF1, IGF2, and IGFBP3. *Endocrine Related Cancer* **17** 51-60
305. Rowlands M-A, Holly J, Gunnell D, Gilbert R, Donovan J, Lane J, *et al.* The relation between adiposity throughout the life course and variation in IGFs and IGFBPs: evidence from the ProtecT (Prostate testing for cancer and Treatment) study. *Cancer Causes and Control* 2010 **21** 1829-1842
306. Blüher S, Kratzsch J, Kiess W. Insulin-like growth factor I, growth hormone and insulin in white adipose tissue. *Best Practice & Research Clinical Endocrinology & Metabolism* 2005 **19** 577-587
307. Bergmann U, Funatomi H, Yokoyama M, Beger HG, Korc M. Insulin-like growth factor 1 overexpression in human pancreatic cancer: evidence for autocrine and paracrine roles. *Cancer Research* 1995 **55** 2007-2011
308. Mitsuo Koenuma TYTT. Insulin and insulin-like growth factor 1 stimulate proliferation of metastatic variants of colon carcinoma. *Cancer Science* 1989 **80** 51-58
309. Lippman ME. Growth regulation of human breast cancer. *Clinical Research* 1985 **33** 375-382.
310. Li S-L, Liang S-J, Guo N, Wu AM, Fujita-Yamaguchi Y. Single-chain antibodies against human insulin-like growth factor I receptor: expression, purification, and effect on tumor growth. *Cancer Immunology, Immunotherapy* 2000 **49** 243-252
311. Reinmuth N, Liu W, Fan F, Jung YD, Ahmad SA, Stoeltzing O, *et al.* Blockade of insulin-like growth factor 1 receptor function inhibits growth and angiogenesis of colon cancer. *Clinical Cancer Research* 2002 **8** 3259-3269
312. Tomizawa M, Shinozaki F, Sugiyama T, Yamamoto S, Sueishi M, Yoshida T. Insulin-like growth factor-I receptor in proliferation and motility of pancreatic cancer. *World Journal of Gastroenterology* 2010 **16** 1854-1858
313. Iihara K, Shiozaki H, Oku K, Tahara H, Doki Y, Oka H, *et al.* Growth-regulatory mechanism of two human esophageal-cancer cell lines in protein-free conditions. *International Journal of Cancer* 1993 **55** 364-370

314. Oku K, Tanaka A, Yamanishi H, Nishizawa Y, Matsumoto K, Shiozaki H, *et al.* Effects of various growth factors on growth of a cloned human esophageal squamous cancer cell line in a protein-free medium. *Anticancer Research* 1991 **11** 1591-1595
315. Hollowood AD LT, Perks CM, Newcomb PV, Alderson D, Holly JMP. IGFBp-3 prolongs the p53 response and enhances apoptosis following UV irradiation. *International Journal of Cancer* 2000 **88** 336-341
316. Brady G, Crean SJ, Naik P, Kapas S. Upregulation of IGF-2 and IGF-1 receptor expression in oral cancer cell lines. *International Journal of Oncology* 2007 **31** 875-881
317. Shimizu A, Tani M, Kawai M, Hirono S, Miyazawa M, Uchiyama K, *et al.* Influence of visceral obesity for postoperative pulmonary complications after pancreaticoduodenectomy. *Journal of Gastrointestinal Surgery* 2011 **15** 1401-1410
318. Van den Berghe G, Wouters P, Weekers F, Verwaest C, Bruyninckx F, Schetz M, *et al.* Intensive insulin therapy in critically ill patients. *New England Journal of Medicine* 2001 **345** 1359-1367
319. Thourani VH, Weintraub WS, Stein B, Gebhart SSP, Craver JM, Jones EL, *et al.* Influence of diabetes mellitus on early and late outcome after coronary artery bypass grafting. *The Annals of Thoracic Surgery* 1999 **67** 1045-1052
320. Umpierrez GE, Isaacs SD, Bazargan N, You X, Thaler LM, Kitabchi AE. Hyperglycemia: An independent marker of in-hospital mortality in patients with undiagnosed diabetes. *Journal of Clinical Endocrinology & Metabolism* 2002 **87** 978-982
321. Thorell A, Nygren J, Ljungqvist O. Insulin resistance: a marker of surgical stress. *Current Opinion in Clinical Nutrition & Metabolic Care* 1999 **2** 69-78
322. Rossini AA. Why control blood glucose levels? *Archives of Surgery* 1976 **111** 229-233
323. Furnary AP, Zerr KJ, Grunkemeier GL, Starr A. Continuous intravenous insulin infusion reduces the incidence of deep sternal wound infection in diabetic patients after cardiac surgical procedures. *The Annals of Thoracic Surgery* 1999 **67** 352-360
324. Gletsu N, Lin E, Zhu J-L, Khaitan L, Ramshaw BJ, Farmer PK, *et al.* Increased plasma interleukin 6 concentrations and exaggerated adipose tissue interleukin 6 content in severely obese patients after operative trauma. *Surgery* 2006 **140** 50-57
325. Tanaka K, Yano M, Motoori M, Kishi K, Miyashiro I, Yamada T, *et al.* Excess visceral fat accumulation is a risk factor for postoperative systemic inflammatory response syndrome in patients with esophageal cancer. *Esophagus* 2008 **5** 75-80
326. Buontempo PJ, Jubin RG, Buontempo CA, Wagner NE, Reyes GR, Baroudy BM. Antiviral activity of transiently expressed IFN-kappa is cell-associated. *Journal of Interferon & Cytokine Research* 2006 **26** 40-52
327. Wu L, LaRosa G, Kassam N, Gordon CJ, Heath H, Ruffing N, *et al.* Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. *The Journal of Experimental Medicine* 1997 **186** 1373-1381

328. Comelli F, Giagnoni G, Bettoni I, Colleoni M, Costa B. The inhibition of monoacylglycerol lipase by URB602 showed an anti-inflammatory and anti-nociceptive effect in a murine model of acute inflammation. *British Journal of Pharmacology* 2007 **152** 787-794
329. Labar G, Wouters J, Lambert DM. A review on the monoacylglycerol lipase: at the interface between fat and endocannabinoid signalling. *Current Medicinal Chemistry* 2010 **17** 2588-2607
330. Bassols J, Moreno-Navarrete JM, Ortega F, Ricart W, Fernandez-Real JM. LIGHT is associated with hypertriglyceridemia in obese subjects and increased cytokine secretion from cultured human adipocytes. *International Journal of Obesity* 2009 **34** 146-156
331. Bassols J, Moreno JM, Ortega F, Ricart W, Fernandez-Real JM. Characterization of Herpes Virus Entry Mediator as a Factor Linked to Obesity. *Obesity* 2009 **18** 239-246
332. Kim H-M, Jeong C-S, Choi H-S, Kawada T, Yu R. LIGHT/TNFSF14 enhances adipose tissue inflammatory responses through its interaction with HVEM. *FEBS letters* 2011 **585** 579-584
333. Browning JL. Inhibition of the lymphotoxin pathway as a therapy for autoimmune disease. *Immunological Reviews* 2008 **223** 202-220
334. Madge LA, Kluger MS, Orange JS, May MJ. Lymphotoxin-alpha1 beta and LIGHT induce classical and noncanonical nf-kb-dependent proinflammatory gene expression in vascular endothelial cells. *The Journal of Immunology* 2008 **180** 3467-3477
335. Lau D, Mollnau H, Eiserich JP, Freeman BA, Daiber A, Gehling UM, *et al.* Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proceedings of the National Academy of Sciences of the United States of America* 2005 **102** 431-436
336. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF. Neutrophils in development of multiple organ failure in sepsis. *Lancet* 2006 **368** 157-169
337. Christman JW, Lancaster LH, Blackwell TS. Nuclear factor kappa B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. *Intensive Care Medicine* 1998 **24** 1131-1138
338. Donatelli F, Cavagna P, Di Dedda G, Catenacci A, Di Nicola M, Lorini L, *et al.* Correlation between pre-operative metabolic syndrome and persistent blood glucose elevation during cardiac surgery in non-diabetic patients. *Acta Anaesthesiologica Scandinavica* 2008 **52** 1103-1110
339. Dolan MJ, Kulkarni H, Camargo JF, He W, Smith A, Anaya JM, *et al.* CCL3L1 and CCR5 influence cell-mediated immunity and affect HIV-AIDS pathogenesis via viral entry-independent mechanisms. *Nature Immunology* 2007 **8** 1324-1336
340. Akpan I, Goncalves MD, Dhir R, Yin X, Pistilli EE, Bogdanovich S, *et al.* The effects of a soluble activin type IIB receptor on obesity and insulin sensitivity. *International Journal of Obesity* 2009 **33** 1265-1273
341. Li L, Shen JJ, Bournat JC, Huang L, Chattopadhyay A, Li Z, *et al.* Activin signaling: effects on body composition and mitochondrial energy metabolism. *Endocrinology* 2009 **150** 3521-3529
342. Kwon BS, Tan KB, Ni J, Lee K-O-OZH, Kim KK, Kim Y-J, *et al.* A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue

