Dedication – For the many inspirational participants who have given their time and support to this trial during such an important period in their lives

The Effect of Thalidomide on Cachexia in Upper Gastrointestinal Cancer

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The thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy of the University of Portsmouth

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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Abstract

Progress made in the palliation of those with terminal cancers has allowed most symptoms to be controlled if not completely alleviated. For many in this situation now, the most overwhelming and unpleasant ongoing problems are related to the accompanying cachexia.

Cachexia - (Greek,kakos-bad, hexis-condition) a wasting syndrome that causes weakness and a loss of weight, fat, and muscle(1)

Cachexia has direct and tangible consequences such as reducing independence to a level where the patient requires institutional end of life care rather than being able to die in their own home or a place of their choosing. It also reduces survival independent of the primary disease histology, stage or the patient's performance score(2)

Upper gastrointestinal adenocarcinomas (i.e. oesophagus, gastric, pancreas, ampullary) often result in profound cachexia. Indeed unexplained weight loss is often the presenting symptom. There are often few other symptoms and consequently diagnosis is often made relatively late in the disease process, when the tumour has spread beyond the possibility of surgical cure. For a proportion of patients, chemotherapy and radiotherapy can offer substantial improvements but side effects often outweigh benefits. Many then either decide never to take these options or elect to stop taking them during the treatment course. A substantial proportion of people in this situation have no acceptable treatment options available to them.

Previous attempts to medically manipulate this condition have been largely unsuccessful. Early small trials using thalidomide have pointed to a possible role in reducing loss of lean body mass but none have demonstrated a functional benefit or investigated underlying mechanisms.

In this trial we aimed to draw definite conclusions as to whether patients with terminal upper gastrointestinal adenocarcinomas would benefit from taking thalidomide. We also investigated the likely biological mechanisms underlying any effects.

One of our major challenges was identifying a practical method for measuring lean body mass in a clinical setting. Measurement of lean body mass by Dual Energy X-ray Absorptiometry (DEXA) is accurate but expensive, bulky, immobile and entails a small radiation dose. Our comparisons between methods of lean body mass measurement showed that anthropometry was a reasonable alternative to DEXA scanning (Gold Standard) but that bio-impedance produced an even more accurate result. We concluded that bioimpedance could be used as a valid alternate to DEXA in this population with the proviso that it as lean body mass falls it will tend to be underestimated by bioimpedance.

We found thalidomide to be well tolerated but to offer no clinical benefit overall. In fact, at the three month visit those in the thalidomide group had a significant greater reduction in measured grip strength and the functional aspect of their quality of life as measured by questionnaire. Neither change was sustained at the six month visit. There was no measurable change in lean body mass between groups. The average survival was slightly higher in the placebo group but this difference was not significant (mean survival thalidomide group 83 days, placebo group 88 days).

There was also a suggestion of some benefit of thalidomide therapy in those presenting with a more inflammatory disease as measured by plasma IL-6 and CRP. Sub-group analysis revealed that the thalidomide treatment group had a significantly longer survival over the placebo treated group if they presented with a higher than average IL-6 and a reduction in weight loss. Survival was significantly shorter with thalidomide treatment for those presenting with a lower than average IL-6. Thalidomide led to a significant suppression of plasma IL-6 levels over time. Unfortunately the survival advantage seen with thalidomide treatment in those with a more inflammatory state was not associated with any improvement in quality of life. The reduction in grip strength and functional quality of life was less marked in the thalidomide treated group but still present.

It may be that thalidomide treatment leads to an overall reduction of muscular strength through its known side effect of somnolence leading to inactivity but that in treating the inflammatory component of cachexia it is able to improve survival. We suggest that future clinical trials of cachexia include measurement of peripheral cytokine measurements which seem to be strongly associated with outcomes. There may be a different anti-inflammatory medication or combination of treatments that could successfully treat the cachexia without the same side effect profile of thalidomide. Clinical benefits may be dictated by the degree of inflammation present at presentation and patients may need to be stratified for future therapies.

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Abbreviations

ADL	Activities of Daily Living
AgRP	Agouti Related Peptide
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
CDP	Calcium dependent proteolytic pathway
CTCAE	National Cancer Institute Common Terminology Criteria for Adverse Events v3.0
CRF	Corticotrophin Releasing Factor
CRP	C Reactive Protein
CART	Cocaine and Amphetamine-Regulated Transcript
CSF	Cerebrospinal Fluid
COX	Cyclooxygenase
DEXA	Dual Energy X-ray Absorptiometry
ELISA	Enzyme-linked immunoabsorbent assay
EORTC QLQ-C30	European Organisation for Research and Treatment of Cancer validated Quality of Life Questionnaire
EPA	Eicosapentaenoic acid
FDA	US Food and Drug Administration
IDMC	Independent Data Monitoring Committee
IFN	Interferon
IGF	Insulin like Growth Factor
IκB	Inhibitory protein κB
IKK	Inducible IκB kinase
IL	Interleukin
MAFbx	Muscle Atrophy F-box
mRNA	messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
MURF	Muscle Ring Finger
MyHC	Myosin Heavy Chain

NFκB	Nuclear factor kappa B
NPY	Neuropeptide Y
LBM	Lean Body Mass
LIF	Leukaemia inhibiting factor
LMF	Lipid mobilising factor
POMC	Pro-opiomelanocortin
PIF	Proteolysis inducing factor
QOL	Quality of Life
REE	Resting Energy Expenditure
RLU	Relative Light Unit
SOP	Standard Operating Procedure
TACE	TNF-α converting enzyme
TNF-α	Tumour necrosis factor alpha
UCP	Uncoupling protein
UPP	Ubiquitin-proteosome proteolytic pathway

Chapter 1 Introduction

“_the shoulders, clavicles, chest and thighs melt away. This illness is fatal_”

—Hippocrates (460–370 BC)

1.1 Clinical aspects of cachexia

1.1.1 The syndrome

Patients with a variety of chronic inflammatory conditions experience a progressive involuntary weight loss that can be profound. Termed cachexia, this syndrome is characteristic of patients with acquired immunodeficiency syndrome (AIDs); tuberculosis; rheumatoid arthritis; cardiac and respiratory failure and is strongly associated with advanced cancers, in particular those of upper gastrointestinal origin.

Initially it was assumed that cancer cachexia may represent a host competition for nutrients by the tumour but doubt was shed on this theory by the recognition that many small tumours caused more intense cachexia than larger volume tumours(3). Loss of appetite is common in those with advanced cancers(4) but the weight loss of cachexia is not simply due to calorie deprivation. Attempts to prevent cachexia solely by improving appetite(5;6) or increasing calorie intake(7;8) have invariably failed. It has now been firmly established that it is an active inflammatory process, governed at least partly by the action of cytokines and tumour factors (see mechanisms section below).

Weight loss due to cachexia results in changes in body composition more similar to infection related weight loss than starvation(9). In starvation the major loss tends be from adipose tissue. Protein is relatively preserved and what is lost is divided equally between skeletal and visceral muscle(10). In cachexia weight is lost preferentially from the skeletal muscle and adipose compartments with relative sparing of visceral proteins(11-13). In fact, the liver often enlarges in cachexia due to production of acute phase proteins(14).

Table 1 Characteristic body composition changes in starvation vs cancer cachexia

	Starvation	Cachexia
Body fat	↓↓	↓↓
Skeletal muscle	↓	↓↓
Visceral muscle	↓	→/↑

Cachexia has been known to physicians for centuries as a ‘signum mali ominis’ (a sign of ill omen).

Of all malignancies it is most commonly seen in those with gastric or pancreatic cancer, with over 80% losing some weight and a third losing greater than 10% of their pre-morbid weight(2). Patients with pancreatic cancer have an average 14% weight loss at diagnosis and 24.5% when death is imminent (15).

1.1.2 Morbidity

Previous studies have suggested that 90% of terminally ill people would choose to die at home but are often deeply concerned about being a burden to caregivers(16). Muscle strength is proportional to muscle mass(17;18) and the weakness associated with cachexia is proportional to the muscle atrophy experienced. In gastrointestinal cancer patients, weight loss of 2.5kg over a 6 to 8 week period is enough to cause a significant deterioration in performance status(19) and of multiple clinical measurements McMillan and colleagues regressed against the Karnofsky performance status in advanced gastrointestinal cancer patients, only lean body mass was an independent predictor of performance score in both sexes(20). This muscular atrophy and the fatigue caused by cachexia lead to difficulty carrying out normal activities, often reducing independence and necessitating institutional end of life care. The associated loss of appetite can also be debilitating. People enjoy eating and the loss of the desire to eat can itself adversely affect quality of life(21). It can also be extremely distressing for the patient and those around them to witness profound weight loss, an all too obvious sign of deterioration.

1.1.3 Mortality

Those with cachexia are significantly less likely to have a good response to chemotherapy and more likely to suffer toxic side effects(2). The cachexia itself is also a major cause of mortality, contributing to death in up to one in five cases(22-24).The quantity of weight lost, and the rapidity with which it is lost, correlates inversely to survival, with death commonly occurring when the individual’s weight has dropped to 70% of previous levels(25). Patients with greater than 15% weight loss have significant impairment of respiratory muscle function, which probably contributes to the decreased survival time in those with the syndrome(2).

1.2 Reduced energy intake

Weight losing cancer patients have a calorie intake around 300kcal per day lower than weight-stable cancer patients(26). It is not uncommon for upper gastrointestinal cancer patients to have physical difficulty ingesting adequate calories: chemotherapy and radiotherapy induce nausea, vomiting and mucositis; tumour bulk and ascites can cause physical obstruction; and tumour invasion or intestinal resection can result in early satiety, shortened transit time, dumping syndromes, bacterial overgrowth and malabsorption.

In addition, anorexia is present in up to 40% of cancer patients at presentation(27) and 64% when terminally ill(28), with the resulting reduction in oral intake contributing to the energy deficit observed in cancer cachexia(29). In healthy humans weight loss is a potent stimulus for increased food intake and is governed by a network of feedback mechanisms. The absence of this response in cancer cachexia patients implies a dysregulation of the normal processes. The hypothalamus is the co-ordinating centre for energy balance. Here peripheral signals converge, bringing information concerning energy availability and requirements. This information is processed by neuropeptide pathways which stimulate or inhibit second-order neurons that convey the information to the periphery and govern eating behaviour.

1.2.1 Key mediators of eating behaviour

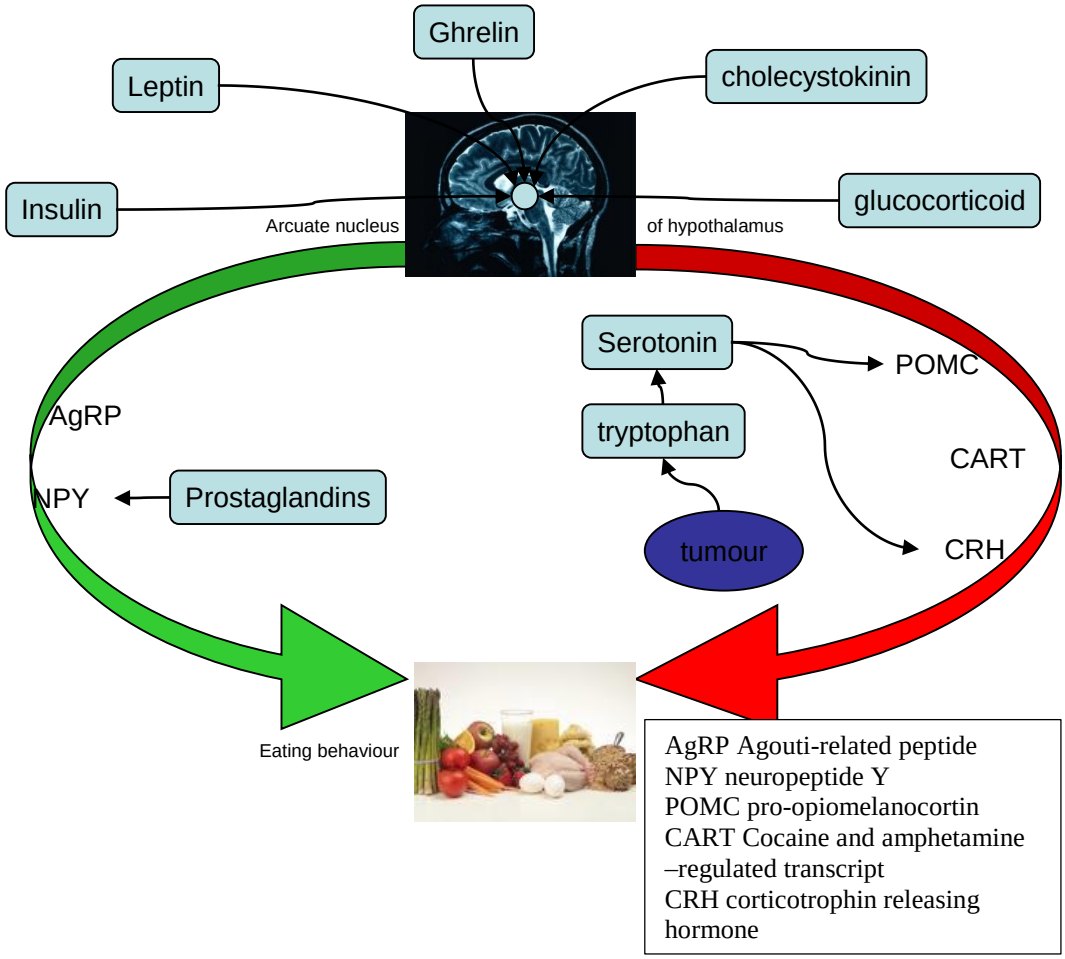


Figure 1 Summary of the key factors in the regulation of the desire to eat in health

1.2.1.1 Orexigenic factors

1.2.1.1.1 Neuropeptide Y

In health neuropeptide Y (NPY) is a powerful prophagic signal, repeated intra-hypothalamic injections causing a six-fold increase in body weight gain in healthy rats(30). It is synthesized in tandem with agouti-related peptide (AgRP, see 1.2.1.1.2) by specific hypothalamic neurons. These AgRP / NPY neurons are stimulated by glucocorticoids and ghrelin but inhibited by leptin and insulin(31). NPY is up-regulated at times of increased energy demand such as starvation, lactation or exercise(32). Anorectic tumour-bearing rats have decreased NPY immunostaining in their hypothalamic nuclei(33) and lack the normal mechanisms regulating NPY levels. If otherwise healthy rats have their food restricted in order that their body weight matches that of tumour bearing rats, then hypothalamic levels of NPY are

increased, stimulating appetite to recover the lost weight but in tumour bearing rats levels remain low(34).

Tumour bearing rats also lack the normal responses to hypothalamic NPY. Hypothalamic receptors(35) and downstream messengers of NPY(36) are abnormal in these animals and the increased food intake resulting from intra-hypothalamic NPY injections in healthy rats is actually reversed. This effect occurs prior to the onset of anorexia but becomes more pronounced in parallel with the progression of the anorexia(37). A human study found significantly lower levels of circulating NYP in cancer patients than in controls but levels did not correlate with the degree of anorexia(38).

1.2.1.1.2 Agouti-related peptide

AgRP is also a potent appetite stimulator. It is a competitive antagonist to alpha-melanocyte stimulating hormone (α-MSH) at melanocortin receptors MC3-R and MC4-R, thereby preventing their usual appetite suppressing effects (see 1.2.1.2)(39;40). Central injection of AgRP in murine models leads to substantial increases in food intake(41).

1.2.1.1.3 Ghrelin

Ghrelin is released by gastric cells when the stomach is empty, approximately doubling before each meal and returning to baseline after eating(42). Baseline serum levels are inversely proportional to body mass index(43). It stimulates receptors on centrally located orexigenic cells(44) causing release of NPY(45) and AgRP via nitric oxide(46). Human studies have confirmed that peripherally administered ghrelin results in increased food intake(47). It has multiple physiological effects relevant to the cachexia syndrome including promotion of gastric emptying, potentially reducing nausea(48); inhibition of pro-inflammatory and stimulation of anti-inflammatory cytokines(49-52) and stimulating endogenous growth hormone and IGF-1, thereby increasing anabolism(53-55). In rats with burn injury it attenuates the usual rise in muscular TNF-α and IL-6 and inhibits skeletal muscular breakdown(56).

Higher baseline levels of activated ghrelin are found in patients with cancer cachexia than either non-cancer controls or non-cachexic cancer patients(57). This is

appropriate for a weight losing state but does not seem to produce the desired peripheral effects, implying that cachexia is a state of ghrelin resistance.

1.2.1.2 Anorexigenic factors

1.2.1.2.1 Alpha-melanocyte stimulating hormone (α-MSH)

Alpha-MSH stimulates melanocortin receptors MC3-R and MC4-R in the hypothalamus resulting in appetite suppression. Blockade of its precursor, pro-opiomelanocortin (POCM), restores food intake in rat model cachexia but does not increase the intake of controls(58).

1.2.1.2.2 Leptin

Leptin is a hormone released from adipocytes(59), levels correlate with body mass index both in cancer cachexia and healthy subjects(60;61). It reduces NPY in the hypothalamus and binds competitively to the NPY receptor (Y-5)(62) producing a reduction in food intake(63). Inhibition of leptin or its signalling pathway leads to excessive eating and dramatic obesity in both mouse and humans(64-66). Generally a difference has not been demonstrated in circulating leptin levels between those with cancer cachexia and healthy subjects other than that expected due to the lower body fat of the cancer patients(61;67;68). The levels of circulating leptin transported across the blood brain barrier is known to vary in different health states (69) which could explain why combined results from human and animal studies have not been able to elicit clarity in the role played by leptin in cancer-related anorexia(70-72).