- distribution and involvement in lymphocyte activation. *Journal of Biological Chemistry* 1997 **272** 14272-14276
343. Ware CF, Crowe PD, Grayson MH, Androlewicz MJ, Browning JL. Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. *The Journal of Immunology* 1992 **149** 3881-3888
 344. Lukashev M, LePage D, Wilson C, Bailly Vr, Garber E, Lukashin A, *et al.* Targeting the lymphotoxin-beta receptor with agonist antibodies as a potential cancer therapy. *Cancer Research* 2006 **66** 9617-9624
 345. Liu GZ, Fang LB, Hjelmstrom P, Gao XG. Enhanced plasma levels of LIGHT in patients with acute atherothrombotic stroke. *Acta Neurologica Scandinavica* 2008 **118** 256-259
 346. Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology* 2010 **49** 1618-1631
 347. Moudgil GC, Pandya AR, Ludlow DJ. Influence of anaesthesia and surgery on neutrophil chemotaxis. *Canadian Anaesthetists' Society Journal* 1981 **28** 232-238
 348. Duignan JP, Collinst PB, Johnson AH, Bouchier-Hayes D. The association of impaired neutrophil chemotaxis with postoperative surgical sepsis. *British Journal of Surgery* 1986 **73** 238-240
 349. Nijhuis J, Rensen SS, Slaats Y, van Dielen FMH, Buurman WA, Greve JWM. Neutrophil activation in morbid obesity, chronic activation of acute inflammation. *Obesity* 2009 **17** 2014-2018
 350. Cottam DR, Schaefer PA, Fahmy D, Shaftan GW, Angus LD. The effect of obesity on neutrophil Fc receptors and adhesion molecules (CD16, CD11b, CD62L). *Obesity Surgery* 2002 **12** 230-235
 351. Rassias AJ, Marrin CAS, Arruda J, Whalen PK, Beach M, Yeager MP. Insulin infusion improves neutrophil function in diabetic cardiac surgery patients. *Anesthesia & Analgesia* 1999 **88** 1011-1016
 352. Gallacher SJ, Thomson G, Fraser WD, Fisher BM, Gemmell CG, MacCuish AC. Neutrophil bactericidal function in diabetes mellitus: evidence for association with blood glucose control. *Diabetic Medicine* 1995 **12** 916-920
 353. Palmblad J, Hallberg D, Engstedt L. Polymorphonuclear (PMN) function after small intestinal shunt operation for morbid obesity. *British Journal of Haematology* 1980 **44** 101-108
 354. Kawashima H, Watanabe N, Hirose M, Li YF, Hirose J, Miyasaka M. Vascular and non-vascular ligands for L-selectin. *Cell Adhesion & Communication* 1998 **6** 135-139
 355. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. *Trends in Immunology* 2011 **32** 452-460
 356. Boyle EM Jr, Pohlman TH, Johnson MC, Verrier ED. Endothelial cell injury in cardiovascular surgery: the systemic inflammatory response. *The Annals of Thoracic Surgery* 1997 **63** 277-284
 357. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF- kappa B and cytokine-inducible enhancers. *The FASEB Journal* 1995 **9** 899-909
 358. Mokart D, Merlin M, Sannini A, Brun JP, Delpero JR, Houvenaeghel G, *et al.* Procalcitonin, interleukin 6 and systemic inflammatory response syndrome

- (SIRS): early markers of postoperative sepsis after major surgery. *British Journal of Anaesthesia* 2005 **94** 767-773
359. Elenkov IJ, Chrousos GP. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends in Endocrinology & Metabolism* 1999 **10** 359-368
 360. Korn T, Oukka M, Kuchroo V, Bettelli E. Th17 cells: Effector T cells with inflammatory properties. *Seminars in Immunology* 2007 **19** 362-371
 361. Reilly CF, McFall RC. Platelet-derived growth factor and transforming growth factor-beta regulate plasminogen activator inhibitor-1 synthesis in vascular smooth muscle cells. *Journal of Biological Chemistry* 1991 **266** 9419-9427
 362. Ingelsson E, Hulthe J, Lind L. Inflammatory markers in relation to insulin resistance and the metabolic syndrome. *European Journal of Clinical Investigation* 2008 **38** 502 - 509
 363. Egdahl RH. Pituitary-adrenal response following trauma to the isolated leg. *Surgery* 1959 **46** 9-21
 364. Mayer M, Rosen F. Interaction of glucocorticoids and androgens with skeletal muscle. *Metabolism* 1977 **26** 937-962
 365. Sheeran P, Hall GM. Cytokines in anaesthesia. *British Journal of Anaesthesia* 1997 **78** 201-219
 366. van Venrooij LMW, Verberne HJ, de Vos R, Borgmeijer-Hoelen MMMJ, van Leeuwen PAM, de Mol BAJM. Postoperative loss of skeletal muscle mass, complications and quality of life in patients undergoing cardiac surgery. *Nutrition* 2012 **28** 40-45
 367. Watters JM, Clancey SM, Moulton SB, Briere KM, Zhu JM, Meakins JL, *et al.* Impaired recovery of strength in older patients after major abdominal surgery. *Annals of Surgery* 1993 **218** 380-393
 368. Marik PE, Zaloga GP. Immunonutrition in high-risk surgical patients: a systematic review and analysis of the literature. *Journal of Parenteral and Enteral Nutrition* 2010 **34** 378-386
 369. Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. *Cytokine & Growth Factor Reviews* 2002 **13** 455-481
 370. Feeney C, Reynolds JV, Hussey J. Preoperative physical activity levels and postoperative pulmonary complications post-esophagectomy. *Diseases of the Esophagus* 2011 **24** 489-494
 371. Zhang Y, Gu Y, Guo T, Li Y, Cai H. Perioperative immunonutrition for gastrointestinal cancer: A systematic review of randomized controlled trials. *Surgical Oncology* 2012 **21** e87-e95
 372. Gianotti L, Braga M, Nespoli L, Radaelli G, Beneduce A, Di Carlo V. A randomized controlled trial of preoperative oral supplementation with a specialized diet in patients with gastrointestinal cancer. *Gastroenterology* 2002 **122** 1763-1770
 373. Ochoa JB, Bernard AC, O'Brien WE, Griffen MM, Maley ME, Rockich AK, *et al.* Arginase I expression and activity in human mononuclear cells after injury. *Annals of Surgery* 2001 **233** 393-399
 374. Munder M, Eichmann K, Morán JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *The Journal of Immunology* 1999 **163** 3771-3777

375. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JWM, *et al.* The effect of dietary supplementation with n—3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *New England Journal of Medicine* 1989 **320** 265-271
376. Caughey GE, Mantzioris E, Gibson RA, Cleland LG, James MJ. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. *American Journal of Clinical Nutrition* 1996 **63** 116-122
377. Wakefield CH, Carey PD, Foulds S, Monson JR, Guillou PJ. Polymorphonuclear leukocyte activation: An early marker of the postsurgical sepsis response. *Archives of Surgery* 1993 **128** 390-395
378. Crooks SW, Stockley RA. Leukotriene B4. *The International Journal of Biochemistry & Cell Biology* 1998 **30** 173-178
379. Modig J, Hallgren R. Pathophysiologic significance of lung granulocytes in human adult respiratory distress syndrome induced by septic or traumatic shock. *Acta Chirurgica Scandinavica* 1987 **153** 267-271
380. Malekshahi Moghadam A, Saedisomeolia A, Djalali M, Djazayeri A, Pooya S, Sojoudi F. Efficacy of omega-3 fatty acid supplementation on serum levels of tumour necrosis factor-alpha, C-reactive protein and interleukin-2 in type 2 diabetes mellitus patients. *Singapore Medical Journal* 2012 **53** 615-619
381. Mohammadi E, Rafrat M, Farzadi L, Asghari-Jafarabadi M, Sabour S. Effects of omega-3 fatty acids supplementation on serum adiponectin levels and some metabolic risk factors in women with polycystic ovary syndrome. *Asia Pacific Journal of Clinical Nutrition* 2012 **21** 511-518
382. Moses AG, Maingay J, Sangster K, Fearon KC, Ross JA. Pro-inflammatory cytokine release by peripheral blood mononuclear cells from patients with advanced pancreatic cancer: relationship to acute phase response and survival. *Oncology Reports* 2009 **21** 1091-1095
383. Nagai T, Anzai T, Mano Y, Kaneko H, Anzai A, Sugano Y, *et al.* Eicosapentaenoic acid suppresses adverse effects of C-reactive protein overexpression on pressure overload-induced cardiac remodeling. *Heart Vessels* 2012
384. Holm T, Berge RK, Andreassen AK, Ueland T, Kjekshus J, Simonsen S, *et al.* Omega-3 fatty acids enhance tumor necrosis factor-alpha levels in heart transplant recipients. *Transplantation* 2001 **72** 706-711
385. Wang J, Yu J-C, Kang W-M, Ma Z-Q. Superiority of a fish oil—enriched emulsion to medium-chain triacylglycerols/long-chain triacylglycerols in gastrointestinal surgery patients: A randomized clinical trial. *Nutrition* 2012 **28** 623-629
386. Larsen BM, Goonewardene LA, Joffe AR, Van Aerde JE, Field CJ, Olstad DL, *et al.* Pre-treatment with an intravenous lipid emulsion containing fish oil (eicosapentaenoic and docosahexaenoic acid) decreases inflammatory markers after open-heart surgery in infants: a randomized, controlled trial. *Clinical Nutrition* 2012 **31** 322-329
387. Vane J, Corin RE. Prostacyclin: a vascular mediator. *European Journal of Vascular and Endovascular Surgery* 2003 **26** 571-578
388. Vachiéry J-L. Prostacyclins in pulmonary arterial hypertension: the need for earlier therapy. *Advances in Therapy* 2011 **28** 251-269
389. Coceani F, Bishai I, Lees J, Sirko S. Prostaglandin E2 and fever: a continuing debate. *The Yale Journal of Biology and Medicine* 1986 **59** 169-174

390. Miles EA, Allen E, Calder PC. In vitro effects of eicosanoids derived from different 20-carbon fatty acids on production of monocyte-derived cytokines in human whole blood cultures. *Cytokine* 2002 **20** 215-223
391. Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN. Lipid mediator class switching during acute inflammation: Signals in resolution. *Nature Immunology* 2001 **2** 612-619
392. Peck LW, Monsen ER, Ahmad S. Effect of three sources of long-chain fatty acids on the plasma fatty acid profile, plasma prostaglandin E2 concentrations, and pruritus symptoms in hemodialysis patients. *The American Journal of Clinical Nutrition* 1996 **64** 210-214
393. Ziboh VA. Omega 3 polyunsaturated fatty acid constituents of fish oil and the management of skin inflammatory and scaly disorders. *World Review of Nutrition & Dietetics* 1991 **66** 425-435
394. Murphy RA, Mourtzakis M, Chu QSC, Baracos VE, Reiman T, Mazurak VC. Nutritional intervention with fish oil provides a benefit over standard of care for weight and skeletal muscle mass in patients with nonsmall cell lung cancer receiving chemotherapy. *Cancer* 2011 **117** 1775-1782
395. van der Meij BS, Langius JAE, Smit EF, Spreeuwenberg MD, von Blomberg BME, Heijboer AC, *et al.* Oral nutritional supplements containing (n-3) polyunsaturated fatty acids affect the nutritional status of patients with stage iii non-small cell lung cancer during multimodality treatment. *The Journal of Nutrition* 2010 **140** 1774-1780
396. Murphy RA, Yeung E, Mazurak VC, Mourtzakis M. Influence of eicosapentaenoic acid supplementation on lean body mass in cancer cachexia. *British Journal of Cancer* 2011 **105** 1469-1473
397. Donohoe CL, Doyle SL, McGarrigle S, Cathcart MC, Daly E, O'Grady A, *et al.* Role of the insulin-like growth factor 1 axis and visceral adiposity in oesophageal adenocarcinoma. *British Journal of Surgery* 2012 **99** 387-396
398. Doyle SL, Lysaght J, Reynolds JV. Obesity and post-operative complications in patients undergoing non-bariatric surgery. *Obesity Reviews* 2010 **11** 875-886
399. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, *et al.* Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992. *Chest* 2009 **136** e28
400. Mica L, Vomela J, Keel M, Trentz O. The impact of body mass index on the development of systemic inflammatory response syndrome and sepsis in patients with polytrauma. *Injury* 2012 [Epub ahead of print]
401. Bautmans I, Njemini R, De Backer J, De Waele E, Mets T. Surgery-induced inflammation in relation to age, muscle endurance, and self-perceived fatigue. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2010 **65A** 266-273

Appendix I

RT² Profiler™ PCR Array Human Inflammatory Response & Autoimmunity 384HT (PAHS-3803Z, SABiosciences, MD, USA)

Position	Unigene	GeneBank	Symbol	Description	Gene Name
A01	Hs.77867	NM_000674	ADORA1	Adenosine A1 receptor	RDC7
A02	Hs.324746	NM_001622	AHSG	Alpha-2-HS-glycoprotein	A2HS, AHS, FETUA, HSGA
A03	Hs.76364	NM_001623	AIF1	Allograft inflammatory factor 1	AIF-1, IBA1, IRT-1, IRT1
A04	Hs.591680	NM_004757	AIMP1	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	EMAP2, EMAPII, SCYE1, p43
A05	Hs.507080	NM_001639	APCS	Amyloid P component, serum	MGC88159, PTX2, SAP
A06	Hs.237658	NM_001643	APOA2	Apolipoprotein A-II	Apo-AII, ApoA-II, apoAII
A07	Hs.474740	NM_030882	APOL2	Apolipoprotein L, 2	APOL-II, APOL3
A08	Hs.474737	NM_145640	APOL3	Apolipoprotein L, 3	APOLIII, CG121
A09	Hs.270833	NM_001657	AREG	Amphiregulin	AR, AREGB, CRDGF, MGC13647, SDGF
A10	Hs.72885	NM_001700	AZU1	Azurocidin 1	AZAMP, AZU, CAP37, HBP, HUMAZUR, NAZC
A11	Hs.478588	NM_001706	BCL6	B-cell CLL/lymphoma 6	BCL5, BCL6A, LAZ3, ZBTB27, ZNF51
A12	Hs.665244	NM_013314	BLNK	B-cell linker	AGM4, BASH, BLNK-S, LY57, MGC111051, SLP-65, SLP65
A13	Hs.1274	NM_006129	BMP1	Bone morphogenetic protein 1	FLJ4432, PCOLC, PCP, PCP2, TLD
A14	Hs.73853	NM_001200	BMP2	Bone morphogenetic protein 2	BMP2A
A15	Hs.387411	NM_001201	BMP3	Bone morphogenetic protein 3	BMP-3A
A16	Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7	OP-1
A17	Hs.529053	NM_000064	C3	Complement component 3	AHUS5, ARMD9, ASP, CPAMD1
A18	Hs.591148	NM_004054	C3AR1	Complement component 3a receptor 1	AZ3B, C3AR, HNFAG09
A19	Hs.56279	NM_021571	CARD18	Caspase recruitment domain family, member 18	ICEBERG, UNQ5804, pseudo-ICE
A20	Hs.440961	NM_001042440	CAST	Calpastatin	BS-17, MGC9402
A21	Hs.72918	NM_002981	CCL1	Chemokine (C-C motif) ligand 1	I-309, P500, SCYA1, S1Se, TCA3
A22	Hs.54460	NM_002986	CCL11	Chemokine (C-C motif) ligand 11	MGC22554, SCYA11
A23	Hs.414629	NM_005408	CCL13	Chemokine (C-C motif) ligand 13	CKb10, MCP-4, MGC17134, NCC-1, NCC1, SCYA13, SCYL1
A24	Hs.10458	NM_004590	CCL16	Chemokine (C-C motif) ligand 16	CKb12, HCC-4, ILINCK, LCC-1, LEC, LMC, MGC117051, Mtn-1, NCC-4, NCC4, SCYA16, SCYL4
B01	Hs.546294	NM_002987	CCL17	Chemokine (C-C motif) ligand 17	A-152E5.3, ABCD-2, MGC138271, MGC138273, SCYA17, TARC

B02	Hs.143961	NM_002988	CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	AMAC-1, AMAC1, Ckb7, DC-CK1, DCCK1, MIP-4, PARC, SCYA18
B03	Hs.50002	NM_006274	CCL19	Chemokine (C-C motif) ligand 19	Ckb11, ELC, MGC34433, MIP-3b, MIP3B, SCYA19
B04	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2	GDGF-2, HCL1, HSMCR30, MCAF, MCP-1, MCP1, MGC9434, SCYA2, SMC-CF
B05	Hs.75498	NM_004591	CCL20	Chemokine (C-C motif) ligand 20	Ckb4, LARC, MIP-3a, MIP3A, SCYA20, ST38
B06	Hs.57907	NM_002989	CCL21	Chemokine (C-C motif) ligand 21	6Ckine, Ckb9, ECL, MGC34555, SCYA21, SLC, TCA4
B07	Hs.534347	NM_002990	CCL22	Chemokine (C-C motif) ligand 22	ABCD-1, DC, B-CK, MDC, MGC34554, SCYA22, STCP-1
B08	Hs.169191	NM_005064	CCL23	Chemokine (C-C motif) ligand 23	CK-BETA-8, Ckb8, Ckb-8, Ckb-8-1, MIP-3, MIP3, MPIF-1, SCYA23
B09	Hs.247838	NM_002991	CCL24	Chemokine (C-C motif) ligand 24	Ckb-6, MPIF-2, MPIF2, SCYA24
B10	Hs.310511	NM_005624	CCL25	Chemokine (C-C motif) ligand 25	Ckb15, MGC150327, SCYA25, TECK
B11	Hs.131342	NM_006072	CCL26	Chemokine (C-C motif) ligand 26	IMAC, MGC126714, MIP-4a, MIP-4alpha, SCYA26, TSC-1
B12	Hs.648124	NM_006664	CCL27	Chemokine (C-C motif) ligand 27	ALP, CTACK, CTAK, ESKINE, ILC, PESKY, SCYA27
B13	Hs.656904	NM_148672	CCL28	Chemokine (C-C motif) ligand 28	CCK1, MEC, MGC71902, SCYA28
B14	Hs.514107	NM_002983	CCL3	Chemokine (C-C motif) ligand 3	GOS19-1, LD78ALPHA, MIP-1-alpha, MIP1A, SCYA3
B15	Hs.75703	NM_002984	CCL4	Chemokine (C-C motif) ligand 4	ACT2, AT744.1, G-26, LAG1, MGC104418, MGC126025, MGC126026, MIP-1-beta, MIP1B, MIP1B1, SCYA2, SCYA4
B16	Hs.514821	NM_002985	CCL5	Chemokine (C-C motif) ligand 5	D175136E, MGC17164, RANTES, SCYA5, SISd, TCP228
B17	Hs.251526	NM_006273	CCL7	Chemokine (C-C motif) ligand 7	FIC, MARC, MCP-3, MCP3, MGC138463, MGC138465, NC28, SCYA6, SCYA7
B18	Hs.271387	NM_005623	CCL8	Chemokine (C-C motif) ligand 8	HC14, MCP-2, MCP2, SCYA10, SCYA8
B19	Hs.301921	NM_001295	CCR1	Chemokine (C-C motif) receptor 1	CD191, CKR-1, CKR1, CMKBR1, HM145, MIP1aR, SCYAR1
B20	Hs.278446	NM_016602	CCR10	Chemokine (C-C motif) receptor 10	GPR2
B21	Hs.511794	NM_001123396	CCR2	Chemokine (C-C motif) receptor 2	CC-CKR-2, CCR2A, CCR2B, CD192, CKR2, CKR2A, CKR2B, CMKBR2, FLJ78302, MCP-1-R, MGC103828, MGC111760, MGC168006
B22	Hs.506190	NM_001837	CCR3	Chemokine (C-C motif) receptor 3	CC-CKR-3, CD193, CKR3, CMKBR3, MGC102841
B23	Hs.184926	NM_005508	CCR4	Chemokine (C-C motif) receptor 4	CC-CKR-4, CD194, CKR4, CMKBR4, ChemR13, HGCN:14099, K5-5, MGC88293
B24	Hs.450802	NM_000579	CCR5	Chemokine (C-C motif) receptor 5	CC-CKR-5, CCCKR5, CD195, CKR-5, CKR5,