1.2.1.2.3 Corticotrophin Releasing Factor

CRF produces a multitude of critical physiological effects, mainly those of the stress response, including appetite inhibition(73).

1.2.1.2.4 Serotonin

Serotonin has been implicated in the process of cancer cachexia for decades(74). Hypothalamic serotonin induces production of corticotrophin-releasing factor (CRF) (75) and stimulates POMC neurons in the arcuate nucleus(76) so reducing appetite. Hypothalamic levels of serotonin are higher in tumour-bearing anorexic rats than in controls and food intake in these animals can be restored by hypothalamic injection of

a serotonin antagonist(77). Results from human studies are similar: levels of tryptophan (serotonin's precursor and a valid substitute marker(78)) are higher in both plasma and CSF in anorectic cancer patients than either healthy controls or those with cancer but no anorexia(79). Upon successful removal of the tumour, both rats and human with cancer cachexia normalise their central and peripheral serotonin/tryptophan levels and food intake is re-established(77;80). Fenfluramine is a serotonin agonist and was widely used as an anti-obesity medication between 1973 and 1997 when withdrawn due to links with cardiac abnormalities.

1.3 Increased energy expenditure

In simple calorie deprivation resting energy expenditure (REE) declines in an attempt to preserve energy(81), both in the total REE and in the per unit REE (which takes account of the weight already lost)(82). Patients with pancreatic cancers have consistently been found to have an increased REE (total and per unit) (83) which is significantly higher in those presenting with an elevated C-reactive protein level(83). Results in those with oesophageal and gastric cancers have generally shown an increased REE(84) but have been a little less consistent(85), perhaps complicated by a partial calorie deprivation in those unable to eat adequately for mechanical reasons. In one study, the REE was increased in the cancer group by 120kcal per day. The amount of weight lost was equivalent to a loss of 120,000kcal (3 years at 120kcal/day). In practise the weight loss in cachexia tends to occur over a period of weeks or months and some patients have been shown to experience weight loss despite a normal energy intake and normal REE(85;86). There must therefore be other factors involved but it seems likely that increased REE significantly contributes.

There is good evidence that the tumour itself is the ultimate cause of this increase in REE. If the tumour is successfully removed the hypermetabolic state is ameliorated but unsuccessful tumour resection aggravates the condition(82).

The mechanisms accounting for the imbalance of the appetite control(1.2.1) also affect the REE. NPY increases parasympathetic activity whereas POMC increases sympathetic activity, increasing REE. The normal compensatory mechanism for an increase in REE would be to increase energy intake but patients with cachexia

struggle to ingest more calories due to the difficulties discussed above. Voluntary energy losses are instead minimised by reductions in activity(87), manifest clinically as fatigue, apathy and depression(83). It has been suggested that if energy losses through REE were less then physical activity could be resumed(87).

1.3.1 Gluconeogenesis

The brain requires energy in the form of glucose or ketones (fatty acids cannot cross the blood brain barrier). At times of starvation glucose is made available by gluconeogenesis, producing glucose from a variety of sources, including amino acids. Although essential this is a costly process, both in terms of energy consumption (each glucose molecule produced uses six high energy phosphate bonds(88)) and the breakdown of skeletal muscle required to provide the amino acid substrate. In health this sacrifice of skeletal muscle is limited by a switch from gluconeogenesis to ketone production from fat but this does not occur in cachexia.

1.3.2 Cori cycle

It has long been known that tumour cells use the anaerobic glycolysis pathway to produce energy in contrast to healthy cell which use the aerobic tricarboxylic acid cycle, a phenomenon known as the ‘the Warburg effect’(89). Conditions within solid tumours become oxygen deficient when they are more than just a few cells in size but in fact this changed metabolism in tumour cells occurs even when they are supplied with plentiful oxygen(90). Pyruvate kinase is key in glycolysis and the recent discovery of an alternative form of this enzyme present in cancer cells suggests a probable mechanism for the effect. It may provide an advantage for the rapid growth required(91). Aerobic metabolism is roughly 40 times more efficient than anaerobic so this switch is one factor in the increased REE.

Glucose metabolism under these conditions leads to the production of large amounts of lactate which is then converted back to glucose by the liver and extra-hepatic tissues by gluconeogenesis in another energy inefficient process known as the Cori cycle(92). Activity of the Cori cycle increases in weight losing colorectal cancer patients(93) and contributes to energy wastage, it has been estimated up to 300kcal/day(94)

Figure 2 Cori Cycle

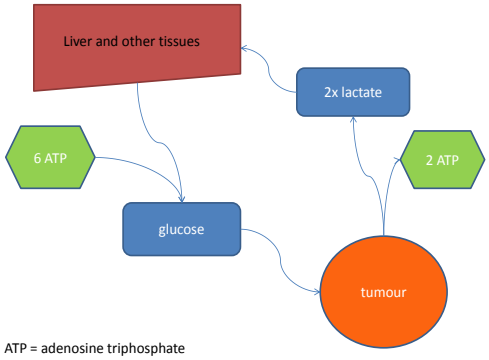


Figure 3 Simplified representation of the Cori cycle demonstrating energy wastage

1.3.3 Uncoupling proteins

Uncoupling proteins are mitochondrial proteins that separate oxidative phosphorylation from ATP production, instead releasing energy as heat. Uncoupling proteins types 2 and 3 are expressed in skeletal muscle and their mRNA is increased during tumour growth(95).

1.4 Structural changes

Cachexia is associated with a multitude of complex metabolic changes, many of which increase REE(96-100). Some of the key changes are summarised in the Figure 4.

Figure 4 Metabolic alterations in cachexia

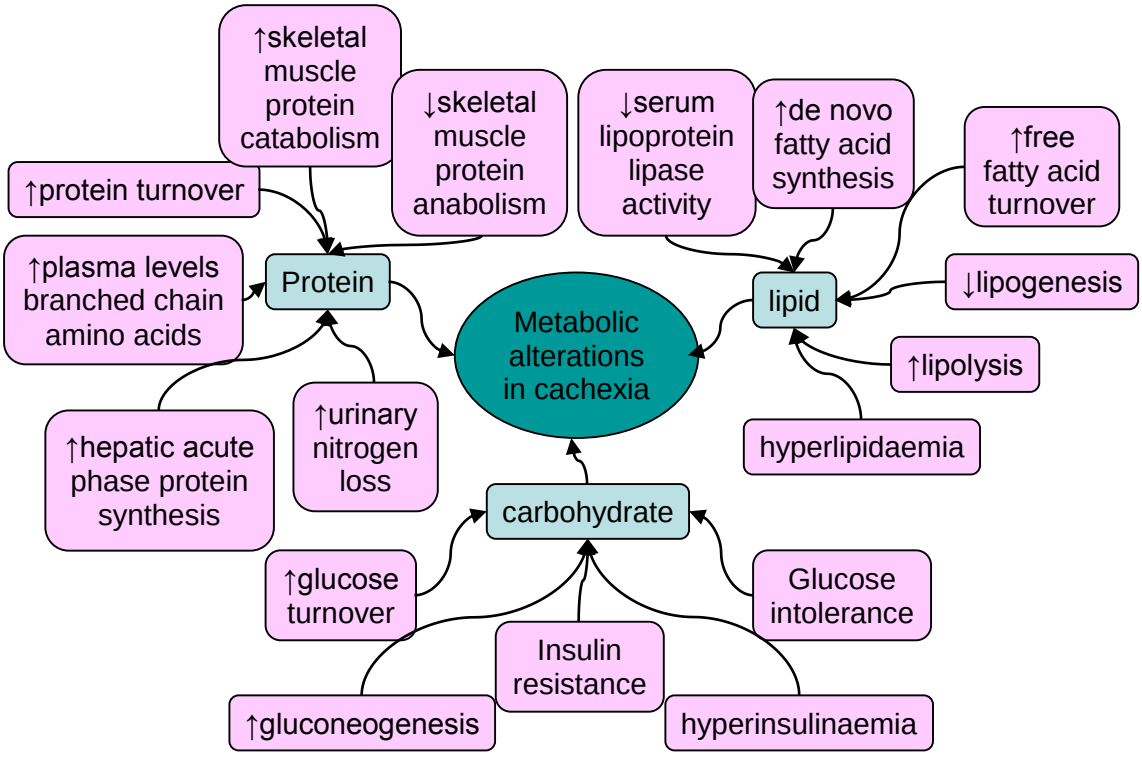


Figure 5 The main physiological changes seen in cancer cachexia subdivided by those affecting different bodily compartments

1.4.1 Loss of lean body mass

Muscle strength is proportional to muscle mass(101). It seems reasonable to assume that the morbidity and mortality associated with cachexia are due primarily to loss of lean body mass rather than body fat. Wasting of respiratory muscles in particular increases the risk of pneumonias(102) and respiratory failure which is so often the terminal event in end stage cancer patients. The muscle wasting associated with cachexia results from both a reduced rate of protein anabolism(103-105) and an increased rate of catabolism(105;106).

1.4.1.1 Reduced anabolism

Synthesis of lean body mass in cachexia may be limited by a lack of substrates as the required amino acids are diverted into increased synthesis of acute phase proteins and gluconeogenesis(107). There is also a vicious cycle of weakness leading to inactivity, which itself leads to reduced synthesis of skeletal muscle(108).

1.4.1.1.1 MyoD

MyoD is a nuclear transcription factor. Its binding to the myosin heavy-chain (MyHC) gene is vital for production of the MyHC(109) that constitutes about 40% of the myofibrillar protein content in normal muscle(110). It is vital to muscle regeneration after injury but is down-regulated in tumour bearing rats with weight loss(111).

1.4.1.1.2 Myostatin

Myostatin suppresses muscle growth by inhibiting myoblast proliferation(112-115). Murine models have demonstrated that systemic overexpression leads to cachexia-like muscular atrophy(116) whereas gene deletion or administration of anti-myostatin antibodies result in skeletal muscular hypertrophy and increased strength(117;118) It may have a role in cancer cachexia but this is so far unproven, possibly in part due to the technical difficulties of human myostatin assays(119).

1.4.1.1.2.1 Eukaryotic initiation factor 2 and Angiotensin II

Eukaryotic initiation factor 2 inhibits the initiation of protein translation(120), angiotensin II acts in a similar way(121) and is capable of inducing a 40-50% depression in protein synthesis in murine myotubes(121) in addition to stimulating muscle protein degradation. Angiotensin II also increases protein breakdown(108)

1.4.1.1.2.2 Insulin like growth factor (IGF)

IGFs are proteins structurally similar to insulin which have a wide range of effects including inducing protein synthesis and preventing activation muscle breakdown pathways, particularly that stimulated by angiotensin II(122;122;123;123-125).

1.4.1.2 Increased catabolism

1.4.1.2.1 Proteolytic pathways

Increased catabolism probably has a greater role than reduced anabolism in the muscle wasting of cancer cachexia(126). There are three major pathway of protein catabolism(126). Components of the first, the lysosomal system, are elevated in skeletal muscle biopsies from patients with lung cancer and minimal weight loss, probably playing a role in early protein breakdown. This pathway does not seem to be involved once the cachexia becomes established(127).

Subsequently muscle catabolism is processed by the other two proteolytic pathways. The calcium-dependent proteolytic pathway(128) destroys structural components of myofibrillar proteins, disassembling them into soluble actin and myosin components. A role for this pathway in cachexia is suggested by a demonstrable increase in soluble muscle filaments in skeletal muscle of rats with muscle atrophy induced by fasting, glucocorticoids or sepsis(129). Also, proteases involved in this pathway have been shown to be over-expressed in the atrophic skeletal muscles of diabetic rats and the appropriate protease inhibitors will attenuate the excessive muscle proteolysis(130). Despite this reasonable evidence for a role of this pathway in animal model, its contribution remains unclear in human cachexia.

1.4.1.2.2 The ubiquitin-proteasome pathway

Once solubilised by the calcium dependent pathway, actin and myosin are destroyed by the ubiquitin-proteasome pathway (UPP)(131), which accounts for up to 85% of protein degradation(126;132;133) in health. In addition to the destruction of these structural proteins, the UPP performs a vital function in removing short lived or abnormal proteins (e.g. damaged or degraded proteins produced by errors in gene transcription, mRNA translation or oxidative stressors(134;135)), thereby controlling cell cycles, signal transmission, immune response, tumour progression and apoptosis(126;136).

A key component of the UPP is a cylindrical protein-degrading complex named the proteasome (the 26S complex), present in cell nuclei and cytoplasm. Prior to entry to the proteasome, target proteins are marked for destruction by the ATP-dependent attachment of chains of a small regulatory protein named ubiquitin. This allows the proximal end of the proteasome (the 19s complex) to recognise the target protein, remove and recycle the ubiquitin, and move the substrate through to the chymotrypsin, trypsin and caspase containing proteasome core (20S complex). These enzymes hydrolyse the protein into oligopeptides (6-8 amino-acids) which are then consumed in other processes.

There is good evidence for this pathway being hyper-stimulated and causing increased destruction of muscular proteins in both animal and human studies of cachexia. Tumour-bearing animal models have demonstrated increased expression of elements

of the UPP (including ubiquitinated proteins) in skeletal muscle associated with an increase in muscle protein destruction compared with controls(137-140) and it has been shown that blockade of the UPP in incubated muscles from cachexic rats prevents their degradation(141;142). Muscle samples from people with gastrointestinal cancer cachexia show increased mRNA for specific proteasome subunits(143). Ubiquitin mRNA and proteasome activity also increases, positively correlating with disease stage and nutritional status (144;145).

A number of steps in this pathway have potential to be regulated in order to control muscular destruction. Binding of the ubiquitin to the target protein requires at least 3 sequential enzymic reactions termed E1, E2 and E3. E1 activates ubiquitin and passes it to the second enzyme, E2. E3 then binds between E2 and the substrate allowing ubiquitin to bind to the target protein. The initiation enzyme, E1, is a ubiquitous enzyme found throughout eukaryotic cells. Studies have shown that the baseline activity of this step is high, supplying ample active ubiquitin for the next stage(146) and that E1 supplementation does not increase ubiquitination(147). E1 is therefore unlikely to be a major control stage. Conversely, the conjugating E2 and E3 ligases exist in a number of different forms, each recognising different substrates. Higher regulation of these would allow fine control of the system(148) and in vitro supplementation of specific subclasses of E2 does up-regulate protein ubiquitination(147). Other subclasses of E2 have been shown to be induced at the mRNA level in rats with muscular atrophy induced by glucocorticoids(149) and in tumour-bearing rats(150). Specific E2 enzymes are suppressed by the anabolic insulin-like growth factor-1(149;151) and mRNA expression correlates with muscle proteolysis(151-153). E3 ligases are another likely target for regulation: specific E3 ligases are up-regulated in muscles atrophying due to sepsis(154), insulin deficiency(126) and fasting(155) and mice lacking the gene for other E3 enzymes show less muscular wasting in response to denervation(156). E3 ligases which have attracted particular attention since their identification in 2001 include muscle ring finger 1 (MuRF1) and muscle atrophy F-box (MAFbx)(157). Knockout mice for MuRF1 and MAFbx genes are partially resistant to denervation induced muscle loss(150;156-158)., also both ligases are up-regulated both in animal models(159) and human conditions associated with muscular atrophy(160). MAFbx controls the degradation of MyoD (see 1.4.1.1.1), thereby controlling muscular anabolism as well

as catabolism(161). Other studies of E2 and E3 enzymes have been less convincing and the overall picture remains unclear(148)

1.4.1.2.2.1 NFκB

NFκB is a nuclear transcription factor expressed in almost all eukaryotic cells which regulates gene expression, so controlling a wide range of processes including immune function, apoptosis and oncogenesis(162;163). In relation to cachexia in particular it controls the UPP and production of MyoD(164). It exists as a trimer in cytoplasm, bound to DNA binding proteins and an inhibitory protein I-κB(165). Inducible IκB kinases (IKKs) phosphorylate IκB so causing its destruction, leaving an active NFκB dimer capable of translocation to the nucleus.

It has been shown to increase in muscle atrophied from disuse and in vitro blockade inhibits protein loss in myotubules but the clearest evidence for its key role in cachexia was provided by an elegant animal model study by Cai(166). His group created genetically modified mice with hyper-activation of NFκB. These mice exhibit profound skeletal muscle atrophy similar to that seen in cachexia and have raised levels of ubiquitin E3 ligase suggesting the increased NFκB stimulates the UPP. Further evidence comes from the complete reversibility of atrophy in these mice with administration of a pharmacological proteasome inhibitor. They also demonstrated that by genetically adding a dominant inhibitory form of IκB alpha, thereby preventing the activation of increased NFκB, the muscle atrophy did not occur. Mice genetically modified with either inactive NFκB or with deletion of an ubiquitin ligase are both resistant to tumour induced cachexia. More recent animal studies have suggested that blockade of NFκB attenuates about half of cancer associated muscle atrophy(167). In health the action of NFκB in muscle provides amino acids for essential energy sources for other tissues. In cachexia it appears to have become uncontrolled.

Figure 6 Summary of the ubiquitin-proteasome proteolytic pathway

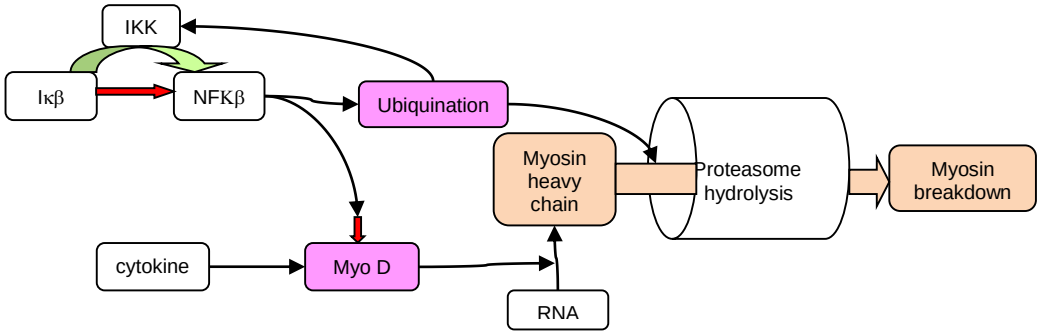


Figure 7 Represention of the breakdown of myosin heavy chains by the ubiquitin-proteosome pathway via ubiquination and then proteosomic breakdown

Figure 8 Three main aspects of cachexia

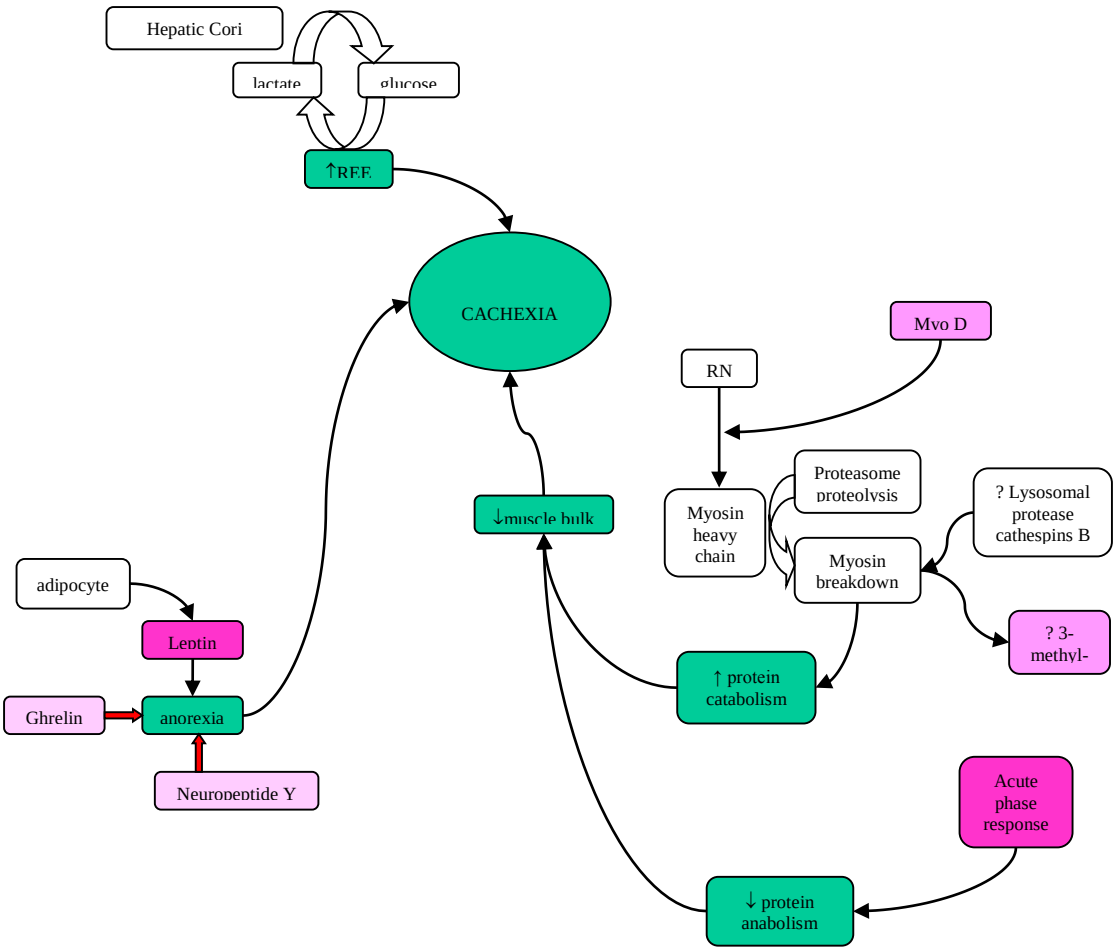


Figure 9 Cachexia is complex, involving loss of muscle mass, energy wastage and reduced appetite

1.5 Biochemical orchestration of cachexia

Three key findings conclusively demonstrate the presence of circulating tumour derived mediators driving cachexia: non-viable preparations of fat depleting mouse tumours transplanted into healthy mice induce fat depletion(168); in parabiotic rats, those paired with tumour bearing mates become cachexic in absence of metastases(169) and serum from tumour-bearing mice injected into healthy mice produces an immediate massive fat mobilisation that does not respond to feeding(170). It has now been firmly established that the cachexia is a para-neoplastic phenomenon caused by substances released either from the tumour or from other tissues as a response to the tumour, rather than from competition for resources from the tumour itself.

Solid tumours are complex tissues, comprising of a variable mixture of primary tumour cells; tissue matrix cells such as fibroblasts and endothelial cells(171); and a variety of immune cells such as M2 macrophages, dentritic cells and T-regulatory leucocytes(172). Together, these cells produce a variety of tumour-derived mediators (proteins released from the tumour cells) and cytokines which promote the cellular proliferation, tissue remodelling and angiogenesis necessary for cancer development and progression(172;173) but also have far reaching consequences in the host. The exact mechanisms underlying these physiological changes are complex and remain incompletely understood.

1.5.1 Pro-cachectic cytokines

Cytokines are small, soluble communicating protein molecules present in every tissue. They are produced mainly from immune cells and regulate a multiple of biological responses. They form complex networks, with each cytokine influencing the production of others. There is substantial overlap in their effects and individual cytokines may have different actions in different environments(171). The tumour microenvironment is awash with cytokines produced both by the tumour cells and by the infiltrating cell populations(174). They are also released from the periphery, including the brain, as part of the host's response to the tumour. Cytokines commonly implicated in the cachexic process include tumour necrosis factor alpha (TNF-α)(175;176), interleukin 6 (IL-6)(177;178), interleukin 1 beta (IL-1β), and interferon

gamma (IFNγ)(179-181). Messenger ribonucleic acid (mRNA) encoding receptors for all of these cytokines can be found in skeletal muscle(182) and they are often raised in the serum of people with a variety of cancers, particularly when associated with cachexia(183). Chronic administration of these cytokines in animal models leads to anorexia, increased protein degradation, decreased protein synthesis and amino acid uptake, resulting in a clinical syndrome closely resembling cachexia. In some cases though, administration of more than one cytokine in tandem is necessary to produce the effects(184).

Research into cytokine effects is complicated by the regulatory effects of one cytokine on another. For example, administration of IL-1 and TNF-α leads to in vivo production of IL-6. It is therefore a challenge to elucidate whether the effect of an individual cytokine is direct or indirectly mediated by the release of others. Animal models of cachexia allow more precise experimental manipulation but these models are artificial and each varies, with different cytokines and proteolytic pathways taking prominence and showing different responses to the same cytokine stimulants or suppressants(175;176;185-190). It is also probable that human cancer cachexia not only differs in its mechanisms from the animal models, but also that different people and different cancers produce different catabolic phenotypes, engaging individual cytokine cascades.

1.5.1.1 Cytokines and anorexia

The anorexia associated with cancer cachexia seems to be governed largely by cytokines(191). Inflammatory cytokines are known to have cerebral receptors, mainly centred in the hypothalamus(192). They can produce central effects by a variety of mechanisms: they can be produced within the brain(193-195); they can travel across the blood brain barrier (BBB) into the brain from the periphery(69;192); they can bind onto the peripheral side of the endothelial cells forming the BBB, stimulating them to release anorexic substances on the central side and they can disrupt the BBB, allowing increased passage of other substances including immune cells(69). All of these systems could be potential methods of regulation, for example a significant proportion of a peripherally administered dose of these cytokines will be transported into the brain across the BBB via selective, saturable systems (69;192) but the proportion transported will be altered in a variety of disease states(69). Studies of chronic

inflammation, including those in non-intestinal cancer cachexia, consistently find an inverse relationship between the leptin level and levels of pro-inflammatory cytokines(72;196;197). Il-6 in particular is similar structurally and functionally to leptin(198) and it has been postulated these cytokines could be reducing appetite by mimicking the anorexic effects of leptin (see 1.2.1.2.2) in the hypothalamus(192;199). In animal models they also increase hypothalamic serotonin activity and CRF levels(200), so stimulating the anorexigenic POMC pathway.

1.5.1.2 Cytokines and the UPP

The same cytokines have the ability to activate NFκB by causing destruction of the IκB inhibitor protein so stimulating muscular breakdown via the UPP. Often the activated NFKB will in turn stimulate production of further pro-inflammatory cytokines creating a positive feedback loop(166).

1.5.2 Tumour necrosis factor alpha (TNF-α)

Figure 10 Flowchart summarising known actions of TNF-alpha in cachexia

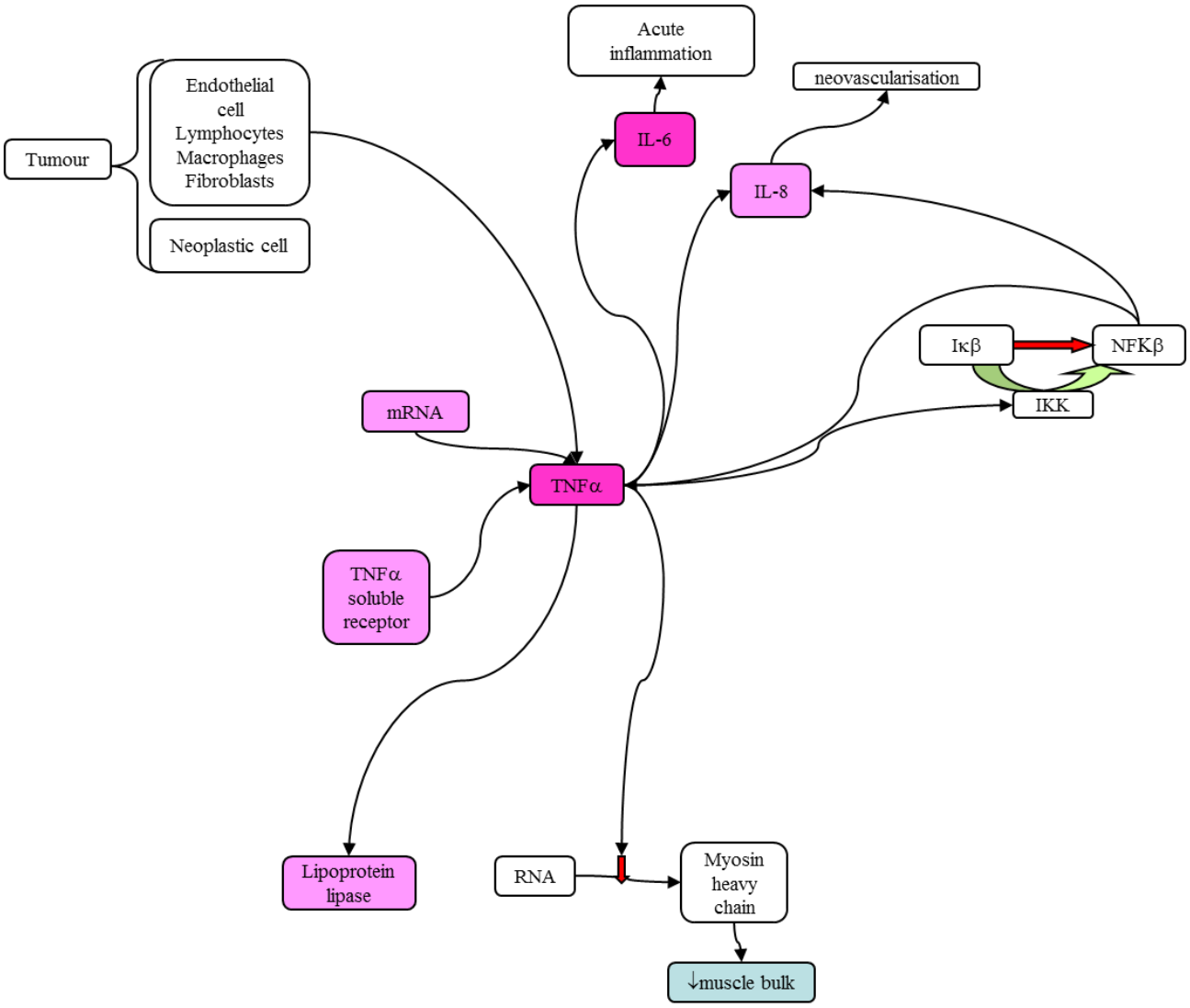


Figure 11 TNF alpha is a direct mediator of many of the process involved in cachexia and affects local and peripheral levels of other cytokines which are mediators in turn.

In 1975 Carswell et al discovered a serum factor capable of inducing haemorrhagic necrosis of tumours and named it Tumour Necrosis Factor alpha (TNF-α)(201). Beutler et.al. separately isolated 'cachectin' secreted by macrophages which induced a hypertriglyceridaemic state through suppression of lipoprotein lipase(202). It was subsequently shown that cachectin and TNF-α were in fact to be the same factor and that administration of this molecule to mice induced anorexia, weight loss and the depletion of whole-body protein. More recently there has been plentiful evidence implicating it as having a role in the syndrome of cancer cachexia but results have been varied and the detail is often conflicting. It is mainly produced by macrophages, T cells and natural killer (NK) cells in response to bacterial toxins, inflammatory products and other invasive stimuli but also, at lower concentrations, by tumour

cells(203). TNF-α binds to one of two receptors, either TNF receptor-1 (TNFR-1, p55 receptor) or TNF receptor-2 (TNFR-2, p75 receptor) which are expressed on virtually all nucleated cells(204) and have distinct but overlapping functions. Binding to either of these receptors releases active NF-κB by stimulating IKK(205;206). It also induces production of other inflammatory cytokines, including IL-1, IL-6 and IFN-γ from macrophages and NK cells(207). TNF-α can be pro- or anti-oncotic, varying according to the situation and the dose of the cytokine. Synthesis of low levels of TNF-α within the tumour itself enhances tumour growth through increasing angiogenesis via IL-8 release(208;209) and by facilitating invasion and migration of tumour cells(210;211). TNF-α deficient mice have been shown to be resistant to skin cancers(212). Conversely, higher TNF-α levels lead to tumour cell death and vascular collapse(213) and TNF-α deficient mice are unable to reject a synthetic fibrosarcoma until TNF-α is replaced(214).

Administration of recombinant TNF-α has been shown to produce a syndrome closely resembling cachexia both clinically and physiologically in mice(215), rats(163;216;217), and humans(218). It has also been demonstrated in mice that transplantation of TNF-α secreting tumours produces a similar syndrome and that anti-TNF-α antibodies attenuate tumour induced cachexia(215;219;220). Artificially inhibiting TNF-α production also attenuates cachexia in a mouse cancer cachexia model (221). In human studies elevated serum TNF-α levels have been found in prostatic(215), lymphoma(222), oat cell cancer(222), ovarian cancer(222), breast(215), lung(223) pancreas(224). It has also been shown that the increase in TNF-α mRNA transcripts seen in pancreatic cancer patients normalises after resection of the tumour(225). In many studies, for example in pancreatic(224;226;226) and prostate cancer(215), TNF-α levels correlate with clinical outcomes such as lower body mass index, weight loss, lower haemoglobin, lower albumin, more advanced disease stage and shorter survival times. Results have not been entirely consistent and have not always correlated with degree of cachexia or tumour stage(227;228). This could be partly due to difficulty in measuring the protein because of its short half-life and at its lability in sub-optimal storage conditions(222;225). Levels may also be affected by a number of gene polymorphisms for TNF-α which affect constitutive and inducible level of TNF-α and susceptibility to, severity and mortality rates of a

number of infective (229-231), and immune diseases(232;233) and associated with prolonged ventilation after surgery(234), These polymorphisms were not found to correlate with serum levels of TNF-α or have any clinical relevance in pancreatic cancer patients(224).

1.5.2.1 TNFR1 and TNFR2

The TNF-α receptors are key in mediation of cachexia. Knockout mice lacking the TNFR1 gene do not develop the cachexia in response to tumour implantation seen in wild type mice(235). The active receptors are membrane bound but the extra-cellular domain is cleaved by TNF-α converting enzyme (TACE) into soluble, circulating receptor fragments (sTNFRs). These behave as natural TNF-α inhibitors, binding and sequestering it, so preventing it binding to their active, membrane bound equivalents(236). In mice with TNF-α secreting tumours, infusion of sTNF receptors allow them to grow and reproduce normally(215). There are a variety of polymorphisms of these receptors. Different variants show different strength of action and circulating levels of the soluble receptors (236). They do seem to have clinical relevance with sTNFR levels and TNFR2 gene polymorphisms being associated with heart failure, obesity & insulin resistance(236). Presence of biologically active TNF-α is reflected by higher sTNFR levels but they are more stable over time and are used in many studies as a substitute marker for TNF-α(237). Similarly to TNF-α, sTNFR levels have been shown to be elevated in weight losing cancer patients(238)

1.5.2.2 TNF-α in altered energy balance

TNF-α administration leads to a reduction in food intake(191;191;239). The exact mechanism for this remains elusive but it can be replicated by intracerebroventricular infusion so presumably is centrally mediated(240). It has also been shown that TNF-α increases expression of genes encoding both for leptin(72) (resulting in anorexia, see 1.2.1.2.2) and uncoupling proteins 2 and 3(95;241) (resulting in energy transfer into heat production rather than anabolism, see 1.3.3). TNF-α induced anorexia can be blocked by cyclooxygenase inhibitors (see 1.6.3.4), suggesting a prostaglandin based mechanism(242).