						CMKBR5, FLJ78003, IDDM22
C01	Hs.46468	NM_004367	CCR6	Chemokine (C-C motif) receptor 6		BN-1, C-C CKR-6, CC-CKR-6, CCR-6, CD196, CKR-L3, CKRL3, CMKBR6, DCR2, DRV6, GPR29, GPRCY4, STRL22
C02	Hs.370036	NM_001838	CCR7	Chemokine (C-C motif) receptor 7		BLR2, CD197, CDw197, CMKBR7, EBI1
C03	Hs.113222	NM_005201	CCR8	Chemokine (C-C motif) receptor 8		CC-CKR-8, CCR-8, CDw198, CKRL1, CMKBR8, CMKBRL2, CY6, GPRCY6, MGC129966, MGC129973, TER1
C04	Hs.225946	NM_006641	CCR9	Chemokine (C-C motif) receptor 9		CDw199, GPR-9-6, GPR28
C05	Hs.729361	NM_016557	CCRL1	Chemokine (C-C motif) receptor-like 1		CC-CKR-11, CCBP2, CCR-11, CCR10, CCR11, CCX CKR, CCX-CKR, CKR-11, PPR1, VSHK1
C06	Hs.535713	NM_003965	CCRL2	Chemokine (C-C motif) receptor-like 2		CKRX, CRAM, CRAM-A, CRAM-B, FLJ55815, HCR, MGC116710, MGC34104
C07	Hs.163867	NM_000591	CD14	CD14 molecule		-
C08	Hs.87205	NM_005582	CD180	CD180 molecule		LY64, Ly78, MGC126233, MGC126234, RP105
C09	Hs.355307	NM_001242	CD27	CD27 molecule		MGC20393, S152, T14, TNFRSF7, Tp55
C10	Hs.591629	NM_006139	CD28	CD28 molecule		MGC138290, Tp44
C11	Hs.631659	NM_000616	CD4	CD4 molecule		CD4mut
C12	Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5		Bp50, CDW40, MGC9013, TNFRSF5, p50
C13	Hs.592244	NM_000074	CD40LG	CD40 ligand		CD154, CD40L, HIGM1, IGM, IMD3, T-BAM, TNFSF5, TRAP, gp39, hCD40L
C14	Hs.501497	NM_001252	CD70	CD70 molecule		CD27L, CD27LG, TNFSF7
C15	Hs.436568	NM_004355	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain		DHLA, HLA, IL, Ia-GAMMA
C16	Hs.171182	NM_006889	CD86	CD86 molecule		B7-2, B7.2, B70, CD28LG2, LAB72, MGC34413
C17	Hs.466039	NM_001784	CD97	CD97 molecule		TM7LN1
C18	Hs.517106	NM_005194	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta		C, EBP-beta, CRP2, IL6DBP, LAP, MGC32080, NF-IL6, TCF5
C19	Hs.15159	NM_181641	CKLF	Chemokine-like factor		C32, CKLF1, CKLF2, CKLF3, CKLF4, UCK-1
C20	Hs.889	NM_001828	CLC	Charcot-Leyden crystal protein		GAL10, Gal-10, LGALS10, LGALS10A, LPPL_HUMAN, MGC149659
C21	Hs.549232	NM_181269	CMTM1	CKLF-like MARVEL transmembrane domain containing 1		CKLFH, CKLFH1, CKLFSF1, MGC71870
C22	Hs.195685	NM_144673	CMTM2	CKLF-like MARVEL transmembrane domain containing 2		CKLFSF2, MGC39436
C23	Hs.129966	NM_001842	CNTFR	Ciliary neurotrophic factor receptor		MGC1774
C24	Hs.709456	NM_000567	CRP	C-reactive protein, pentraxin-related		MGC149895, MGC88244, PTX1

D01	Hs.591402	NM_000757	CSF1	Colony stimulating factor 1 (macrophage)	MCSF, MGC31930
D02	Hs.1349	NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	GMCSF, MGC131935, MGC138897
D03	Hs.520937	NM_006140	CSF2RA	Colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)	CD116, CDw116, CSF2R, CSF2RAX, CSF2RAY, CSF2RX, CSF2RY, GM-CSF-R-alpha, GMCSFR, GMR, MGC3848, MGC4838
D04	Hs.592192	NM_000395	CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	CD131, CDw131, IL3RB, IL5RB
D05	Hs.2233	NM_000759	CSF3	Colony stimulating factor 3 (granulocyte)	C17orf33, CSF3OS, GCSF, MGC45931
D06	Hs.524517	NM_000760	CSF3R	Colony stimulating factor 3 receptor (granulocyte)	CD114, GCSFR
D07	Hs.483811	NM_001330	CTF1	Cardiotrophin 1	CT-1, CT1
D08	Hs.531668	NM_002996	CX3CL1	Chemokine (C-X3-C motif) ligand 1	ABCD-3, C3Xkine, CX3C, CX3C, NTN, NTT, SCYD1, fractalkine, neurotactin
D09	Hs.78913	NM_001337	CX3CR1	Chemokine (C-X3-C motif) receptor 1	CCR11, CMKBRL1, CMKDRI, GPR13, GPRV28, V28
D10	Hs.789	NM_001511	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	FSP, GRO1, GROa, MGSA, MGSA-a, NAP-3, SCYB1
D11	Hs.632586	NM_001565	CXCL10	Chemokine (C-X-C motif) ligand 10	C7, IFI10, INP10, IP-10, SCYB10, crg-2, gIP-10, mob-1
D12	Hs.632592	NM_005409	CXCL11	Chemokine (C-X-C motif) ligand 11	H174, I-TAC, IP-9, IP9, MGC102770, SCYB11, SCYB9B, b-R1
D13	Hs.522891	NM_000609	CXCL12	Chemokine (C-X-C motif) ligand 12	IRH, PBSF, SCYB12, SDF1, SDF1A, SDF1B, TLSF, TPAR1
D14	Hs.100431	NM_006419	CXCL13	Chemokine (C-X-C motif) ligand 13	ANGIE, ANGIE2, BCA1, BCA1, BLC, BLR1L, SCYB13
D15	Hs.483444	NM_004887	CXCL14	Chemokine (C-X-C motif) ligand 14	BMAC, BRAK, KEC, KS1, MGC10687, MIP-2g, MIP2G, NJAC, SCYB14
D16	Hs.708201	NM_022059	CXCL16	Chemokine (C-X-C motif) ligand 16	CXCLG16, SR-PSOX, SRPSOX
D17	Hs.590921	NM_002089	CXCL2	Chemokine (C-X-C motif) ligand 2	CINC-2a, GRO2, GROb, MGSA-b, MIP-2a, MIP2, MIP2A, SCYB2
D18	Hs.89690	NM_002090	CXCL3	Chemokine (C-X-C motif) ligand 3	CINC-2b, GRO3, GROg, MIP-2b, MIP2B, SCYB3
D19	Hs.89714	NM_002994	CXCL5	Chemokine (C-X-C motif) ligand 5	ENA-78, SCYB5
D20	Hs.164021	NM_002993	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CKA-3, GCP-2, GCP2, SCYB6
D21	Hs.77367	NM_002416	CXCL9	Chemokine (C-X-C motif) ligand 9	CMK, Humig, MIG, SCYB9, crg-10
D22	Hs.194778	NM_000634	CXCR1	Chemokine (C-X-C motif) receptor 1	C-C, C-C-CKR-1, CD128, CD181, CDw128a, CKR-1, CMKAR1, IL8R1, IL8RA, IL8RBA
D23	Hs.846	NM_001557	CXCR2	Chemokine (C-X-C motif) receptor 2	CD182, CDw128b, CMKAR2, IL8R2, IL8RA, IL8RB
D24	Hs.198252	NM_001504	CXCR3	Chemokine (C-X-C motif) receptor 3	CD182, CD183, CKR-L2, CMKAR3, GPR9, IP10-R, Mig-R, MigR