1.5.2.3 TNF-α in altered protein metabolism

Skeletal muscle of weight losing patients shows increased expression of TNF-α and TNFRs(243;244), resulting in both reduced protein synthesis and increased protein breakdown. TNF-α inhibits synthesis of myosin heavy chains both in vitro and in vivo, it also blocks the anabolic effects of IGF-1(245) and growth hormone(246). In cardiac cachexia, IGF-1 mRNA levels negatively correlate with TNF-α levels in skeletal muscle(247). Also, in vitro, TNF-α acts synergistically with IFN-γ to inhibit activation of mRNA for MyHC(119;132) and in animal models TNF-α administration leads to decreased MyHC mRNA(248), mediated by a reduction of MyoD mRNA(249;249)(see 1.4.1.1.1).

A role for TNF-α in increased protein breakdown is convincing and seems to be mediated mainly via the UPP(138) at a number of different stages in the pathway. TNF-α activates NF-κB through degradation of IκB(249-253) leading to a loss of myosin which can be blocked by over-expression of IκB(250). TNF-α also increases ubiquitin gene expression and ubiquitin in skeletal muscle(254-257), TNF-α antibodies reduce ubiquitin gene expression in skeletal muscle(258-260) and prevent the associated increase in protein catabolism(139). Administration of TNF-α also leads to induction of specific E2 and E3 ligases both in vitro and in the resultant atrophying skeletal muscle in vivo(139;261;262).

Again results from different studies have been inconsistent. Human studies are difficult to carry out and animal models are by their nature artificial. In some animal models anti-TNF-α antibodies are entirely ineffective in attenuating cachexia but other anti-cytokine antibodies (e.g. anti-IL6(263) or anti IFNγ(264)) work well.

1.5.3 Interleukin 6 (IL-6)

Il-6 is produced as part of the acute phase response in the liver and levels often mirrors C reactive protein (CRP)(265). It is also produced by most cell types at a local level in response a number of inflammatory and infective stimuli including TNF-α(266;267). In mice, subcutaneous tumour implantation causes splenic IL-6 levels to increase a few days before the onset of cachexia(267) and monocytes from pancreatic cancer patients with cachexia (but not those from patients without cachexia) stimulate

production of unusually high levels of IL-6 from cancer cell lines in vitro(227;268;269). Reports are fairly consistent that Il-6 is elevated in cancer patients locally at the tumour site(270), in the serum(223;238;265;271;272) and in skeletal muscle(244), often in tandem with elevated TNF-α(268). Levels generally correlate with tumour stage, degree of cachexia, nutritional markers, increased resting energy expenditure and survival(238;265;268).

In vitro, IL-6 causes destruction of myotubules(273) and artificially elevating circulating Il-6 levels in animal models to above physiological levels leads to atrophy of previously healthy muscle(274-279). Mice genetically engineered to have high levels of IL-6 show muscle atrophy which is reversible by IL-6 receptor antibody(280); rats treated with IL-6 undergo increased muscle breakdown(275) and microinfusion of IL-6 directly into a rat leg muscle leads to 9% reduction in protein with a 17% reduction in myofibrillar content compared to the contralateral muscle after a fortnight(278;279). Whether these effects occur with chronic low dose administration more akin to the levels seen in cancer cachexia is less certain.

The cachexia produced in one of the major animal models, the murine Colon-26 adenocarcinoma, is IL-6 dependent and is preventable by IL-6 receptor antagonists or anti-IL-6 monoclonal antibodies(187;187;190;263). If the tumour is resected then IL-6 levels reduce and cachexia is reversed(177). Interestingly the clones of colon-26 that do not produce cachexia also do not produce IL-6(281). In this model anti-TNF-α therapies have no beneficial effect(263). Anti-IL-6 antibodies are also protective against cachexia produced by inoculating mice with human prostatic and melanoma tumours(190). The $APC^{min/+}$ mouse model has a mutated adenomatous polyposis coli gene and develops dysplastic colonic polyps by 4 weeks of age. By 6 months these mice have a 45% reduction in gastrocnemius muscle mass compared with wild type(282). In this model a higher IL-6 serum level correlates with a higher degree of cachexia. The cachexia is prevented by genetic deletion of IL-6 but reinstated by systemic IL-6 replacement(283). In this model Il-6 mRNA is not elevated in muscle, suggesting a distant source of the cytokine(284). Similar to TNF-α there are polymorphisms of IL-6, some of which have been found to have clinical significance in a variety of situations(285-288), including general longevity(289) and susceptibility

to breast cancer(290). Early data are suggesting a relevance of some polymorphisms to susceptibility to the development of cancer cachexia(291).

1.5.3.1 IL-6 in altered energy balance

It is long established that IL-6 causes anorexia. Il-6 is closely related to leptin(267) and it has been postulated that it mimicks its hypothalamic effects(292). This may account for the lack of compensatory increased intake usually seen in response to weight loss but is unlikely to be the primary cause of the cachexia. Interestingly, IL-6 knockout mice have increased susceptibility to intracellular infections, impaired wound healing and defective inflammatory responses but do not become obese in the way that leptin deficient animals do. Il-6 does not obviously account for the asthenia often associated with cachexia. Administration of supraphysiological doses of IL-6 to rats, adequate to cause muscle atrophy, have no effect on physical activity(276). In an animal model, IL-6 and TNF-α induced anorexia is prevented by cyclooxygenase inhibitors, suggesting that a prostaglandin such as PGE_2 may be involved in the pathway (242).

1.5.3.2 IL-6 in altered protein metabolism

Administration of systemic IL-6 to healthy human volunteers results in a 50% reduction in muscle turnover acutely, mainly due to a reduction in protein synthesis(293). Effects of chronic administration are not known. Mechanisms of action of IL-6 in muscle atrophy are not as clearly defined as those for TNF-α. Any effect seems to be indirect or synergistic as incubation of muscle in vitro with pure IL-6 does not cause proteolysis (294). In a mouse model, levels of atrogin-1, an E3 ligase, rise with both cachexia and with artificially increased IL-6(284), suggesting involvement of the UPP.

1.5.4 Interleukin 1 beta (IL-1β)

IL-1β is part of the IL-1 superfamily and is closely related to IL-1α with overlapping pro-inflammatory effects. Both cytokines produce their effects through the same IL-1 receptor. Blocking this receptor in tumour bearing mice attenuates loss of body fat and lean tissue and reduces systemic IL-6 levels(219;295). IL-1β is necessary for murine tumour growth. Implantation of melanoma, prostate or mammary cancer cells into IL-1β knockout mice does not develop into either the local tumour or lung

metastases seen in wild type mice. This is seems to be due to an absence of angiogenesis which is restored by addition of IL-1β and replicated by addition of an antagonist to the IL1 receptor in wild type mice(296). Evidence for IL-1β having a key role in angiogenesis is increasing(297;298). It has also been shown that certain IL-1 gene polymorphisms significantly affect survival in pancreatic cancer patients(299).

1.5.4.1 IL-1 receptor antagonist

IL-1RA is a naturally occurring antagonist to IL-1 which competitively binds to the IL-1 receptor(300). It behaves as an inhibitor to IL-1 mediated inflammation. Nine infants have been reported who were born with genetically defective IL-1ra. They all had widespread inflammatory consequences, including septic-like organ failure but with sterile cultures. IL-1ra is now commercially available as anakinra (Kineret®) and was effective and life-saving in these infants(301;302). Anakinra is now used therapeutically to control inflammation in rheumatoid arthritis patients. There are known polymorphisms of the IL-1ra which have been shown to affect survival times in human colorectal cancer(300)

1.5.4.2 IL-1β in altered energy balance

Similar to IL-6, IL-1β has long been established as a cause of anorexia(303;304). The effect seems to be mediated centrally(240), cerebral IL-1 levels in tumour bearing rats negatively correlate with food intake(239;305). Recombinant human IL-1α and IL-1β, and murine IL-1α administered systemically to rats causes anorexia(216;217) and hypothalamic IL-1ra injections causes an increase in food intake(306). The exact mechanism is unclear but anorexia in rats induced by IL-1β administration can be abolished by pre-treatment with either ibuprofen or fish oil, again suggesting a prostaglandin based mechanism(242). Intracerebral infusion of IL-1β leads to an increase in hypothalamic IL-1β and IL-1ra mRNA but also a reduction in hypothalamic NPY mRNA and increase in serotonin, both possible mechanisms of appetite suppression(307). Lastly there are complex interactions between IL-1 and leptin. Injection of leptin to rats both peripherally and centrally induces an increase in hypothalamic IL-1β and an anorexia which is preventable with co-administration of either IL-1ra or a cyclooxygenase inhibitor, suggesting leptin, IL-1β and prostaglandin are factors in the same pathway(308). Equally administration of IL-1 to

mice, hamsters and to healthy human volunteers causes an increase in serum leptin, although this response wears off over time(309)

1.5.4.3 IL-1 in altered protein metabolism

The bulk of evidence for IL-1β in cachexia relates to a role in anorexia rather than structural changes but there is evidence that it indirectly influences levels of other mediators such as IGF-1 which in turn affect muscle turnover(247).

1.5.5 Interferon gamma (IFN-γ)

IFN-γ is produced by activated T cells and natural killer cells. Nude mice inoculated with IFN-γ producing cells develop cachexia and IFN-γ antibodies reverse cachexia in murine and rat models(264;310).

1.5.6 Vascular endothelial growth factor (VEGF)

VEGF induces the angiogenesis vital for tumour growth(311-313). Several studies have shown that serum VEGF levels rise in a variety of cancers, correlate with the stage of disease and normalise after successful tumour resection(314-316). More recently it has become clear that VEGF has a larger role in cancer development than solely in controlling angiogenesis. Importantly, it dampens the response of immune cells in the tumour microenvironment, partly through recruitment of T regulatory cells, permissing tumour growth(317) and sustains the self-renewal of cancer stems(318). TNF-α stimulates VEGF production via NFκB(319).

1.5.7 Proteolysis inducing factor (PIF)

PIF is a glycoprotein produced by tumour cells which was first isolated relatively recently by Tisdale's group from a cachexia inducing murine MAC 16 adenocarcinoma model(320). Again this factor seems to be specific to certain animal models and is of no consequence in the widely used Yoshida ascites hepatoma model(321) but it does seem to be important in human studies. In patients with gastrointestinal cancers, PIF is produced specifically by those tumours associated with weight loss(322) and is detectable in the urine only at times of weight loss(323). Also the presence of PIF in urine of pancreatic cancer patients correlates with the amount of weight lost(324) and when the substance was isolated from human urine it is

capable of inducing cachexia in mice (an effect that can be prevented by prior administration of the mouse monoclonal antibody)(325).

1.5.7.1 PIF in altered energy balance

PIF does not have known direct effects on energy balance but it may produce indirect effects through induction of other cytokines, IL-6 in particular(326;327).

1.5.7.2 PIF in altered protein metabolism

Injection of PIF into healthy mice causes an increase in gastrocnemius ubiquitin mRNA and a variety of proteasome subunits in association with muscular atrophy(328) In vitro, PIF induces NF-κB via breakdown of IκB, increased activity of an E2 ligase and increased proteolytic activity of the proteasome(328-332). The increased NF-κB also leads to increased TNF-α and IL-6 from Kupffer cells and monocytes(333). The proteolysis mediated by PIF in cachexia is specific to skeletal muscle, not affecting cardiac or renal muscle in the way that other cytokines such as TNF-α do(334;335).

1.5.7.3 PIF controversy

There are conflicting results in studies of every cytokine and tumour factor but the role of PIF in cachexia is questioned even more than others reviewed here, possibly due to its novelty. Despite the convincing evidence in the studies reviewed above others have found less positive results. It has been shown to be unrelated to weight loss, anorexia, survival or even the presence of cancer by several groups(336-341) although some of these negative studies assessed a non-active form of the substance(342).

Figure 12 Summary of cytokine interactions

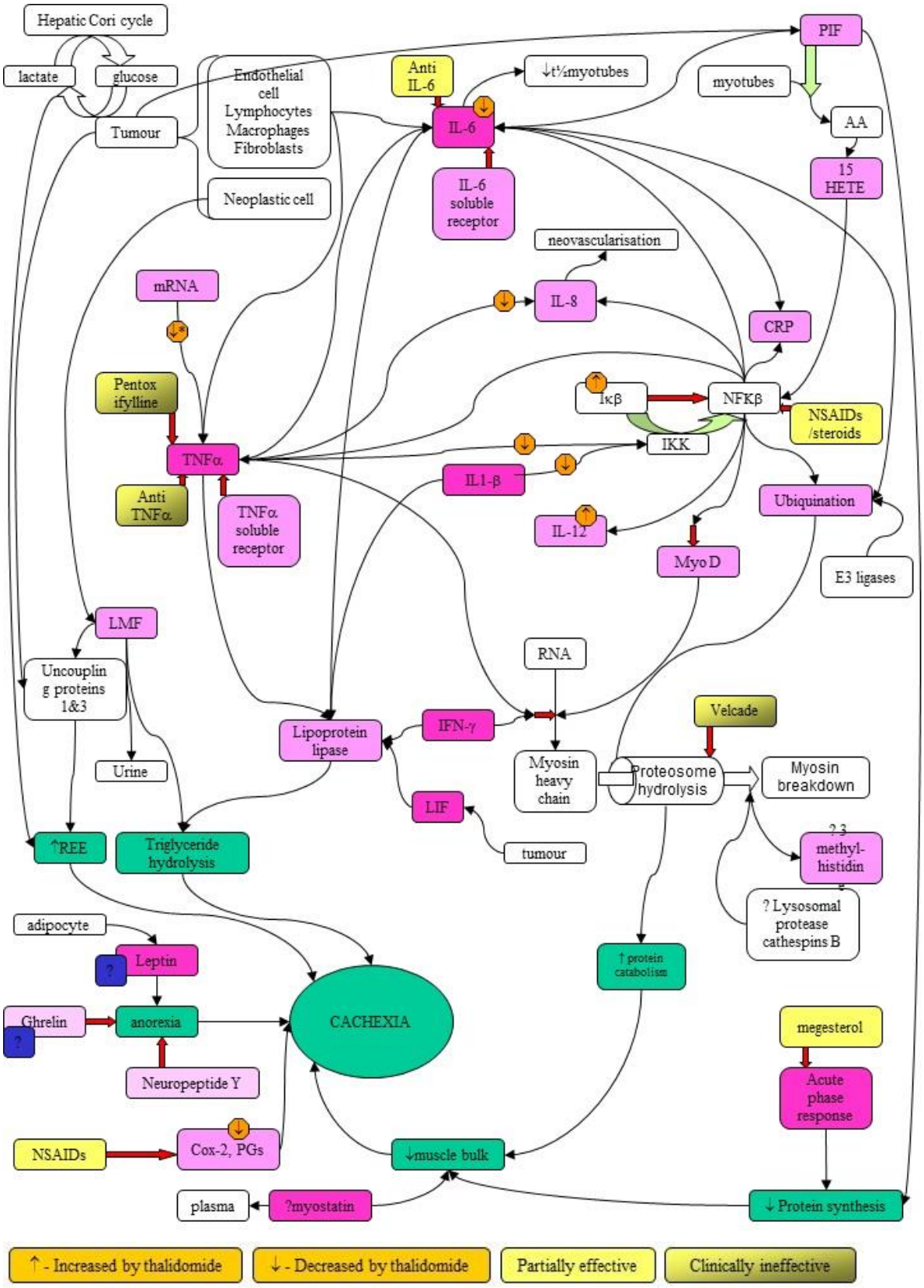


Figure 13 summary of the interactions of the cytokine network leading to the syndrome of cachexia including those parts of the process targeted by therapeutic agents including thalidomide

1.6 Potential therapies

1.6.1 Increased calorie intake

Various methods have been investigated

1.6.1.1 High calorie diets

1.6.1.1.1 Dietary counselling

Attempts to prevent cachexia through dietary counselling have been invariably unsuccessful(8)

1.6.1.1.2 Parenteral and enteral feeding

Artificial nutrition by the enteral route leads to weight gain, decreased inpatient stays and improved mortality in malnourished older patients(343). Its role in cancer and cachexia is less clear. It can produce weight gain but this is mainly fat rather than useful lean body mass(344) and generally does not translate into benefits in morbidity or mortality(344-351) although one RCT in 300 patients with solid tumours and cachexia did suggest improved survival and exercise capacity if parenteral nutrition in combination with cyclooxygenase and erythropoietin was instigated when voluntary intake fell below 70% of recommended (30kcal/kg/day). Although forced calorie increase does not seem to be beneficial, it maybe that specific nutrients contained in parental nutrition therapy such as arginine, glutamine and fatty acids have benefit. Concerningly, other data suggest that these nutrients may prevent apoptosis and stimulate tumour growth(352)

1.6.1.2 Orexigenic agents (appetite stimulants)

1.6.1.2.1 Corticosteroids and progestagens

Corticosteroids and progestagens (Medroxyprogesterone Acetate and Megesterol Acetate) are well known to produce increases in appetite and body fat (but not lean body mass) both in health and in cancer cachexia(353-356;356-360). Mechanisms remain incompletely understood but there is up-regulation of NPY and down-regulation of serotonin and anti-inflammatory cytokines such as TNF and Il-1(361-364). They can cause a short term feeling of well-being but are equally likely to cause

aggression or confusion. They do not produce any long term clinical benefits(357;365-369) and have troublesome medium to long-term consequences including insulin resistance(370) and muscle atrophy, probably via up-regulation of the UPP(371;372).

1.6.1.2.2 Cannabinoids

Dronabinol, a synthetic form of the active ingredient in marijuana, stimulates appetite in advanced HIV disease(373;374), working through cytokines or direct stimulation of endocannabinoid receptors(375-377). Unfortunately it does not reliably increase appetite in cancer patients(184), has no proven clinical benefit(370) (378;379;379) and has a number of negative effects such as loss of concentration and coordination, fluid retention and impotence(365). There is no additional benefit in using steroids and cannabinoids in combination(378).