E01	Hs.593413	NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4	CD184, D2S201E, FB22, HM89, HSY3RR, LAP3, LCRI, LESTR, NPY3R, NPYR, NPYRL, NPYV3R, WHIM
E02	Hs.113916	NM_001716	CXCR5	Chemokine (C-X-C motif) receptor 5	BLR1, CD185, MDR15, MGC117347
E03	Hs.34526	NM_006564	CXCR6	Chemokine (C-X-C motif) receptor 6	BONZO, CD186, STRL33, TYMSTR
E04	Hs.292356	NM_000397	CYBB	Cytochrome b-245, beta polypeptide	CGD, GP91-1, GP91-PHOX, GP91PHOX, NOX2, p91-PHOX
E05	Hs.91546	NM_019885	CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	CYP26A2, DKFZp686G0638, MGC129613, P450RAI-2
E06	Hs.586174	NM_004946	DOCK2	Dedicator of cytokinesis 2	FLJ46592, KIAA0209
E07	Hs.501452	NM_005755	EBI3	Epstein-Barr virus induced 3	IL-27B, IL27B
E08	Hs.105407	NM_001399	EDA	Ectodysplasin A	ED1, ED1-A1, ED1-A2, EDA1, EDA2, HED, ODT1, STHAGX1, XHED, XLHED
E09	Hs.212088	NM_001979	EPHX2	Epoxide hydrolase 2, cytoplasmic	CEH, SEH
E10	Hs.2303	NM_000799	EPO	Erythropoietin	EP, MGC138142, MVCD2
E11	Hs.631624	NM_000121	EPOR	Erythropoietin receptor	EPO-R, MGC138358
E12	Hs.446352	NM_004448	ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	CD340, HER-2, HER-2, neu, HER2, MLN 19, NEU, NGL, TKR1
E13	Hs.591774	NM_018695	ERBB2IP	ErbB2 interacting protein	ERBIN, LAP2
E14	Hs.517293	NM_016946	F11R	F11 receptor	CD321, JAM, JAM1, JAMA, JCAM, KAT, PAM-1
E15	Hs.655207	NM_000506	F2	Coagulation factor II (thrombin)	PT
E16	Hs.62192	NM_001993	F3	Coagulation factor III (thromboplastin, tissue factor)	CD142, FLJ17960, TF, TFA
E17	Hs.654450	NM_000132	F8	Coagulation factor VIII, procoagulant component	AHF, DXS1253E, F8B, F8C, FVIII, HEMA
E18	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)	APT1LG1, CD178, CD95-L, CD95L, FASL, TNFSF6
E19	Hs.483635	NM_000800	FGF1	Fibroblast growth factor 1 (acidic)	AFGF, ECGF, ECGF-beta, ECGFA, ECGFB, FGF-alpha, FGFA, GLIO703, HBGF1
E20	Hs.664499	NM_004465	FGF10	Fibroblast growth factor 10	-
E21	Hs.584758	NM_021032	FGF12	Fibroblast growth factor 12	FGF12B, FHF1
E22	Hs.284244	NM_002006	FGF2	Fibroblast growth factor 2 (basic)	BFGF, FGFb, HBGF-2
E23	Hs.567268	NM_002009	FGF7	Fibroblast growth factor 7	HBGF-7, KGF
E24	Hs.11392	NM_004469	FIGF	C-fos induced growth factor (vascular endothelial growth factor D)	VEGF-D, VEGFD
F01	Hs.428	NM_001459	FLT3LG	Fms-related tyrosine kinase 3 ligand	FL, FLT3L
F02	Hs.203717	NM_002026	FN1	Fibronectin 1	CIG, DKFZp686F10164, DKFZp686H0342, DKFZp686I1370, DKFZp686O13149, ED-B, FINC,

									FN, FNZ, GFND, GFND2, LETS, MSF
F03	Hs.728789	NM_005252	FOS	FBJ murine osteosarcoma viral oncogene homolog				AP-1, C-FOS	
F04	Hs.753	NM_002029	FPR1	Formyl peptide receptor 1				FMPLP, FPR	
F05	Hs.279463	NM_016204	GDF2	Growth differentiation factor 2				BMP-9, BMP9	
F06	Hs.86232	NM_020634	GDF3	Growth differentiation factor 3				KFS3, MCOP7, MCOPCB6	
F07	Hs.1573	NM_000557	GDF5	Growth differentiation factor 5				BMP14, CDMP1, LAP4, OS5, SYNS2	
F08	Hs.492277	NM_001001557	GDF6	Growth differentiation factor 6				BMP13, CDMP2, KFM, KFS, KFS1, KFSL, MCOP4, MCOPCB6, MGC158100, MGC158101, SCDO4, SGM1	
F09	Hs.25022	NM_005260	GDF9	Growth differentiation factor 9				-	
F10	Hs.388347	NM_005264	GFRA1	GDNF family receptor alpha 1				DKFZp313E1012, DKFZp686J0156, FLJ10561, FLJ31546, GDNFR, GDNFRA, GFR-ALPHA-1, MGC23045, RET1L, RETL1, TRNR1	
F11	Hs.441202	NM_001495	GFRA2	GDNF family receptor alpha 2				GDNFRB, NRTNR-ALPHA, NTNRA, RETL2, TRNR2	
F12	Hs.125180	NM_000163	GHR	Growth hormone receptor				GHP	
F13	Hs.49105	NM_053274	GLMN	Glomulin, FKBP associated protein				FAP, FAP48, FAP68, FKBPAP, GLML, GVM, VMGLOM	
F14	Hs.466471	NM_000175	GPI	Glucose-6-phosphate isomerase				AMF, DKFZp686C13233, GNPI, NLK, PGI, PHI, SA-36, SA36	
F15	Hs.8882	NM_003485	GPR68	G protein-coupled receptor 68				GPR12A, MGC111379, MGC156983, OGR1	
F16	Hs.40098	NM_013372	GREM1	Gremlin 1				CKTSF1B1, DAND2, DRM, GREMLIN, IHG-2, MGC126660	
F17	Hs.98206	NM_022469	GREM2	Gremlin 2				CKTSF1B2, DAND3, PRDC	
F18	Hs.514220	NM_002087	GRN	Granulin				GEP, GP88, PCDGF, PEPI, PGRN	
F19	Hs.20516	NM_006037	HDAC4	Histone deacetylase 4				AHO3, BDMR, HA6116, HD4, HDAC-A, HDACA, KIAA0288	
F20	Hs.438782	NM_005474	HDAC5	Histone deacetylase 5				FLJ90614, HD5, NY-CO-9	
F21	Hs.200063	NM_001098416	HDAC7	Histone deacetylase 7				DKFZp586J0917, FLJ99588, HD7A, HDAC7A	
F22	Hs.196054	NM_178425	HDAC9	Histone deacetylase 9				DKFZp779K1053, HD7, HD7b, HD9, HDAC, HDAC7, HDAC7B, HDAC9B, HDAC9FL, HDRP, KIAA0744, MITR	
F23	Hs.1570	NM_000861	HRH1	Histamine receptor H1				H1-R, hisH1	
F24	Hs.37026	NM_024013	IFNA1	Interferon, alpha 1				IFL, IFN, IFN-ALPHA, IFN-alphaD, IFNA13, IFNA@, MGC138207, MGC138505, MGC138507	
G01	Hs.93907	NM_002172	IFNA14	Interferon, alpha 14				IFN-alphaH, LEIF2H, MGC125756, MGC125757	
G02	Hs.211575	NM_000605	IFNA2	Interferon, alpha 2				IFN-alphaA, IFNA, INF2, MGC125764,	

G03	Hs.1510	NM_021068	IFNA4	Interferon, alpha 4	IFN-alpha4a, INFA4, MGC142200
G04	Hs.73890	NM_002170	IFNA8	Interferon, alpha 8	IFN-alphaB
G05	Hs.529400	NM_000629	IFNAR1	Interferon (alpha, beta and omega) receptor 1	AVP, IFN-alpha-REC, IFNAR, IFNBR, IFRC
G06	Hs.708195	NM_000874	IFNAR2	Interferon (alpha, beta and omega) receptor 2	IFN-R, IFN-alpha-REC, IFNABR, IFNARB
G07	Hs.93177	NM_002176	IFNB1	Interferon, beta 1, fibroblast	IFB, IFF, IFNB, MGC96956
G08	Hs.682604	NM_176891	IFNE	Interferon, epsilon	IFN-E, IFNE1, IFNT1, MGC119018, MGC119020, PRO655
G09	Hs.856	NM_000619	IFNG	Interferon, gamma	IFG, IFI
G10	Hs.520414	NM_000416	IFNGR1	Interferon gamma receptor 1	CD119, FLJ45734, IFNGR
G11	Hs.634632	NM_005534	IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	AF-1, IFGR2, IFNGT1
G12	Hs.591083	NM_020124	IFNK	Interferon, kappa	RP11-27J8.1
G13	Hs.73010	NM_002177	IFNW1	Interferon, omega 1	-
G14	Hs.458096	XM_378052	IFNWP2	Interferon, omega 1 pseudogene 2	-
G15	Hs.421245	NM_006083	IK	IK cytokine, down-regulator of HLA II	CSA2, MGC59741, RED
G16	Hs.193717	NM_000572	IL10	Interleukin 10	CSIF, IL-10, IL10A, MGC126450, MGC126451, TGIF
G17	Hs.504035	NM_001558	IL10RA	Interleukin 10 receptor, alpha	CDW210A, HIL-10R, IL-10R1, IL10R
G18	Hs.654593	NM_000628	IL10RB	Interleukin 10 receptor, beta	CDW210B, CRF2-4, CRFB4, D21S58, D21S66, IL-10R2
G19	Hs.467304	NM_000641	IL11	Interleukin 11	AGIF, IL-11
G20	Hs.591088	NM_004512	IL11RA	Interleukin 11 receptor, alpha	MGC2146
G21	Hs.673	NM_000882	IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	CLMF, IL-12A, NFSK, NKSF1, P35
G22	Hs.674	NM_002187	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	CLMF, CLMF2, IL-12B, NKSF, NKSF2
G23	Hs.567294	NM_005535	IL12RB1	Interleukin 12 receptor, beta 1	CD212, IL-12R-BETA1, IL12RB, MGC34454
G24	Hs.479347	NM_001559	IL12RB2	Interleukin 12 receptor, beta 2	-
H01	Hs.845	NM_002188	IL13	Interleukin 13	ALRH, BHR1, IL-13, MGC116786, MGC116788, MGC116789, P600
H02	Hs.496646	NM_001560	IL13RA1	Interleukin 13 receptor, alpha 1	CD213A1, IL-13Ra, NR4
H03	Hs.336046	NM_000640	IL13RA2	Interleukin 13 receptor, alpha 2	CD213A2, CT19, IL-13R, IL13BP
H04	Hs.654378	NM_000585	IL15	Interleukin 15	IL-15, MGC9721
H05	Hs.524117	NM_002189	IL15RA	Interleukin 15 receptor, alpha	MGC104179