1.6.1.2.3 Serotonin antagonists

Cyproheptadine may be beneficial in cachexia related to carcinoid(380) but has not proven helpful in other cancers(370;381). Ondansetron is helpful for nausea but does not lead to weight gain(382).

1.6.2 Anabolic agents

1.6.2.1.1 Androgens

Androgens are a promising treatment with exciting recent trial results. They are not particularly effective as appetite stimulants(359) but they are able to increase net protein synthesis and increase lean body mass at the expense of body fat(383)

Addition of nandrolone deconate to chemotherapy in non-small cell lung cancer led to attenuation of weight loss(384) in a prospective randomised trial. Oxandrolone in combination with dietary advice and exercise led to an average 4lb increase in lean body mass and increase in functional ability (average Eastern Cooperative Oncology Group performance score dropped from two to one) over 4 months in a 131 patient open label trial(385). In a prospective randomised phase 3 trial comparing oxandrolone to megesterone acetate on 155 patients with solid tumours and cachexia receiving chemotherapy, oxandrolone treatment was associated with increased LBM

and reduction in body fat whereas megesterone acetate was associated with increase in appetite, weight and body fat but not LBM. It was suggested that a combination of these agents could be beneficial(386). Similarly positive results have also been shown in muscle wasting in the otherwise healthy elderly(387), HIV disease(388) and chronic obstructive airways disease(389) but there are real concerns over unwanted peripheral effects.

Enobosarm is a first in class non-steroidal selective androgen receptor modulator. It acts on androgen receptors in the skeletal muscle with a more limited effect on those in the liver, skin and prostate with the intention of limiting side effects. A recent phase 2 trial with randomised 159 patients with cancer cachexia (varied primaries) to enobosarm or placebo. Those in the enobosarm group showed a relative gain in LBM (by DEXA) and improved physical function with few side effects(390).

1.6.2.1.2 Growth Hormone

Growth hormone may increase skeletal muscle mass at the expense of tumour growth in animals(391) but increases mortality in critically ill human studies, possibly due to a blunting of the acute phase response through diversion of amino acids substrates to muscle synthesis(392). It may be that using ghrelin to cause the release of endogenous growth hormone is a safer alternative(see 1.6.2.1.3)(392;393)

1.6.2.1.3 Ghrelin

Both animal model studies(394) and human trials of ghrelin in cachexia have been positive(54;393;395). It increases food intake and grip strength in cardiac cachexia patients(396). In cancer cachexia patients offered a buffet lunch, administration of ghrelin led to a 31% increased intake and a 23% increase in the perceived pleasantness of the meal compared with administration of a placebo(54). Ghrelin requires parenteral administration and is expensive. There as yet unproven and conflicting links between ghrelin and mitosis(397-403).

1.6.3 Anti-cytokine treatments

The background information presented above presents a compelling rationale for the therapeutic use of anti-inflammatory cytokines (e.g. IL-4, IL-10, IL-12, IL-15) or neutralisation of pro-inflammatory cytokines (e.g. TNF-α, IL-6, IL-1) in the treatment

of cachexia. Animal model work has shown promising results but, as described above, models vary in the aetiology of the cachexia they seek to reproduce and do not always accurately reflect the complex reality of human disease.

1.6.3.1 Individual cytokine targets

IL-12 and IL-15 administration both individually reduce tissue wasting in tumour bearing animals, probably at least partly via reduction in IL-6(404-407). Equally, specific antibodies or receptor antagonists to TNF-α, IL-6, IL-1 and IFN-γ have all been effective in preventing or attenuating at least some aspects of cachexia in a variety of animal models(180;406;408-410). In contrast, human trials of therapies targeting individual cytokines have been small in number and invariably disappointing. Trials are limited by the expense of these medications and safety concerns over blocking vital cytokine functions. Anti-TNF-α therapy has been most thoroughly investigated, partly due to the proven safety record and widespread use of this medication in other inflammatory diseases such as arthritis and inflammatory bowel disease. Etanercept (Enbrel®) is a soluble recombinant fusion protein comprised of two TNF-R2 receptors bound to the Fc portion of human IgG1. Each etanercept molecule binds two TNF-α molecules, preventing them binding to the native receptor. Infliximab (Remicade®) and adalimumab (Humira®) are both monoclonal antibodies which bind and neutralise TNF-α. A small study added either etanercept or placebo to docetaxel chemotherapy in advanced cancer patients and found an improvement in fatigue in the etanercept group(411). In a trial of 89 patients with advanced pancreatic cancer patients were given standard gemcitabine therapy with either infliximab or placebo. There was a slight trend toward improvement in the QOL and LBM in the infliximab group but this did not reach significance(412). A similar trial using docetaxel +/- infliximab in poor performance non-small cell lung cancer patients was stopped early because the addition infliximab did not attenuate the weight loss(413). A placebo controlled trial of etanercept in cancer cachexia showed no clinical benefit(406) and two small trials of etanercept in breast and ovarian cancer have not shown definite clinical benefit(204;414). One small trial of anti-IL-6 monoclonal antibody in patients with acquired immunodeficiency syndrome and lymphoma suggested a possible improvement in fever and body weight(415).

1.6.3.2 Pentoxyfylline

Pentoxyfylline is a phosphodiesterase-4 inhibitor which reduces plasma levels of TNF-α by reducing its production by monocytes and T lymphocytes(416;417). It is widely used clinically for a variety of indications including intermittent claudication and alcoholic hepatitis. Again pentoxyfylline has been successful in preventing muscle atrophy in the yoshida-sarcoma bearing rat(418) but not in preventing human cachexia(419). It is interesting that pentoxyfylline is ineffective in the treatment of Crohn's disease despite the enormous success of other anti-TNF-α therapies in that situation.

1.6.3.3 Eicosapentaenoic acid (EPA)

EPA is an alpha-3 omega fatty acid found in fish oil. It down-regulates pro-inflammatory cytokines, PIF, the UPP(420;421) and protein catabolism(422) in murine models. It prevents cachexia in Walker 256 tumour-bearing rats(423) and protects otherwise healthy rats from the cachexia-like symptoms induced by starvation(424) or IL-1 administration(242). The first major study of EPA in human disease suggested that adequate amounts led to increases in lean body mass and quality of life(425). Unfortunately two follow up trials, one by the same group, failed to replicate the positive results(426;427).

1.6.3.4 Cyclooxygenase-2 (Cox-2) inhibitors

Cyclooxygenase-2 inhibitors block the synthesis of prostaglandin from arachidonic acid. This leads in turn to a reduction in TNF-α and IL-6 expression. Animal studies using a number of different models have demonstrated an anti-cachexic effect(428-430). Small human trials using COX-2 inhibitors alone are also promising(366;431) (REFS 15,16,17). McMillan gave either megesterol acetate alone or megesterol acetate with ibuprofen (a COX-2 inhibitor) to patients with advanced gastrointestinal cancer and weight loss. There was a weight reduction in the megesterone acetate group and a weight increase in the group taking ibuprofen in addition, leading to a significant difference between groups(432). Mantovani led an interesting non-randomised study in which 39 patients with advanced cancer were given a combination of celecoxib (a COX-2 inhibitor), medroxyprogesterone acetate, and nutritional support including EPA supplementation. The combination seemed

effective in reducing TNF-α and IL-6, fatigue and REE and increasing appetite, LBM and QOL(433). He went on to show that celecoxib alone improved LBM and reduced TNF-α in cancer cachexia(434)

1.6.3.4.1 Direct UPP inhibitors

Bortezomib (Velcade®) binds specifically with the 20S proteasome complex, so blocking the UPP. A study of 46 patients with advanced pancreatic cancer showed no benefit in single agent bortezomib therapy(256) and a trial of bortezomib, paclitaxel and carboplatin in advanced oesophageal or gastric cancer was closed early due to lower than expected chemotherapy response rates(435)

1.6.3.5 Metabolic therapies

1.6.3.5.1 Hydrazine sulphate

Hydralazine is a cori-cycle inhibitor but three placebo-controlled trials have shown no benefit in cancer cachexia(436-438) and side effects can be serious(439).

1.6.3.5.2 Insulin

One human study using insulin therapy in 138 patients with advanced gastrointestinal malignancy showed some measureable metabolic improvements (although no increase in lean body mass or significantly improved survival in the insulin treated patients(440).

1.6.4 Miscellaneous

The search for a successful treatment for cachexia has seen infusions of adenosine 5'-triphosphate (ATP) leading to improvements in lean body tissue and survival in advanced non-small cell lung cancer(441-443) and melatonin (which down-regulates TNF-α(443)) attenuating cachexia and improving survival in advanced non-small cell lung cancer(444;445), although in other cancer trials this it has not been successful(365). Many other substances have been trialled but with limited or no success.

1.7 **Thalidomide**

Thalidomide was originally marketed in 1956 as a sedative, relaxant and anti-emetic for pregnancy associated nausea. It was however withdrawn from the European market in 1961 following a relatively high incidence of previously rare limb abnormalities in children born to women who had taken the drug, even in very small amounts, during their pregnancies. In all approximately 10,000 people were affected. During this time the US Food and Drug Administration (FDA) did not approve it due to concerns over long term side effects. In 1965 it re-emerged as a treatment for erythema nodosum leprosum (ENL)(446), a painful, vasculitic complication of leprosy, gaining FDA approval for this indication in 1998. During these last three decades the only noted major side effect for the non-pregnant patient has been an infrequent peripheral neuropathy which occurred in 3 out of 49 patients treated for six months in one study(447) and in 1 out of 23 patients in another(448). Renewed interest was stimulated in 1991 by the discovery of thalidomide's powerful in vitro suppression of TNF-α production from lipopolysaccharide stimulated monocytes(449), exerting its effects by enhancing degradation of its mRNA(450;451). Subsequent work has shown thalidomide to modulate several other factors including IFN-γ, IL-10, IL-12, cyclooxygenase 2 (COX-2)(452). It also blocks TNF-α and IL-1β activation of NFκB(253). This is at least partially the basis for its well documented immunomodulatory and anti-inflammatory properties(453;454). Previous studies have found thalidomide to be effective in the management of a wide variety of clinical conditions, including HIV associated wasting(455) and the weight loss experienced in pulmonary tuberculosis(456).

Figure 14 Effects of thalidomide on TNF-alpha.

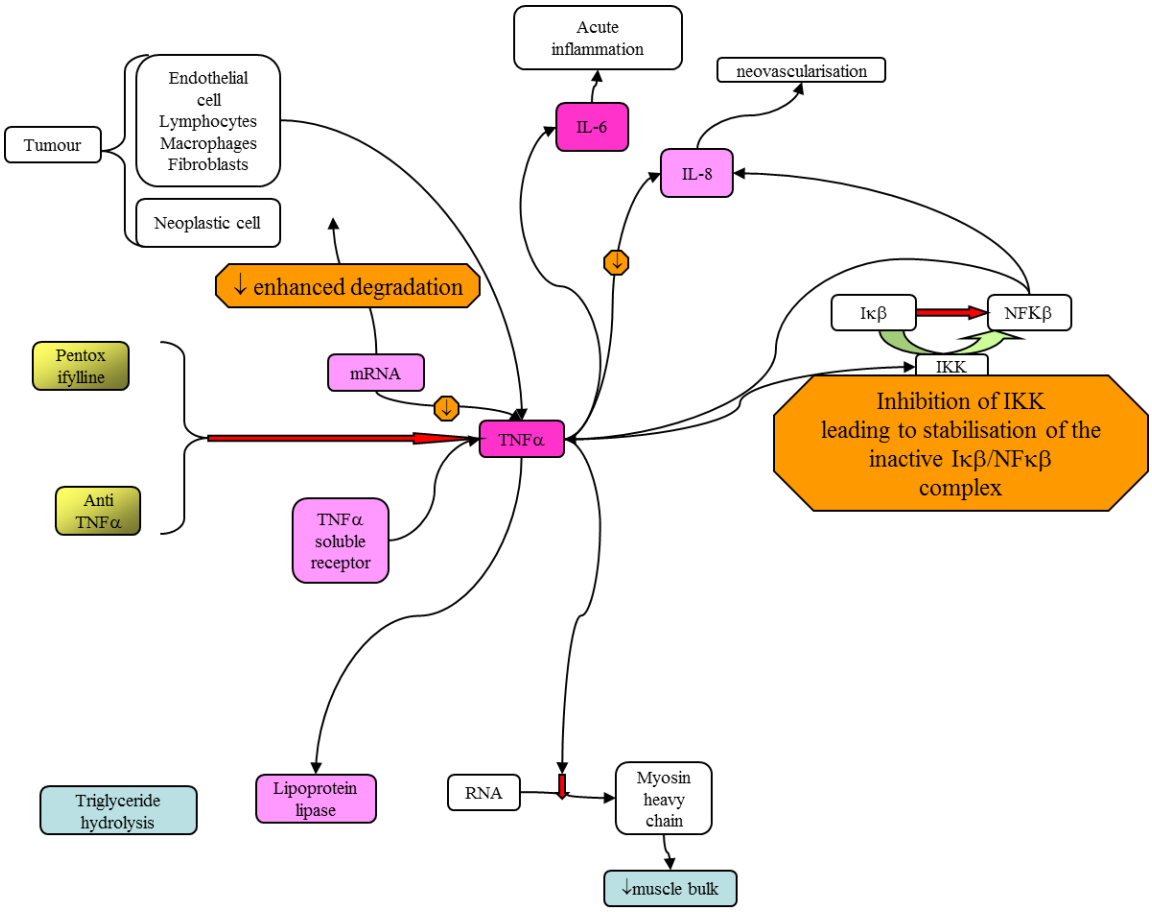


Figure 15 The proven effect s of thalidomide in cachexia (in orange) are mainly directed towards the TNF alpha mediated processes

There has recently been a great deal of interest in the use of anti-angiogenic agents as adjuncts to standard chemotherapy in both haematological and solid organ malignancies. Bevacizumab (a humanised monoclonal antibody directed against vascular endothelial growth factor) was the first angiogenesis inhibitor to market after it was given US FDA approval for use as a first-line treatment for patients with metastatic colorectal cancer in 2004. Thalidomide is known to be anti-angiogenic, possibly by reducing NFκB mediated production of IL-8, a required cytokine for angiogenesis(253) or possibly through inhibition of VEGF. It has been shown to inhibit VEGF secretion in cell lines in vitro(457) and depletes VEGF receptors in zebra fish embryos(458) but does not affect local VEGF increases after liver injury in rats(458;459). This anti-angiogenesis led to trials into its use as an anti-cancer agent. At present, few phase III trials have been completed but data suggest a benefit in multiple myeloma(460), refractory Waldenström's macroglobulinaemia(461),

myelodysplasia(462), advanced prostate cancer(463), renal-cell carcinoma(464), high-grade glioma(465), melanoma(466) and colorectal cancer(467), in some instances resulting in reduction of tumour bulk. Thalidomide is approved in some countries for the treatment of multiple myeloma after the failure of standard therapies and the acute treatment of cutaneous manifestations of moderate to severe ENL. It has been shown that the response rate in multiple myeloma cannot be explained only by the reduction of angiogenesis(468). It is possible that some of the anti-tumour effect is mediated by thalidomide's inactivation of NFκB, which is known to activate the expression of genes involved in cell growth and suppression of apoptosis(249;253). Thalidomide also reduces production of Cox-2(452), which is thought to play an important role in cancer therapy through angiogenesis, immune surveillance and apoptosis(469). In metastatic, chemotherapy resistant colon cancer, the addition of thalidomide to irinotecan chemotherapy improved response rates from 12-21% to 29%. Thalidomide's sedative and anti-emetic effects also allowed patients improved toleration of the irinotecan(470). Phase III studies are currently in progress. Lenalidomide is a thalidomide analogue with similar but more potent effects and a more favourable toxicity profile (less constipation and neurotoxicity). In many cases it is now prescribed as an alternative to thalidomide.