H06	Hs.459095	NM_004513	IL16	Interleukin 16	FLJ16806, FLJ42735, FLJ44234, LCF, NIL16, PRIL16, prIL-16
H07	Hs.41724	NM_002190	IL17A	Interleukin 17A	CTLA8, IL-17, IL-17A, IL17
H08	Hs.156979	NM_014443	IL17B	Interleukin 17B	IL-17B, IL-20, MGC138900, MGC138901, NIRF, ZCYTO7
H09	Hs.278911	NM_013278	IL17C	Interleukin 17C	CX2, IL-17C, IL-21, MGC126884, MGC138401
H10	Hs.655142	NM_138284	IL17D	Interleukin 17D	FLJ30846, IL-17D, IL-22, IL-27, IL27
H11	Hs.272295	NM_052872	IL17F	Interleukin 17F	IL-17F, ML-1, MLI1
H12	Hs.129751	NM_014339	IL17RA	Interleukin 17 receptor A	CD217, CDw217, FU32274, FU36453, IL-17RA, IL17R, MGC10262, hIL-17R
H13	Hs.654970	NM_018725	IL17RB	Interleukin 17 receptor B	CRL4, EVI27, IL17BR, IL17RH1, MGC5245
H14	Hs.83077	NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)	IGIF, IL-18, IL-1g, IL1F4, MGC12320
H15	Hs.469521	NM_003855	IL18R1	Interleukin 18 receptor 1	CD218a, CDw218a, IL-1Rrp, IL18RA, IL1RRP
H16	Hs.158315	NM_003853	IL18RAP	Interleukin 18 receptor accessory protein	ACPL, CD218b, CDw218b, IL18RB, MGC120589, MGC120590
H17	Hs.661017	NM_013371	IL19	Interleukin 19	IL-10C, MDA1, NG-1, ZMDA1
H18	Hs.1722	NM_000575	IL1A	Interleukin 1, alpha	IL-1A, IL1, IL1-ALPHA, IL1F1
H19	Hs.126256	NM_000576	IL1B	Interleukin 1, beta	IL-1, IL1-BETA, IL1F2
H20	Hs.306974	NM_173161	IL1F10	Interleukin 1 family, member 10 (theta)	FIL1-theta, IL-1HY2, IL1-theta, MGC119831, MGC119832, MGC119833
H21	Hs.701982	NM_000877	IL1R1	Interleukin 1 receptor, type I	CD121A, D2S1473, IL-1R-alpha, IL1R, IL1RA, P80
H22	Hs.25333	NM_004633	IL1R2	Interleukin 1 receptor, type II	CD121b, IL1RB, MGC47725
H23	Hs.478673	NM_002182	IL1RAP	Interleukin 1 receptor accessory protein	C3orf13, FU37788, IL-1RACp, IL1R3
H24	Hs.675519	NM_017416	IL1RAPL2	Interleukin 1 receptor accessory protein-like 2	IL-1R9, IL1R9, IL1RAPL-2, TIGIRR-1
I01	Hs.66	NM_016232	IL1RL1	Interleukin 1 receptor-like 1	DER4, FIT-1, IL33R, MGC32623, ST2, ST2L, ST2V, T1
I02	Hs.659863	NM_003854	IL1RL2	Interleukin 1 receptor-like 2	IL1R-rp2, IL1RRP2
I03	Hs.81134	NM_000577	IL1RN	Interleukin 1 receptor antagonist	DIRA, ICIL-1RA, IL-1RN, IL-1ra, IL-1ra3, IL1F3, IL1RA, IRAP, MGC10430, MVCD4
I04	Hs.89679	NM_000586	IL2	Interleukin 2	IL-2, TCGF, lymphokine
I05	Hs.272373	NM_018724	IL20	Interleukin 20	IL-20, IL10D, MGC96907, ZCYTO10
I06	Hs.445868	NM_014432	IL20RA	Interleukin 20 receptor, alpha	FU40993, IL-20R1, ZCYTOR7
I07	Hs.567559	NM_021803	IL21	Interleukin 21	IL-21, Zai11
I08	Hs.210546	NM_021798	IL21R	Interleukin 21 receptor	MGC10967, NILR
I09	Hs.287369	NM_020525	IL22	Interleukin 22	IL-21, IL-22, IL-D110, IL-TIF, MGC79382, MGC79384, TIFIL-23, TIFa, zcyto18

I10	Hs.110915	NM_021258	IL22RA1	Interleukin 22 receptor, alpha 1	CRF2-9, IL22R, IL22R1
I11	Hs.126891	NM_052962	IL22RA2	Interleukin 22 receptor, alpha 2	CRF2-10, CRF2-S1, CRF2X, IL-22BP, IL-22RA2, MGC150509, MGC150510
I12	Hs.98309	NM_016584	IL23A	Interleukin 23, alpha subunit p19	IL-23, IL-23A, IL23P19, MGC79388, P19, SGRF
I13	Hs.677426	NM_144701	IL23R	Interleukin 23 receptor	-
I14	Hs.411311	NM_006850	IL24	Interleukin 24	C49A, FISP, IL10B, MDA7, MOB5, ST16
I15	Hs.302036	NM_022789	IL25	Interleukin 25	IL17E
I16	Hs.272350	NM_018402	IL26	Interleukin 26	AK155, IL-26
I17	Hs.528111	NM_145659	IL27	Interleukin 27	IL-27, IL-27A, IL27A, IL27p28, IL30, MGC71873, p28
I18	Hs.221375	NM_173065	IL28RA	Interleukin 28 receptor, alpha (interferon, lambda receptor)	CRF2, 12, IFNLR, IFNLR1, IL-28R1, LICR2
I19	Hs.406745	NM_172140	IL29	Interleukin 29 (interferon, lambda 1)	IFNL1, IL-29
I20	Hs.231367	NM_000417	IL2RA	Interleukin 2 receptor, alpha	CD25, IDDM10, IL2R, TCGFR
I21	Hs.474787	NM_000878	IL2RB	Interleukin 2 receptor, beta	CD122, IL15RB, P70-75
I22	Hs.84	NM_000206	IL2RG	Interleukin 2 receptor, gamma	CD132, CIDX, IL-2RG, IMD4, P64, SCIDX, SCIDX1
I23	Hs.694	NM_000588	IL3	Interleukin 3 (colony-stimulating factor, multiple)	IL-3, MCGF, MGC79398, MGC79399, MULTI-CSF
I24	Hs.55378	NM_139017	IL31RA	Interleukin 31 receptor A	CRL, CRL3, GLM-R, GLMR, GPL, IL-31RA, MGC125346, PRO21384, hGLM-R
J01	Hs.943	NM_004221	IL32	Interleukin 32	IL-32alpha, IL-32beta, IL-32delta, IL-32gamma, NK4, TAIF, TAIFa, TAIFb, TAIFc, TAIFd
J02	Hs.278910	NM_014440	IL36A	Interleukin 36, alpha	FIL1, FIL1(EPSILON), FIL1E, IL-1F6, IL1(EPSILON), IL1F6, MGC129552, MGC129553
J03	Hs.278909	NM_173178	IL36B	Interleukin 36, beta	FIL1, FIL1-(ETA), FIL1H, FIL1-(ETA), IL-1F8, IL-1H2, IL1-ETA, IL1F8, IL1H2, MGC126880, MGC126882
J04	Hs.211238	NM_019618	IL36G	Interleukin 36, gamma	IL-1F9, IL-1H1, IL-1RP2, IL1E, IL1F9, IL1H1, IL1RP2
J05	Hs.516301	NM_012275	IL36RN	Interleukin 36 receptor antagonist	FIL1, FIL1(DELTA), FIL1D, IL1F5, IL1HY1, IL1L1, IL1RP3, IL36RA, MGC29840
J06	Hs.166371	NM_173205	IL37	Interleukin 37	FIL1, FIL1(ZETA), FIL1Z, IL-1F7, IL-1H, IL-1H4, IL-1RP1, IL-37, IL1F7, IL1H4, IL1RP1
J07	Hs.632790	NM_002183	IL3RA	Interleukin 3 receptor, alpha (low affinity)	CD123, IL3R, IL3RAY, IL3RX, IL3RY, MGC34174, hIL-3Ra
J08	Hs.73917	NM_000589	IL4	Interleukin 4	BCGF-1, BCGF1, BSF-1, BSF1, IL-4, MGC79402
J09	Hs.513457	NM_000418	IL4R	Interleukin 4 receptor	CD124, IL4RA
J10	Hs.2247	NM_000879	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	EDF, IL-5, TRF
J11	Hs.68876	NM_000564	IL5RA	Interleukin 5 receptor, alpha	CD125, CDw125, HSIL5R3, IL5R, MGC26560

J12	Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)	BSF2, HGF, HSF, IFNB2, IL-6
J13	Hs.709210	NM_000565	IL6R	Interleukin 6 receptor	CD126, IL-6R-1, IL-6RA, IL6RA, MGC104991, gp80
J14	Hs.532082	NM_002184	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	CD130, CDW130, DKFzp564F053, GP130, IL-6RB
J15	Hs.591873	NM_000880	IL7	Interleukin 7	IL-7
J16	Hs.591742	NM_002185	IL7R	Interleukin 7 receptor	CD127, CDW127, IL-7R-alpha, IL7RA, ILRA
J17	Hs.624	NM_000584	IL8	Interleukin 8	CXCL8, GCP-1, GCP1, LECT, LUCT, LYNAP, MDNCF, MONAP, NAF, NAP-1, NAP1
J18	Hs.960	NM_000590	IL9	Interleukin 9	HP40, IL-9, P40
J19	Hs.406228	NM_002186	IL9R	Interleukin 9 receptor	CD129
J20	Hs.407506	NM_002191	INH1A	Inhibin, alpha	-
J21	Hs.583348	NM_002192	INH1A	Inhibin, beta A	EDF, FRP
J22	Hs.1735	NM_002193	INH1B	Inhibin, beta B	MGC157939
J23	Hs.654579	NM_000207	INS	Insulin	IDDM2, ILPR, IRDN, MODY10
J24	Hs.401013	NM_002460	IRF4	Interferon regulatory factor 4	LSIRF, MUM1
K01	Hs.166120	NM_001572	IRF7	Interferon regulatory factor 7	IRF-7H, IRF7A
K02	Hs.375957	NM_000211	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	CD18, LAD, LCAMB, LFA-1, MAC-1, MF17, MF17
K03	Hs.518000	NM_002218	ITIH4	Inter-alpha (globulin) inhibitor H4 (plasma kallikrein-sensitive glycoprotein)	DKFzp686G21125, GP120, H4P, IHRP, ITI-HC4, ITIH1, PK-120, PK120
K04	Hs.1048	NM_003994	KITLG	KIT ligand	DKFzp686F2250, FPH2, KL-1, Kitl, MGF, SCF, SF, SHEP7
K05	Hs.77741	NM_000893	KNG1	Kininogen 1	BDK, KNG
K06	Hs.154078	NM_004139	LBP	Lipopolysaccharide binding protein	MGC22233
K07	Hs.656214	NM_020997	LEFTY1	Left-right determination factor 1	LEFTB, LEFTYB
K08	Hs.520187	NM_003240	LEFTY2	Left-right determination factor 2	EBAF, LEFTA, LEFTYA, MGC46222, TGFB4
K09	Hs.705413	NM_002303	LEPR	Leptin receptor	CD295, DKFzp686B1731, LEP-R, OB-R, OBR
K10	Hs.2250	NM_002309	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	CDF, DIA, HILDA
K11	Hs.133421	NM_002310	LIFR	Leukemia inhibitory factor receptor alpha	CD118, FLJ98106, FLJ99923, LIF-R, SJS2, STWS, SWS
K12	Hs.36	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	LT, TNFB, TNFSF1
K13	Hs.376208	NM_002341	LTB	Lymphotoxin beta (TNF superfamily, member 3)	TNFC, TNFSF3, p33
K14	Hs.655431	NM_181657	LTB4R	Leukotriene B4 receptor	BLT1, BLTR, CMKRL1, GPR16, LTB4R1, LTBR1, P2RY7, P2Y7
K15	Hs.153563	NM_002349	LY75	Lymphocyte antigen 75	CD205, CLEC13B, DEC-205, GP200-MR6, LY-75
K16	Hs.653138	NM_004271	LY86	Lymphocyte antigen 86	MD-1, MMD-1, dJ80N2.1
K17	Hs.660766	NM_015364	LY96	Lymphocyte antigen 96	ESOP-1, MD-2, MD2, ly-96

K18	Hs.82045	NM_002391	MDK	Midkine (neurite growth-promoting factor 2)	FLJ27379, MK, NEGF2
K19	Hs.632221	NM_000243	MEFV	Mediterranean fever	FMF, MEF, MGC126560, MGC126586, TRIM20
K20	Hs.277035	NM_007283	MGLL	Monoglyceride lipase	HU-K5, HUK5, MAGL, MGL
K21	Hs.407995	NM_002415	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	GIF, GLIF, MMIF
K22	Hs.654979	NM_022468	MMP25	Matrix metalloproteinase 25	MMP-25, MMP20, MMP20A, MMP11, MT-MMP6, MT-MMP6, MT6-MMP, MT6MMP, MTMMP6
K23	Hs.82906	NM_005373	MPL	Myeloproliferative leukemia virus oncogene	C-MPL, CD110, MPLV, TPOR
K24	Hs.41565	NM_005259	MSTN	Myostatin	GDF8
L01	Hs.369646	NM_004532	MUC4	Mucin 4, cell surface associated	ASGP, HSA276359, MUC-4
L02	Hs.82116	NM_002468	MYD88	Myeloid differentiation primary response gene (88)	MYD88D
L03	Hs.489615	NM_005746	NAMPT	Nicotinamide phosphoribosyltransferase	1110035O14Rik, DKFzp666B131, MGC117256, PBEF, PBEF1, VF, VISFATIN
L04	Hs.509513	NM_147130	NCR3	Natural cytotoxicity triggering receptor 3	1C7, CD337, LY117, MALS, NKp30
L05	Hs.436677	NM_145912	NFAM1	NFAT activating protein with ITAM motif 1	CNAIP, FLJ40652, bk126B4.4
L06	Hs.632209	NM_004555	NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	NFAT4, NFATX
L07	Hs.77810	NM_004554	NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	NF-ATc4, NFAT3
L08	Hs.514284	NM_003204	NFE2L1	Nuclear factor (erythroid-derived 2)-like 1	FLJ00380, LCR-F1, NRF1, TCF11
L09	Hs.654408	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	DKFzp686C01211, EBP-1, KBF1, MGC54151, NF-kappa-B, NF-kappaB, NFKB-p105, NFKB-p50, NFkappaB, p105, p50
L10	Hs.530539	NM_006165	NFRKB	Nuclear factor related to kappaB binding protein	DKFzp547B2013, INO80G
L11	Hs.413074	NM_002504	NFX1	Nuclear transcription factor, X-box binding 1	DKFzp779G2416, MGC20369, NFX2
L12	Hs.631573	NM_033297	NLRP12	NLR family, pyrin domain containing 12	CLR19.3, FCAS2, NALP12, PAN6, PYPAF7, RNO, RNO2
L13	Hs.54483	NM_004688	NMI	N-myc (and STAT) interactor	-
L14	Hs.370414	NM_018055	NODAL	Nodal homolog (mouse)	MGC138230
L15	Hs.709191	NM_000625	NOS2	Nitric oxide synthase 2, inducible	HEP-NOS, INOS, NOS, NOS2A
L16	Hs.657932	NM_024505	NOX5	NADPH oxidase, EF-hand calcium binding domain 5	MGC149776, MGC149777
L17	Hs.122926	NM_000176	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	GCCR, GCR, GR, GRL
L18	Hs.412484	NM_002543	OLR1	Oxidized low density lipoprotein (lectin-like) receptor 1	CLEC8A, LOX1, LOXIN, SCARE1, SLOX1

L19	Hs.248156	NM_020530	OSM	Oncostatin M	MGC20461
L20	Hs.120658	NM_003999	OSMR	Oncostatin M receptor	MGC150626, MGC150627, MGC75127, OSMRB
L21	Hs.718412	NM_006437	PARP4	Poly (ADP-ribose) polymerase family, member 4	ADPRTL1, PARP-4, PARPL, PH5P, VAULT3, VPARP, VWA5C, p193
L22	Hs.535898	NM_002607	PDGFA	Platelet-derived growth factor alpha polypeptide	PDGF-A, PDGF1
L23	Hs.1976	NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide	FLJ12858, PDGF2, SIS, SSV, c-sis
L24	Hs.72933	NM_002620	PF4V1	Platelet factor 4 variant 1	CXCL4L1, CXCL4V1, PF4-ALT, PF4A, SCYB4V1
M01	Hs.137583	NM_005091	PGLYRP1	Peptidoglycan recognition protein 1	MGC126894, MGC126896, PGLYRP, PGRP, PGRP-S, PGRPS, TAG7, TNFSF3L
M02	Hs.189507	NM_012400	PLA2G2D	Phospholipase A2, group IID	SPLASH, sPLA2S
M03	Hs.584823	NM_005084	PLA2G7	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	LDL-PLA2, LP-PLA2, PAFAH
M04	Hs.2164	NM_002704	PPBP	Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	B-TG1, Beta-TG, CTAP-III, CTAP3, CTAPIII, CXCL7, LA-PF4, LDGF, MDGF, NAP-2, PBP, SCAR10, SCYB7, TC1, TC2, TGB, TGB1, THBGB, THBGB1
M05	Hs.502823	NM_181652	PRDX5	Peroxiredoxin 5	ACR1, AOEB166, B166, MGC117264, MGC142283, MGC142285, PLP, PMP20, PRDX6, PRXV
M06	Hs.153310	NM_020820	PREX1	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	KIAA1415, P-REX1
M07	Hs.512633	NM_002728	PRG2	Proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein)	BMPG, MBP, MBP1, MGC14537
M08	Hs.251386	NM_006093	PRG3	Proteoglycan 3	MBP2, MBPH, MGC126662, MGC141971
M09	Hs.1905	NM_000948	PRL	Prolactin	-
M10	Hs.368587	NM_000949	PRLR	Prolactin receptor	hPRLrI
M11	Hs.647450	NM_006404	PROCR	Protein C receptor, endothelial	CCCA, CCD41, CD201, EPCR, MGC23024, bA4204.2
M12	Hs.528665	NM_021935	PROK2	Prokineticin 2	BV8, KAL4, MIT1, PK2
M13	Hs.709174	NM_000952	PTAFR	Platelet-activating factor receptor	PAFR
M14	Hs.196384	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	COX-2, COX2, GRIPGHS, PGG, HS, PGHS-2, PHS-2, hCox-2
M15	Hs.371249	NM_002825	PTN	Pleiotrophin	HARP, HBGf8, HBNF, NEGF1
M16	Hs.269577	NM_002836	PTPRA	Protein tyrosine phosphatase, receptor type, A	HEPTP, HLP, HPTPA, HPTPalpha, LRP, PTPA, PTPRL2, R-PTP-alpha, RPTPA
M17	Hs.591286	NM_002852	PTX3	Pentraxin 3, long	TNFAIP5, TSG-14
M18	Hs.430299	NM_018663	PXMP2	Peroxisomal membrane protein 2, 22kDa	FLJ54922, PMP22