Figure 16 Known actions of thalidomide in cancer

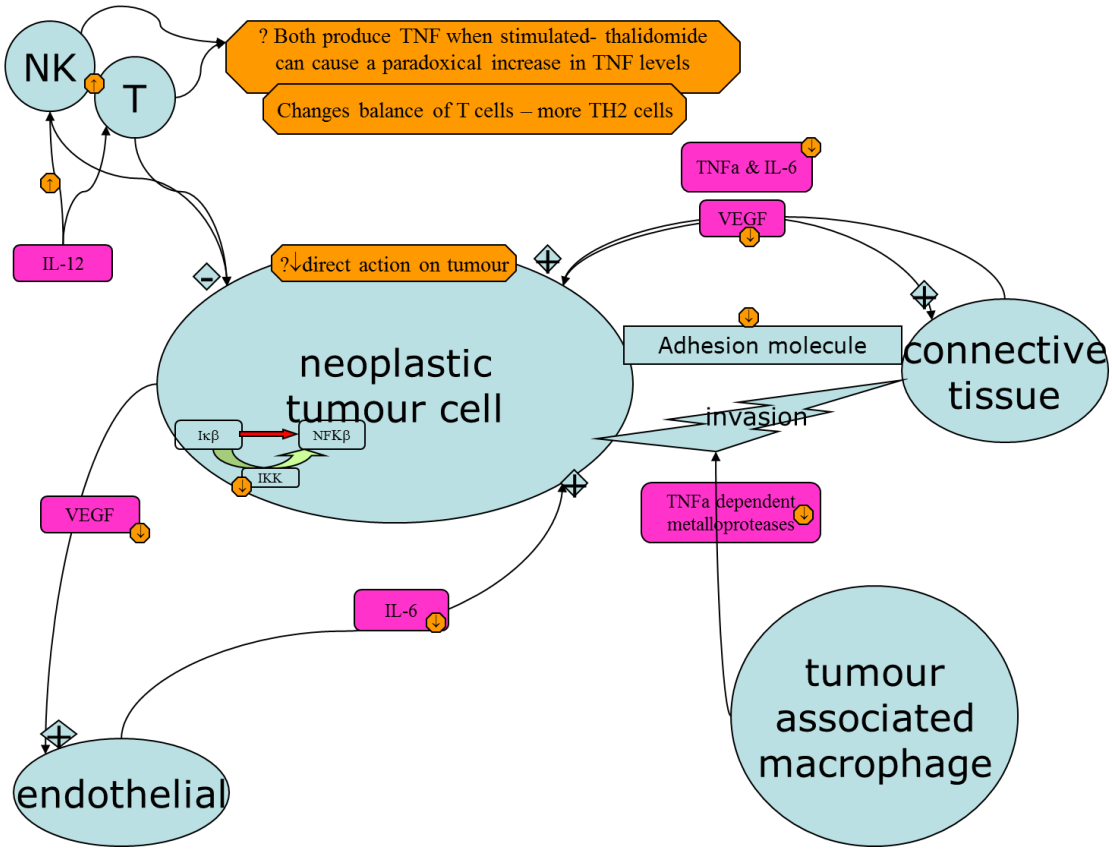


Figure 17 Thalidomide also has direct effects (in orange) on the processes governing the growth and spread of cancers

1.8 Previous clinical trials of thalidomide in cancer cachexia

To date there are five published human trials evaluating the use of thalidomide in cancer cachexia. Bruera(471) showed in an uncontrolled study involving 37 patients with terminal malignancy that thalidomide's anti-emetic, analgesic, and sedative properties were effective in the palliation of otherwise intractable symptoms in patients with terminal malignancy. Khan et al.(472) have reported an open label pilot study of thalidomide in the treatment of cachexia in eleven patients with inoperable oesophageal cancer. In this study thalidomide reversed weight loss over the two week period of the trial, and this was associated with an increase in lean body mass. The same group went on to randomise 16 end stage oesophageal cancer patients to thalidomide and 16 patients to placebo. They found thalidomide to be poorly tolerated due to skin rashes, hyper-somnolence, paraesthesia, constipation, headache and neutropenia. They were not able to demonstrate any benefit from the treatment.

Our research group published the results of the first randomised placebo controlled trial of thalidomide in the treatment of cancer cachexia(454). In this study we demonstrated that thalidomide is safe and effective in attenuating weight loss in patients with cachexia secondary to advanced pancreatic cancer. In this study 50 patients were recruited to either thalidomide (200mg per day) or placebo. Of these 33 patients (17 thalidomide, 16 placebo) were available for assessment at four weeks and (12 thalidomide, 8 placebo) at eight weeks. At four weeks, those who received thalidomide had gained on average 0.37 kg in weight and 1.0 cm3 in arm muscle mass (AMA) compared with a loss of 2.21 kg (absolute difference 22.59 kg [95% confidence interval (CI) 24.3 to 20.8]; p = 0.005) and 4.46 cm3 (absolute difference 25.6 cm3 [95% CI 28.9 to 22.2]; p = 0.002) in the placebo group. At eight weeks, patients in the thalidomide group had lost 0.06 kg in weight and 0.5 cm3 in AMA compared with a loss of 3.62 kg (absolute difference 23.57 kg (95% CI 26.8 to 20.3); p = 0.034) and 8.4 cm3 (absolute difference 27.9 cm3 (95% CI 214.0 to 21.8); p = 0.014) in the placebo group. Improvement in physical functioning correlated positively with weight gain (r = 0.56, p = 0.001).

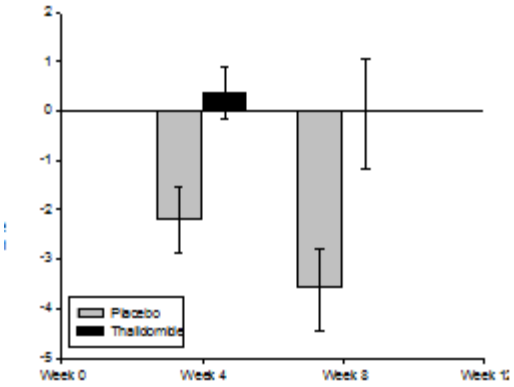


Figure 18 The change in weight seen in pancreatic cancer patients randomised to either thalidomide or placebo in our previous trial

Thalidomide n=17 week 4, n=12 week 8; Placebo n=16 week 4, n=8 week 8. Between groups p=0.005 at 4 weeks, and 0.034 at 8 weeks.

The general weight loss correlated with a reduction in loss of lean body mass as measured by anthropometric techniques. There was also a trend towards prolonged

life expectancy with a median survival of 148 days in the thalidomide group compared to 110 days in the placebo group. This survival benefit is similar to that seen in recent trials using gemcitabine as single agent chemotherapy. The thalidomide was well tolerated; two patients (9%) complained of peripheral neuropathy that resolved on stopping the drug, and two patients (9%) developed a rash that necessitated withdrawing from the trial. A further four patients (17%) complained of severe daytime somnolence that required a reduction in drug dosage in two patients, and cessation of the drug in the other two. Conversely, those in the placebo arm suffered significantly more from insomnia ($p=0.023$). Constipation was the only other side effect experienced to significant levels ($p=0.04$). Further studies are required to investigate whether it is possible to generalise these results to cancer cachexia caused by other cancers, and whether there is a true survival benefit. There is also a lack of human data on the underlying biological processes of this condition and the effect thalidomide has on these.

Mantovani ran a complex phase III trial randomising 332 patients with advanced cancer and loss of >5% of their ideal or pre-illness body weight to five different therapy arms: 1 progestagen; 2 EPA; 3 L-carnithine (an amino acid derivative involved in transporting long chain fatty acids to mitochondrial for energy production); 4 thalidomide 5 progestagen + EPA + L-carnithine + thalidomide. Arms 1 (progestagen) and 2 (EPA) were stopped after interim analyses demonstrated inferiority. All treatments were well tolerated and patient compliant was good. Thalidomide resulted in a significant reduction in IL-6, Glasgow Prognostic Score (GPS) and ECOG PS with no adverse effects. The combination arm (5) was the most effective for all measured end points: IL-6, TNF-α, GPS and ECOG PS.

1.9 Measurement of lean body mass

One of the major obstacles into cachexia research has been measurement of lean body mass. There are accurate techniques available, for example underwater weighing or isotope dilution but all involve expensive or bulky equipment and are not practical outside a laboratory research setting. Anthropometric techniques such as measurement of weight, triceps skin fold thickness, mid-arm circumference are simple and validated but have an average error of around 7-8%(473). Dual-energy x-ray

absorptiometry (DEXA) scanning uses X-rays of two energy levels that are attenuated by different tissues to different extents to offer a precise and non-invasive method which makes no assumptions of the chemical constancy of lean tissue mass(474-476). It is however expensive, involves a small radiation dose and necessitates immobile equipment. Bio-impedance is a widely used but relatively new technology that relies on mathematical equations validated in specific patient groups to determine body composition data from raw bioimpedance values. The bioimpedance value is largely influenced by the type of tissue the current is travelling through (e.g. fat, water, muscle) but will also be influenced by other factors such as extracellular water and cell membrane integrity. Bio-impedance is relatively cheap, portable, easy to use and safe. Equations have been validated in many varying patient groups but not specifically for cachectic patients who will have inevitable changes in electrolyte composition, body water compartmentalisation and cell membrane integrity(477;478).

Chapter 2 Aims and Objectives

2.1 Aims

Based on our previous work we hypothesised that thalidomide can attenuate or reverse both total weight loss and loss of lean body mass in the cachexia associated with incurable upper gastrointestinal adenocarcinomas.

In addition we wished to investigate whether this was associated with an improved quality of life or survival benefit; to obtain a profile of the serum factors implicated in the development of cachexia and investigate how these are affected by thalidomide and to obtain a safety profile for thalidomide in this patient group.

2.2 Objectives

2.2.1 Primary objectives

To evaluate the ability of thalidomide, as compared to a placebo, to attenuate loss of weight in patients with incurable upper gastrointestinal adenocarcinomas.

2.2.2 Secondary objectives

1. To assess any impact on functional or overall quality of life
2. To calculate any change in overall survival.
3. To calculate any change in lean muscle mass
4. To calculate any change in grip strength
5. To obtain serum profiles of factors previously implicated in the development of cachexia for both the control and treated group
6. To document the safety and tolerability of thalidomide in patients with incurable upper gastrointestinal adenocarcinomas.

Chapter 3 Patients, materials and methods

3.1 Trial type

This was a non-commercial, NHS sponsored double-blind, placebo controlled clinical trial. A placebo was chosen for the control group as there is no currently accepted standard or effective treatment for cachexia. The trial period for each patient was six months.

3.2 Trial conduct and sites

Patients were recruited from 7 sites across London and the South of England between December 2005 and February 2011.
The study protocol was approved by Southampton and South West Hampshire Research Ethics Committees(B) in Aug 2005; by the Medicines and Healthcare Products Regulatory Agency in September 2005; by the Research and Development Management committee of each individual site and registered with the International Standard Randomised Controlled Trial Number Register (ISRCTN51456701).
Twice during the trial un-blinded results were reviewed by an Independent Trial Monitoring committee.

3.3 My own role in the trial

I conceived, set up and ran the trial as Chief Investigator. I successfully gained ethics, and MHRA approval, funding and drug supply as well as taking responsibility for all aspects of trial management. I also developed the laboratory assays and ran the samples.

3.4 Participants

Patients over the age of 18 years with incurable upper gastrointestinal adenocarcinomas and weight loss of over 5% of their pre-morbid weight or actively losing at least 1kg per month were identified at gastroenterology or oncology clinics or through multidisciplinary team cancer meetings. The diagnosis was required to be

confirmed cytologically or histologically other than in pancreatic cancers where biopsy is often technically difficult and uncomfortable, in these patients the diagnosis was accepted if felt to be unequivocal clinically and radiologically. Patients were not be recruited within four weeks of receiving either chemotherapy or radiotherapy. Those with clinically detectable ascites or oedema were not included due to potentially complicating weight measurements. Those with evidence of peripheral neuropathy, severe constipation, vertigo or vestibular disease were not included due to known potential thalidomide toxicity. Premenopausal women were included but required to comply with strict guidelines and submit a monthly pregnancy test. Those using megesterol acetate at a stable dose for at least a month were included but asked not to adjust their dose during the trial. Use of corticosteroids and nutritional supplements was unrestricted but documented at clinic visits.

3.5 Sample size

The sample size to detect a 2kg difference in weight change between the two groups at 4 weeks, assuming a between subject standard deviation of 4.3 (based on our previous study(479)) with 80% power required 74 subjects per treatment group. Allowing for 20% attrition we planned to recruit a total of 90 subjects per group. Initially the trial was planned as a single centre. Analysis of eligible patients over the previous three years and our experience of the proportion of patients agreeing to participate in our previous trial suggested that the trial should be fully recruited within 18 months. The launch of the trial coincided with a dramatic change in practice in non-curable upper gastrointestinal cancer patients. During recruitment for our previous trial there were no other worthwhile medical treatments available and patients were generally given trial information at the same outpatient appointment at which they were told their diagnosis. Gemcitabine was licenced for palliative use in terminal pancreatic cancer in 2001(480) and became common practice at our and many other institutions around the time of the launch of this trial. This dramatically reduced the pool of patients with no other acceptable options available to them. It also meant that it was generally inappropriate to recruit to the trial at the first outpatient appointment as time was required for the patient to consider their, now very real, options. If they even wanted to consider gemcitabine therapy they were required to have a biopsy which would generally take about 2-3 weeks to be taken and analysed.

In many cases, by the time gemcitabine had been considered and sometimes tried but rejected, patients who would previously have been ideal candidates for the trial were either ineligible due to a life-expectancy now less than the required 8 weeks or had simply had enough of medical intervention and were not interested in even considering participating. It was also a considerable challenge to identify patients who may be eligible for the trial in the future and keeping track of their progress until such a time as they were appropriate for consideration. Consequently recruitment was substantially slower than anticipated. The planned single centre trial was extended to a total of seven sites in an attempt to reach the required sample size but the trial was eventually closed due to slow progress when 63 participants had enrolled.

3.6 Trial intervention

Each subject was asked to take thalidomide 200mg (a dose used by ourselves and others in previous studies(471;472;481)) or identical placebo once every evening for a period of 26 weeks. Thalidomide is prepared in capsules of 50mg, this therefore entailed taking four capsules per day. Participants were asked to take all four capsules just before bedtime to reduce somnolence but after recruitment of 46 patients, it was noted that drowsiness after the initial dose was limiting tolerance in some patients. A protocol amendment was therefore made and from that point participants were asked to take one capsule on the first day and then increase by 50mg each day until taking the full 200mg or their maximum tolerated dose, whichever was the lesser. If side effects were troublesome to the patient and not easily controlled by conventional means (e.g. anti-emetics or laxatives) then the dose was reduced to 100mg, if they continued despite dose reduction the drug was stopped and the patient was withdrawn from the trial. Drug compliance was assessed by direct questioning and pill count at each patient visit.

Thalidomide 50mg capsules and identical placebo were manufactured by Penn Pharmaceuticals Services limited, Tredegar, Gwent NP22 3AA and supplied free of charge from Pharmion Ltd, Riverside House, Riverside Walk, Windsor, Berkshire SL4 1NA (now Celgene Corporation, 86 Morris Avenue, Summit, New Jersey, USA). The drug was supplied in 28 capsule blister packs labelled with the contact details of the co-ordinating investigator and ‘for clinical trial use only’. The placebo capsule

and packaging was identical to the active medication in every way other than that it did not contain the active ingredient (thalidomide). At each visit patients were supplied with enough capsules from their allocated box to last until their next appointment. Patients were asked to return any surplus drug and this was disposed of through the hospital pharmacy.

We were able to continue to supply trial medication after the 6 month period for patients who wished to continue to take it. Only after careful discussion with each individual patient with consideration of potential long-term side effects (peripheral neuropathy in particular) was this considered. Full details of thalidomide's international licensing are contained in the Investigator's Brochure.

3.7 **Randomisation process**

Medication was supplied by the manufacturing company in blocks or four, each consisting of supply for two participants on active drug and two on placebo. Enough drug for each subject for the six month course of the trial was boxed and labelled from 1-180 to correspond to individual patient trial numbers. The sequence was generated by a standard computerised randomisation procedure at the manufacturing site. Stratification was applied for pancreatic and non-pancreatic origin because previous trial information related to pancreatic alone and we were keen to be able to analyse these results separately, also because the prognosis of pancreatic cancer is generally poorer than other upper gastrointestinal cancers. Stratification was also applied for trial site. Each trial site held two blocks of four at any time, one for pancreatic patients and one for non-pancreatic. Once a block of four was started, it was completed with patients of the appropriate primary cancer site (pancreatic or non-pancreatic) and at the same trial site. Prior to a block of four being completed a further block of four would be allocated and delivered to that trial site. Packaging of active drug and placebo was identical, keeping the investigators, pharmacists and patients blind to the allocation. New participants were simply allocated the next available number in the appropriate block.

The medication was supplied in bulky boxes meaning that the handling pharmacy at the Queen Alexandra Hospital could only accept relatively small batches due to

volume storage restrictions. The drug was short dated and an agreement was made with the pharmaceutical supplier (Pharmion ltd) that unfinished blocks would be replenished when necessary to allow them to be completed. Unfortunately Pharmion was taken over by Celgene just after the trial launched and on two occasions supplied new drug as new blocks of four rather than replenishments for uncompleted blocks. Had recruitment been completed, this would have been of no consequence but due to the sub-optimal number of participants, meant that they were scattered through the planned blocks of four with resulting uneven spread between groups. 36 were allocated active medication and 27 to placebo.

3.8 Schedule of events

Trial visits were conducted at baseline, 1 month, 2 months, 3 months and 6 months. All subjects were given the opportunity to see a dietician for general nutritional advice. Patients were contacted by telephone at 7 months and questioned about any adverse events. At each trial visit any adverse events were recorded using the Common Terminology Criteria for Adverse Events (CTCAE) system(482) Any new symptoms were explored with details concerning time of onset, action taken and outcome. A neurological examination was conducted and sensation to pinprick, light touch, vibration and proprioception documented. All clinical measurements were taken (see 3.9). Bloods were taken at baseline, 1 month, 3 months and 6 months (visits 1, 2, 4 and 5).

Patients developing symptoms or signs of peripheral neuropathy or neutropenia with less than 500 cells/mm^3 was withdrawn from the trial. Patients requiring chemotherapy or radiotherapy after trial enrolment was be withdrawn from the study. No patient was replaced.

Table 2 Schedule of events

Events	Initial clinic appointment	Month 0	Month 1	Month 2	Month 3	Month 6	Month 7
Visit number	0	1	2	3	4	5	Home
Week		0	4	8	12	26	30

Assess eligibility	✓						
Give information sheet	✓						
Informed consent		✓					
Randomisation		✓					
Detailed history		✓					
Blood sample		✓	✓		✓	✓	
Neurological exam		✓	✓	✓	✓	✓	
Anthropometry		✓	✓	✓	✓	✓	
Bio-impedance		✓	✓	✓	✓	✓	
DEXA scan		As opportunity arises					
QOL questionnaire		✓	✓	✓	✓	✓	
Prescribe medication		✓	✓	✓	✓	✓	
Check compliance			✓	✓	✓	✓	
Record adverse events			✓	✓	✓	✓	
Pregnancy test		✓	✓	✓	✓	✓	✓
Phone call for adverse effects							✓

3.9 **Clinical measurements**

All equipment was used according to the manufacturer's instruction manuals. All trial practitioners involved in taking the clinical measurements were trained by the same investigator.

3.9.1.1 Grip strength

Grip strength was measured in the non-dominant hand to detect smaller changes more easily. Measurements were taken in triplicate with the average reading being taken. A Jamar Hydraulic Hand Dynamometer was used for grip strength evaluation due to its proven accuracy(483) and robust and portable design.

3.10 Lean Body Mass

We chose to use anthropometry, bioimpedance and DEXA scanning to assess LBM. We were aware that only a proportion of patients would be able to undergo DEXA imaging as it was only available to us at the Queen Alexandra Hospital. We presented the DEXA to the participants as an additional voluntary investigation that was not part of a standard clinic visit as it required extra time and a walk to radiology. We were sensitive to the possibility of this putting people off attending visits at all when all our other investigations were quick and simple. We hoped that obtaining comparative DEXAs for even a proportion of the anthropometry readings would allow us to judge the accuracy of these two other portable methods in our patient population.

3.10.1.1 Weight

Measured without shoes and wearing light clothing only

3.10.1.2 Anthropometry

Mid upper arm circumference (MAC) was measured using stretch resistant tape
Triceps skin-fold thickness (TSF) was measured using Harpenden skinfold callipers – (the only caliper CE marked under the Medical Devices Directive 93/42/EEC for a Class 1 device with measuring function)
Bone free arm muscle area (AMA), a validated marker of lean muscle mass, was then be calculated from MAC and TSF using the formula $(MAC - \pi TSF)^2 / 4\pi$ minus a correction factor of 10 for male sex or 6.5 for female sex(473).

3.10.1.3 Bioimpedance

We chose to use a tetrapolar multi-frequency machine (Bodystat® Quadscan 4000 Multi-frequency Bioelectrical Impedance Analyser) for optimised accuracy(484-486). Measurements were taken according to the manufacturer's instructions in the supplied

manual and data was interpreted using standard equations on commercial software from Bodystat® Ltd

3.10.1.4 DEXA

Dual-energy X-Ray absorptiometry (DEXA) scanning was only available at one study site using a Hologic Discovery A Model scanner.

3.10.2 Quality of Life

Quality of life was measured using the EORTC QLQ-C30 questionnaire(487) and analysed using the published scoring manual(488). The questionnaire is composed of 30 questions. Each assesses either functional capacity, symptomatology or overall global health. There is no overlap, with the answer to each question then being analysed in only one of the three scales.
The functional scale is composed of questions 1-7 and 20-27; symptom scale questions 8-19 and 28; and global health score questions 29 and 30.

The raw score given by the patient on the functional questions will give a higher score with increased difficulty carrying out activities, a higher score therefore representing a lower level of functioning. In the symptom questions the higher the raw score the more unpleasant symptoms the patient is reporting and for global health score the higher the score the better the overall reported quality of life.

The following linear transformations were therefore applied to convert the raw score into a standardised score from 1-100 where a higher score represented a higher response level(488).

Functional	Standardised score = (1-(raw score-1/range))x 100
Symptoms	Standardised score = (raw score-1/range)x 100
Global Health	Standardised score = (raw score-1/range)x 100

After transformation a higher score represents:

Functional	a higher, more healthy functional level. An improvement in functioning will equate to an increase in the standardised functional score
Symptoms	a higher, less healthy amount of symptomatology. An improvement in symptoms will equate to a reduction in the standardised symptom score
Global Health	an improved quality of life. Improvements in quality of life will equate to an increase in the standardised global health score

3.11 Laboratory Measurements

3.11.1 Collection of blood samples

Sterile collection of blood samples was undertaken by venepuncture using Vacutainer® passive shielding blood collection needles. Blood for measurement of full blood count, urea and electrolytes, liver function tests, thyroid function tests, albumin and C-reactive protein (CRP) was drawn into the standard bottles used at that hospital site. Blood for cytokine estimation and retrieval of monocytes and lymphocytes was drawn into 7.5ml S-monovette containers containing 1.6mg ETDA for anti-coagulation (Sarstedt, Germany). These tubes were selected because they are compatible with Vacutainer® needles and are guaranteed to be free of pyrogens including endotoxin (which has been previously shown to affect cytokine levels, particularly TNF-α)(489;490).

Full blood count, urea and electrolytes, liver function tests, thyroid function tests, albumin and CRP were measured through the standard hospital system at each individual trial site on fresh samples.

Samples for cytokine estimation and monocyte / lymphocyte estimation were put on ice immediately and processed within 2 hours to avoid cytokine breakdown(491), particularly TNF-α, which is known to deplete in samples stored at room temperature or for prolonged periods of time(492). The Monovette container was centrifuged at 1000g for 10minutes. The plasma layer at the top of the sample was then carefully removed using a sterile Pasteur pipette and separated into 500µl aliquots prior to freezing at -80^{0}c and stored at that temperature until analysis. Evidence suggests that

cytokine levels including TNF-α are resistant to freeze-thaw cycles(493) but these were avoided none the less.

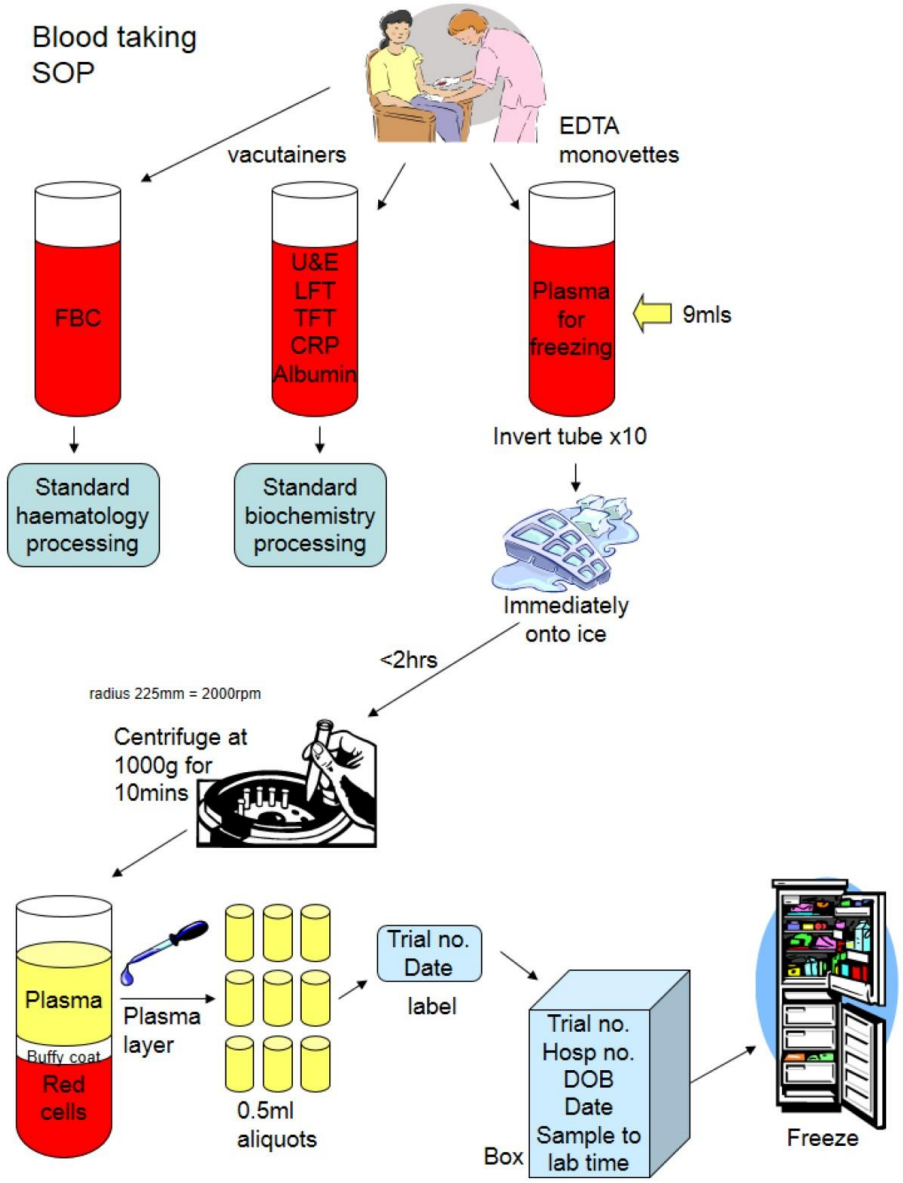


Figure 19 Standard Operating Procedure for processing blood samples. This was provided to the trial nurses at each site after one to one training sessions

3.11.2 Enzyme-linked immunosorbent assays (ELISAs)

We measured levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 to investigate any links between peripheral levels and clinical findings. Both TNF-α and IL-1β(300) have proven difficult to detect by previous investigators as they are present at low levels in the serum and TNF-α in particular seems sensitive to collection and storage technique. We therefore also performed ELISAs for TNFR2

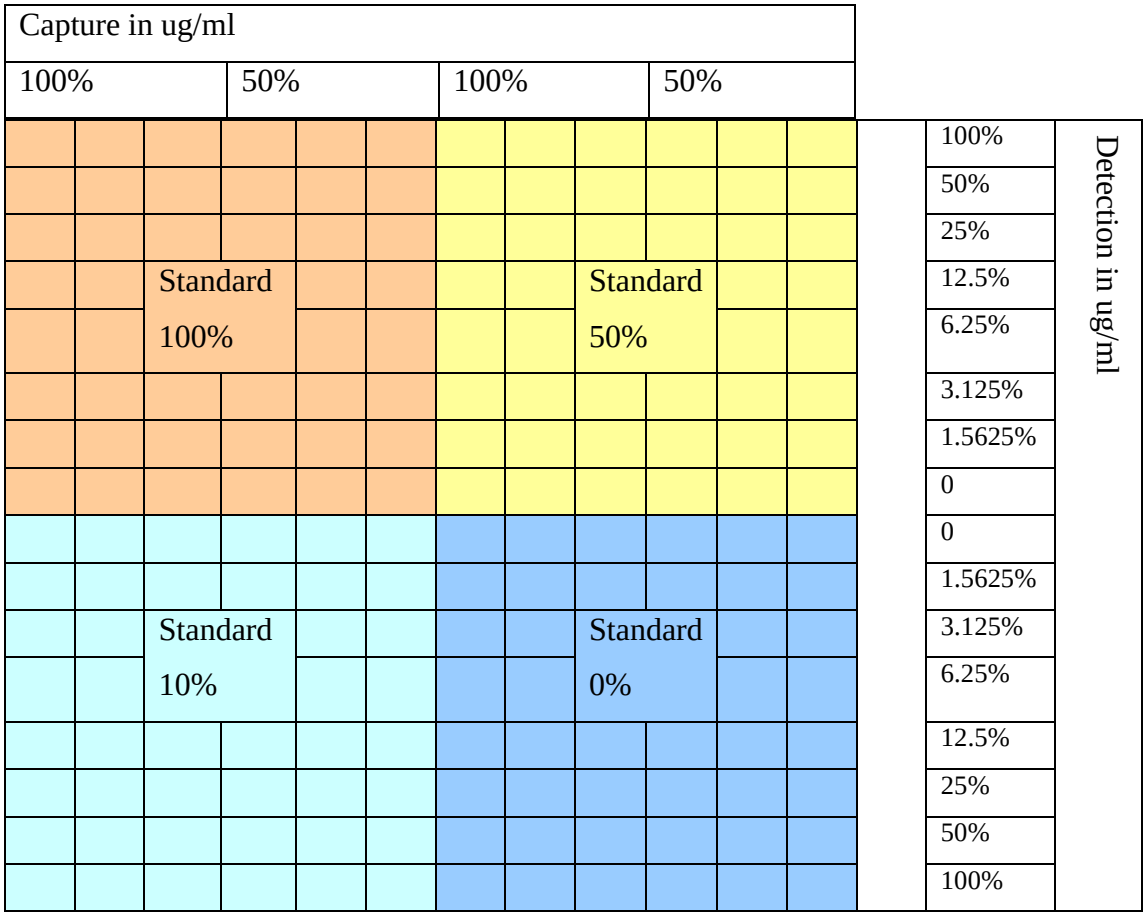
and IL-1ra as surrogate markers(300). We measured VEGF levels as a marker of angiogenesis.

All cytokine levels were measured using sandwich ELISAs. One of the major difficulties was the measurement of TNF-α in human plasma. Previous authors have shown a significant difference in the results obtained by commercial kits available from different companies and often low serum levels have been undetectable(494-496). We therefore chose to develop our own ELISA for each analyte rather than use a commercially available kit to allow us to optimise the technique in order to detect very low levels, particularly of TNF-α. The basic sandwich ELISA used was a combination of that recommended by RnD Systems(497) and that developed from previous experience in our laboratory (with special thanks to Marta Polak). Previous authors have published studies attempting to improve the sensitivity of TNF ELISAs in human plasma(498;499). Our method was based on that described by Kittigul, taken from his original paper and on the work of Innis et al.(500-502). 384 well microplates were used throughout (rather than the standard 96 well) so that all samples could be fitted onto one plate with room for control lines with serum spiked with recombinant standard on the same plate to improve accuracy. Microplates were coated with a capture antibody specific to the analyte of interest. Blocking buffer was then added to block all unbound sites on the microplate, thereby preventing later non-specific binding of detection antibody or horseradish peroxidase (HRP) and so reducing background noise. The sample was then added causing the analyte to be indirectly bound to the plate by the capture antibody. A second antibody was added which binds to a different epitope of the analyte and is itself labelled with biotin, completing the sandwich. The biotin tag was then labelled with HRP which has an extremely high affinity for biotin and so enhances the signal increasing low level detectability. Luminol was then added. HRP oxidises luminol to 3-aminophthalate emitting light proportional to the amount of analyte present. The emitted light was quantified in relative light units (RLUs) using a luminometer. This chemiluminescence method has been proven by others capable of detecting analyte at very low concentrations(503). All antibodies and standard proteins were purchased from RnD systems ltd and the dilutent used to reconstitute each individual analyte were as recommended from the manufacturer. Washes between steps were with phosphate buffered saline (PBS, Sigma-Aldrich®) – a formulation of buffers and slats

which is isotonic and aids in maintaining a constant pH at 7.4. 0.05% tween was added to all washes as a detergent to reduce background. ELISA grade Bovine Serum Albumin (BSA) was used as the blocking agent to prevent non-specific binding of the antigens and antibodies to the microplate. Sodium azide (NaN^3 0.08%) was added as a biocide to all dilutents until the detection stage to prevent bacterial contamination. Repeated runs of the TNF-α ELISAs showed the signal (the luminescence from the standard containing cells) to noise (the luminescence from the cells containing 0% standard) ratios were consistently better without NaN^3 so it was not used for TNF-α ELISAs. NaN_3 works by stopping peroxidase working and so affects HPR which could have caused the problem, although it seemed to benefit all other ELISAs by reducing background and should be completely washed from the microplate prior to adding HRP. All washes and dilutents were produced by hand fresh just prior to use to prevent contamination.

Before any patient samples were processed, the ELISA standard operating procedure (SOP) for each individual analyte was optimised. Initially the ELISA technique was used to find the most effective dilution combination of the capture and detection antibodies using a checkerboard technique:

Figure 20 Checkerboard technique used for ELISAs



Graphs were plotted of the results. These were used to select the concentrations producing the best signal to noise ratio. Once the optimum antibody concentrations had been established the ELISA was run multiple times for each analyte, altering various factors such as incubation times, BSA product, with or without NaN^3. Once the ELISA was optimised it was re-run using serial dilutions of spiked serum from healthy volunteers to minimise matrix effects. A checkerboard style to establish the optimal dilution for the final run using patient samples. The dilutent used was always identical that used to prepare the standards for the specific analyte. Trials runs using serum usually used my own as I found it to have undetectable levels of all pro-inflammatory cytokines on every trial, possibly due to a coincidental pregnancy.

Once ELISA SOPs had been established, each sample was analysed for analysed for TNF-α, sTNF RII, IL-6, IL-1b, IL1RA and VEGF,. Each sample was analysed in triplicate and the mean of the three samples used. Because the trial was prolonged,

samples were stored at -80^{0}c for up to five years. Freeze thaw cycles were avoided by dividing the original sample into small (0.5ml) aliquots prior to the initial freeze. All samples available in 2006 were analysed at that time. Different aliquots of the same samples were re-analysed with all samples in 2011 and these were the results presented. Coefficients of variation (CV) between repeat determination of identical samples in 2006 and 2011 were all under 15 and highly correlated ($p<0.001$) suggesting the storage was effective in keeping specimens stable and adding validity to our ELISA SOP. Softmax pro® was used to plot standard curves and interpret serum samples. For ELISA results below the limit of detection the extrapolation method was used as this is recommended as the most accurate method. Results below the limit of extrapolation were classed as zero(504). Mean coefficient of variation for the final results run were TNF-α 12.3, sTNF RII 3.4, IL-6 3.7, IL-1b 12.83, Il-RA 6, VEGF 3.6.

Full details of the establishment of the ELISA in appendix 8.1. The full IL-6 SOP is presented in appendix 8.4.

3.12 **Survival**

While actively involved in the trial dates of death were noted in the trial case report forms. For those who survived past the end of the trial survival was monitored using the hospital computer system with phone calls to the patient's general practitioner for clarification if necessary

3.13 **End of Trial**

The end of the trial was defined as the last phone call to the last patient. After the end of the trial survival times were monitored through the hospital or general practitioner's records.

3.14 **Statistical analysis**

Analysis was performed on an intention to treat basis. Demographic data was interpreted using Fisher's Exact Test for categorical data, the Mann-Whitney Test for

ordinal or skewed data and the independent samples t-test for normally distributed continuous variables.