M19	Hs.567312	NM_002580	REG3A	Regenerating islet-derived 3 alpha	FLJ18565, HIP, PAP, PAP-H, PAP1, PBCGF, REG-III, REG3
M20	Hs.447084	NM_198448	REG3G	Regenerating islet-derived 3 gamma	MGC118998, MGC118999, MGC119001, PAP IB, PAP1B, PAPIB, REG-III, UNQ429
M21	Hs.103755	NM_003821	RIPK2	Receptor-interacting serine-threonine kinase 2	CARD3, CARDIAK, CCK, GIG30, RICK, RIP2
M22	Hs.19413	NM_005621	S100A12	S100 calcium binding protein A12	CAAF1, CAGC, CGRP, ENRAGE, MRP6, p6
M23	Hs.416073	NM_002964	S100A8	S100 calcium binding protein A8	60B8AG, CAGA, CFAG, CGLA, CP-10, L1Ag, MA387, MIF, MRP8, NIF, P8
M24	Hs.422181	NM_006272	S100B	S100 calcium binding protein B	NEF, S100, S100-B, S100beta
N01	Hs.654493	NM_006512	SAA4	Serum amyloid A4, constitutive	C-SAA, CSAA
N02	Hs.133995	NM_173050	SCUBE1	Signal peptide, CUB domain, EGF-like 1	-
N03	Hs.200804	NM_005625	SDCBP	Syndecan binding protein (syntenin)	MDA-9, ST1, SYCL, TACIP18
N04	Hs.558009	NM_003004	SECTM1	Secreted and transmembrane 1	K12
N05	Hs.89546	NM_000450	SELE	Selectin E	CD62E, ELAM, ELAM1, ESEL, LECAM2
N06	Hs.525557	NM_000295	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	A1A, A1AT, AAT, MGC23330, MGC9222, PI, PI1, PRO2275, alpha1AT
N07	Hs.534293	NM_001085	SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	AACT, ACT, GIG25, MGC88254
N08	Hs.159509	NM_000934	SERPINF2	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	A2AP, AAP, ALPHA-2-PI, API, PLI
N09	Hs.253495	NM_003019	SFTPD	Surfactant protein D	COLEC7, PSP-D, SFTP4, SP-D
N10	Hs.501624	NM_021805	SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	MGC110992, TIR8
N11	Hs.31869	NM_023068	SIGLEC1	Sialic acid binding Ig-like lectin 1, sialoadhesin	CD169, DKFzp667F058, FLJ00051, FLJ00055, FLJ00073, FLJ00411, FLJ32150, SIGLEC-1, SN, dJ1009E24.1
N12	Hs.46440	NM_021094	SLCO1A2	Solute carrier organic anion transporter family, member 1A2	OATP, OATP-A, OATP1A2, SLC21A3
N13	Hs.103505	NM_020427	SLURP1	Secreted LY6/PLAUR domain containing 1	ANUP, ARS, ArsB, LY6LS, MDM
N14	Hs.485572	NM_003877	SOC2	Suppressor of cytokine signaling 2	CIS2, Cish2, SOCS-2, SSI-2, SSI2, STAT12
N15	Hs.434112	NM_173847	SPACA3	Sperm acrosome associated 3	1700025M08Rik, ALLP17, CT54, LYC3, LYLZ13, MGC119058, SLLP1
N16	Hs.313	NM_000582	SPP1	Secreted phosphoprotein 1	BNSP, BSPI, ETA-1, MGC110940, OPN
N17	Hs.525781	NM_152594	SPRED1	Sprouty-related, EVH1 domain containing 1	FLJ33903, NFLS
N18	Hs.450763	NM_020762	SRGAP1	SLIT-ROBO Rho GTPase activating protein 1	ARHGAP13, FLJ22166, KIAA1304
N19	Hs.301989	NM_015136	STAB1	Stabilin 1	CLEVER-1, FEEL-1, FELE-1, FEX1, KIAA0246, STAB-1

N20	Hs.463059	NM_003150	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	APRF, FLJ20882, HIES, MGC16063
N21	Hs.371720	NM_003177	SYK	Spleen tyrosine kinase	DKFZp313N1010, FLJ25043, FLJ37489
N22	Hs.633301	NM_001058	TACR1	Tachykinin receptor 1	NK1R, NKIR, SPR, TAC1R
N23	Hs.1166	NM_000460	THPO	Thrombopoietin	MGC163194, MGDF, MKCSF, ML, MPLLG, TPO
N24	Hs.537126	NM_001039661	TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	FLJ42305, Mal, wyatt
O01	Hs.654532	NM_003263	TLR1	Toll-like receptor 1	CD281, DKFZp547I0610, DKFZp564I0682, KIAA0012, MGC104956, MGC126311, MGC126312, TIL, TIL. LPRS5, rsc786
O02	Hs.120551	NM_030956	TLR10	Toll-like receptor 10	CD290, MGC104967, MGC126398, MGC126399
O03	Hs.519033	NM_003264	TLR2	Toll-like receptor 2	CD282, TIL4
O04	Hs.657724	NM_003265	TLR3	Toll-like receptor 3	CD283
O05	Hs.174312	NM_138554	TLR4	Toll-like receptor 4	ARMD10, CD284, TOLL, hToll
O06	Hs.604542	NM_003268	TLR5	Toll-like receptor 5	FLJ10052, MGC126430, MGC126431, SLEB1, TIL3
O07	Hs.662185	NM_006068	TLR6	Toll-like receptor 6	CD286
O08	Hs.659215	NM_016562	TLR7	Toll-like receptor 7	-
O09	Hs.660543	NM_138636	TLR8	Toll-like receptor 8	CD288, MGC119599, MGC119600
O10	Hs.87968	NM_017442	TLR9	Toll-like receptor 9	CD289
O11	Hs.241570	NM_000594	TNF	Tumor necrosis factor	DIF, TNF-alpha, TNFA, TNFSF2
O12	Hs.437322	NM_007115	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	TSG-6, TSG6
O13	Hs.81791	NM_002546	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	MGC29565, OCIF, OPG, TR1
O14	Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	APO2L, Apo-2L, CD253, TL2, TRAIL
O15	Hs.333791	NM_003701	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	CD254, ODF, OPGL, OPTB2, RANKL, TRANCE, hRANKL2, sOdf
O16	Hs.54673	NM_003808	TNFSF13	Tumor necrosis factor (ligand) superfamily, member 13	APRIL, CD256, FLJ57090, TALL-2, TALL2, TRDL-1, ZTNF2
O17	Hs.525157	NM_006573	TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b	BAFF, BLYS, CD257, DTL, TALL-1, TALL1, THANK, TNFSF20, ZTNF4
O18	Hs.129708	NM_003807	TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14	CD258, HVEML, LIGHT, LTg, TR2
O19	Hs.241382	NM_005118	TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	MGC129934, MGC129935, TL1, TL1A, VEGI, VEGI192A
O20	Hs.248197	NM_005092	TNFSF18	Tumor necrosis factor (ligand) superfamily, member 18	AITRL, GITRL, MGC138237, TL6, hGITRL
O21	Hs.181097	NM_003326	TNFSF4	Tumor necrosis factor (ligand) superfamily,	CD134L, CD252, GP34, OX-40L, TXGP1

				member 4		
O22	Hs.654445	NM_001244	TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8	CD153, CD30L, CD30LG, MGC138144	
O23	Hs.1524	NM_003811	TNFSF9	Tumor necrosis factor (ligand) superfamily, member 9	4-1BB-L, CD137L	
O24	Hs.368527	NM_019009	TOLLIP	Toll interacting protein	FLJ33531, IL-1RAcPIP	
P01	Hs.421194	NM_003596	TPST1	Tyrosylprotein sulfotransferase 1	-	
P02	Hs.30345	NM_016292	TRAP1	TNF receptor-associated protein 1	HSP75, HSP90L	
P03	Hs.134602	NM_003319	TTN	Titin	CMD1G, CMH9, CMPD4, DKFZp451N061, EOMFC, FLJ26020, FLJ26409, FLJ32040, FLJ34413, FLJ39564, FLJ43066, HMERF, LGMD2J, TMD	
P04	Hs.592212	NM_001953	TYMP	Thymidine phosphorylase	ECGF, ECGF1, MEDPS1, MNGIE, MTDP51, PDECGF, TP, hPD-ECGF	
P05	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A	MGC70609, MVCD1, VEGF, VPF	
P06	Hs.78781	NM_003377	VEGFB	Vascular endothelial growth factor B	VEGFL, VRF	
P07	Hs.443750	NM_007259	VPS45	Vacuolar protein sorting 45 homolog (S. cerevisiae)	H1, H1VPS45, VPS45A, VPS45B, VPS54A, VSP45, VSP45A	
P08	Hs.546295	NM_002995	XCL1	Chemokine (C motif) ligand 1	ATAC, LPTN, LTN, SCM-1, SCM-1a, SCM1, SCM1A, SCVC1	
P09	Hs.248116	NM_005283	XCR1	Chemokine (C motif) receptor 1	CCXCR1, GPR5	
P10	Hs.706890	NM_003680	YARS	Tyrosyl-tRNA synthetase	CMTDIC, TYRRS, YRS, YTS	
P11	Hs.520640	NM_001101	ACTB	Actin, beta	PS1TP5BP1	
P12	Hs.534255	NM_004048	B2M	Beta-2-microglobulin	-	
P13	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, MGC88685	
P14	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT, HPRT	
P15	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0	L10E, LP0, MGC111226, MGC88175, P0, PRLP0, RPP0	
P16	N/A	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A	
P17	N/A	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A	
P18	N/A	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A	
P19	N/A	SA_00104	RTC	Reverse Transcription Control	RTC	
P20	N/A	SA_00104	RTC	Reverse Transcription Control	RTC	
P21	N/A	SA_00104	RTC	Reverse Transcription Control	RTC	
P22	N/A	SA_00103	PPC	Positive PCR Control	PPC	
P23	N/A	SA_00103	PPC	Positive PCR Control	PPC	
P24	N/A	SA_00103	PPC	Positive PCR Control	PPC	