Comparison of methods for measuring lean body mass was made by correlating the results, comparing the average difference with the paired samples t-test and by assessing the agreement between methods using the Bland Altman and intra-class correlation coefficient (ICC) methods. The Bland Altman limits of agreement method was chosen to analyse size of differences between pairs. In this the measure is obtained by first calculating the difference between the methods for each individual observation. The 95% limits of agreement (within which 95% of all differences between methods should occur) are then calculated as follows:

Mean difference +/- 1.96(standard deviation of the differences)

The ICC method examines the total variability in outcome values and divides it into two components: that due to patient differences and that due to difference between methods for the same patient. The ICC value is the proportion of the difference which is due to between patient differences. If there is good agreement between methods then the ICC value will be close to 1. This can be calculated on SPSS using an 'absolute agreement' option, meaning the values should be identical, or using a 'consistency' option meaning there is a consistent linear relationship even if the absolute values are not identical. We felt this was reasonable for our purposes and used the 'consistency' option.

The Quality of Life Questionnaire used produces results in three domains: Global Health Score, Functional scales and Symptom scales. Results from each section were analysed as well as 'physical functioning', a subdivision of the functional scale which we felt to be particularly relevant(482). Results were transformed into standardised scores (see quality of life methodology, section 3.10.1.4) and analysed as recommended in the EORTC QLQ-c30 Scoring Manual (488). Results were compared using the independent samples t-test.

The independent samples t-test was also used for comparison of clinical data between groups other than frequency of adverse events. Because the number of these was

small the Fisher's Exact Test was used to compare groups. Kaplan Meir was used to analyse survival data.

Due to the skewed results, Spearman's rank correlation was used to compare cytokine levels between groups.

A significance level of 0.05 was used throughout other than when multiple similar comparisons were made, when a Bonferroni correction was applied.

Chapter 4 Results

4.1 Patient flow

Figure 21 Patient flow

Of the 38 patients who withdrew during the course of the trial, the most common reason (20 patients) was a general physical deterioration or change in circumstance (e.g. being admitted to a hospice) leading to withdrawal of active or unnecessary treatment. Mean survival in the group that withdrew was 33 days after leaving the

trial (median 25 days). Anecdotally, many wondered whether they felt non-specifically less well after the drug was started, which often contributed to their decision to withdraw. The study population were inevitably deteriorating over time due to their terminal cancer and it was difficult to be specific as to whether decline was in any way drug related. Participants who withdrew generally felt no better on stopping the drug and there were no demonstrable differences between the groups in those who withdrew due to concerns over drug side effects after un-blinding.

4.2 **Demographics**

The groups were well matched in all of the variables measured on recruitment to the trial other than weight which was significantly higher in the thalidomide group and nodal stage. There were significantly more with N0 disease (no lymph nodes involved) in the thalidomide group and more with N1 disease (local lymph nodes involved) in the placebo group. Tumour stage and metastatic stage were not significantly different.

	Thalidomide	Placebo	p-value
Gender	75%	67%	0.58*
Age (years)	76.3 (8.1)	74.0 (10.6)	0.379**
Tumour site			
Ampullary	3%	4%	
Oesophageal	25%	11%	
Gastric	19%	44%	
Small bowel	0%	4%	
Pancreatic	52%	37%	
Tumour stage			0.70***
0	7%	5%	
1	17%	0%	
2	24%	35%	
3	21%	40%	
4	31%	20%	
Nodal Stage			0.02***
0	53%	19%	

1	47%	76%	
2	0%	5%	
Metastatic Stage			1.00***
…0	47%	43%	
…1	53%	57%	
Functional QOL score	69.4 (16.4)	67.0 (19.0)	0.60**
Physical functioning QOL score	63.9 (22.0)	59.2 (25.9)	0.45**
Symptoms QOL score	32.1 (13.7)	34.1 (14.2)	0.58**
Global health score	48.8 (22.3)	49 (19.5)	0.97**
Grip strength	25.0 (8.4)	22.5 (9.6)	0.273**
Weight (kg)	69.0 (14.6)	61.6 (12.9)	0.037**
Arm muscle area	34.0 (10.7)	29.6(10.0)	0.099**
DEXA Lean	77.3% (7.3)	75.1% (5.2)	0.29**
Bioimpedance Lean	71.0% (7.8)	69.0% (7.2)	0.14**
CRP (mg/l)	42 (40)	42 (41)	0.913***
IL-6 (pg/ml)	57 (45.6)	54.6 (50.7)	0.676***

Mean (standard deviation)

* Fisher's Exact Test

** T-Test

*** Mann-Whitney

Table 3 Baseline characteristics of the study population presented with thalidomide and placebo groups separately

One patient described his origin as black. All others described themselves as white British

4.3 Safety and tolerability

During the trial one patient taking thalidomide developed a deep vein thrombosis and one taking placebo had a myocardial infarction. Multiple adverse events were reported during the course of the trial. Any reported by the same subject on more than one visit were counted once only. Those that pre-dated the trial or had an obvious cause other than medication (e.g. dysphagia due to blocked oesophageal stent) were discounted and similar symptoms were then grouped into categories by hand without

knowledge of the treatment group. The only striking difference was more infections in the thalidomide group. Using the Chi squared test the produces a significant difference (p=0.04) but we felt a Fischer's Exact Test to be more appropriate due to the low numbers and this suggests a non-significant difference (p=0.071). The larger number of participants allocated to thalidomide led to a greater amount of total patient time being spent taking active treatment than placebo. The total number of new symptoms per patient month was therefore calculated, i.e. total number of symptoms divided by total patient trial days per group (placebo 2229, thalidomide 3047) x 28. There was no significant difference between groups in the rate of infections per patient month.

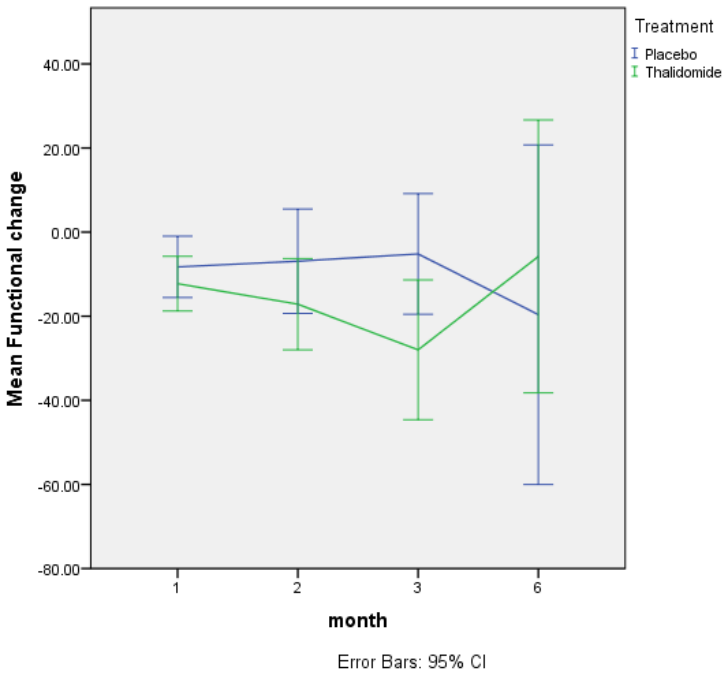
Symptom reported	**Frequency**	
	Thalidomide	Placebo
Anorexia	12	11
Constipation	9	5
Diarrhoea	5	4
Infections total (p=0.071)	12	3
Chest infection	7	1
Urinary tract infection	2	0
Cholangitis	2	1
Cellulitis	0	1
Pyrexia unknown origin	1	0
Fatigue	9	9
Insomnia	4	4
Nausea or vomiting	12	5
Sensory neuropathy	4	3
Pain (Abdo / head / chest / back)	17	14
Rash	7	2
Shortness of Breath	4	2
Weakness	4	3
Other (dysphagia, bloating, dizziness etc)	14	10
Total	113	75
Total per patient day on trial	(113/3047)x28=1.04	(75/2229)x28=0.924

Table 4 Showing all symptoms reported during the course of the trial presented with placebo and thalidomide groups separately

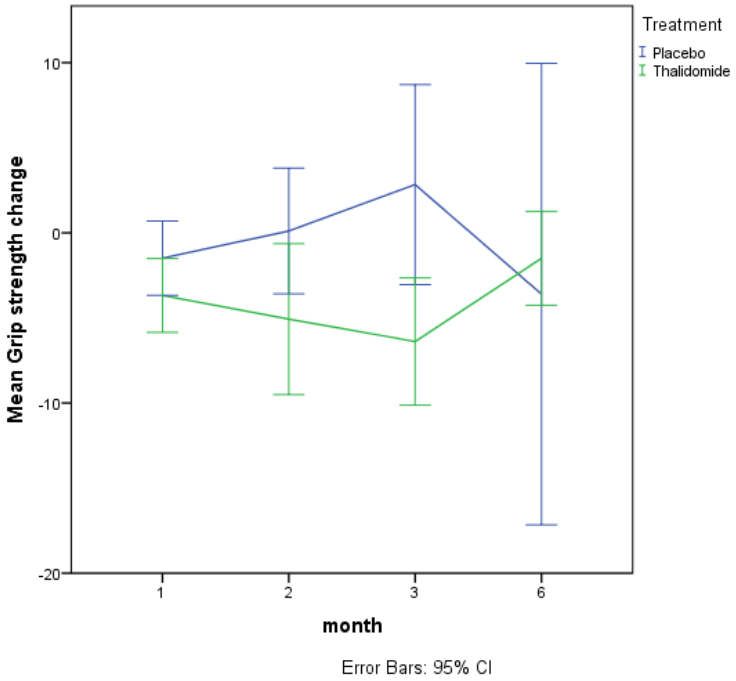
Of the 10 patients who completed the entire 6 months of the trial, two chose to stay on the medication after completion of the trial.

4.4 Overall clinical results

There was no significant difference between groups in the change from baseline in weight, lean body mass (measured by anthropometry, bio-impedance or by DEXA), symptoms or global health score at any time point. At 3 months there was a significantly greater deterioration in functional capacity and in grip strength in the thalidomide group. Neither change was sustained at 6 months.

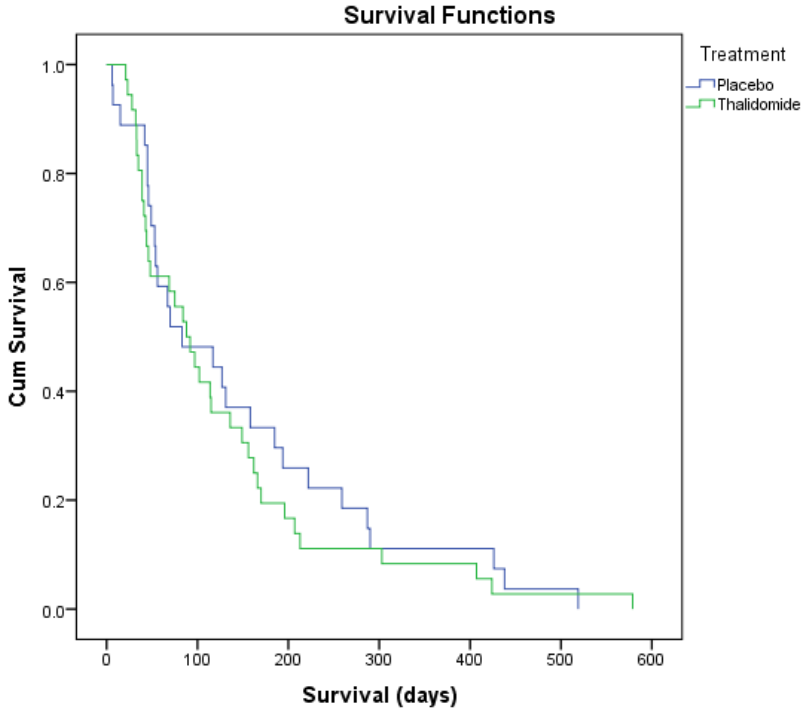


Graph 1 Showing the difference between groups in change of functional QOL from baseline, demonstrating a significantly greater reduction in the thalidomide group at 3 months that is not sustained at 6 months (1 month p=0.403; 2 months p=0.194; 3 months p=0.048; 6 months p=0.513).



Graph 2 Showing the difference between groups in change of grip strength from baseline, demonstrating a significantly greater reduction in the thalidomide group at 3 months that is not sustained at 6 months (1 month p=0.157; 2 months p=0.086; 3 months 0.005; 6 months 0.0.718).

There was no difference in survival time between groups (thalidomide mean 128.0 days s.e. 21.0; placebo mean 147.8 days s.e. 27.090).



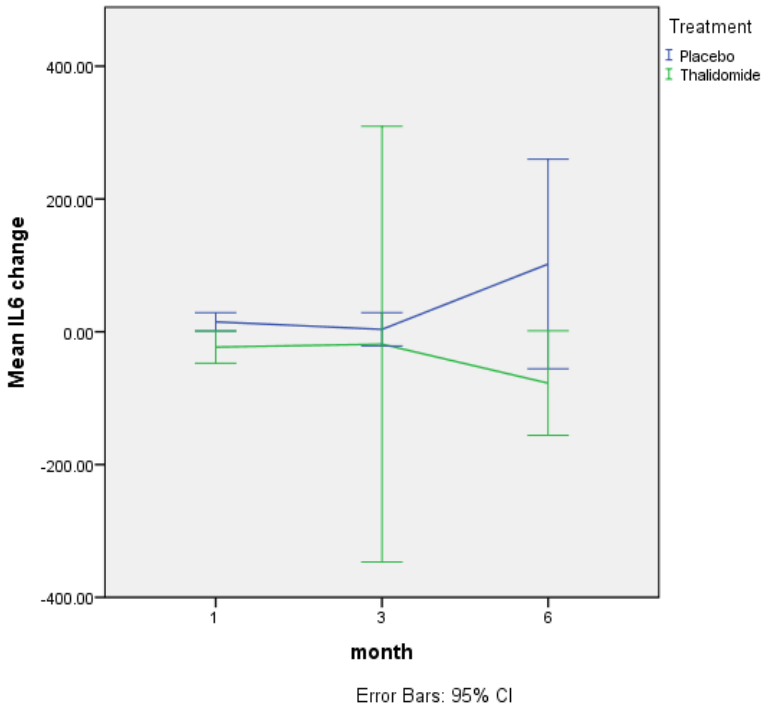
Graph 3 Kaplan Mier survival curve demonstrating no significance difference in survival between groups overall (P=0.509)

4.5 Effect of thalidomide on cytokine levels

4.5.1 Effect of thalidomide on IL-6

IL-6 gradually increased over the course of the trial in the placebo group but in the thalidomide group it gradually decreased from baseline.

There was a significant difference between groups in the change in IL-6 level from baseline at the 1 month (p=0.021) and 6 month (p=0.01) time-points.

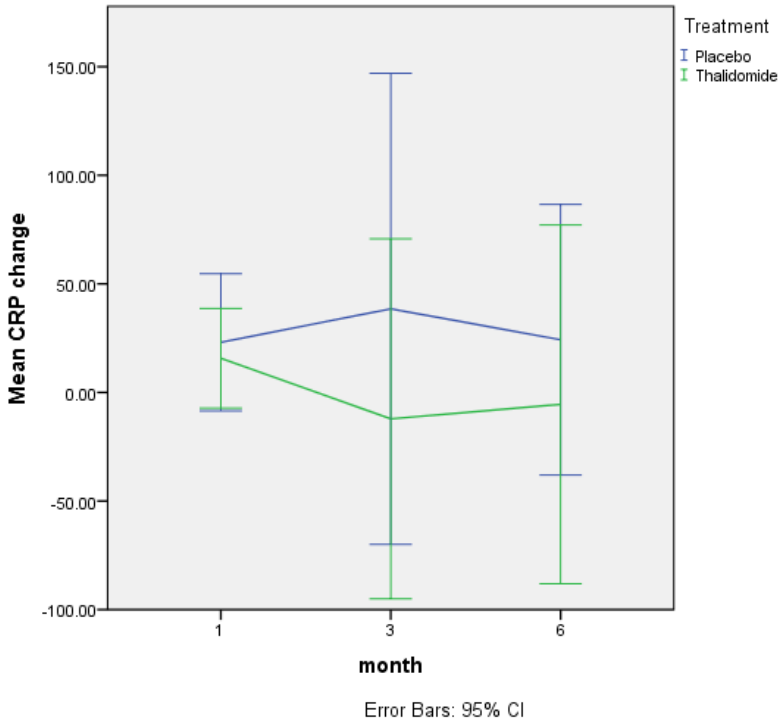


Graph 4 Showing the mean IL-6 change from baseline at each time point with thalidomide and placebo groups presented separately. Levels in the placebo group gradually increase whereas in the thalidomide group IL-6 is reduced from baseline at each time point

In the thalidomide group the baseline level of IL-6 significantly correlated with the fall in IL-6 level at 1 month (r=-0.891, p<0.0001) and at 3 months (r=-0.94, p=0.005), that is the IL-6 dropped more in those who presented with higher levels. There were no correlations in the placebo group.

4.5.2 Effect of thalidomide on CRP

Graphs of change in CRP level from baseline also suggest that thalidomide treatment may have reversed the natural increase in CRP level as the disease progressed but the difference between groups was not significant at any time point



Graph 5 Showing the mean CRP change from baseline at each time point with thalidomide and placebo groups presented separately. Levels in the placebo group gradually increase whereas in the thalidomide group CRP is reduced from baseline at each time point

In the thalidomide group the baseline level of CRP significantly correlated with the fall in CRP level at 1 month (r=-0.627, p=0.002) and almost significantly at 3 months (r=-0.748, p=0.053). There were no correlations in the placebo group.

4.5.3 Effect of thalidomide on other cytokines

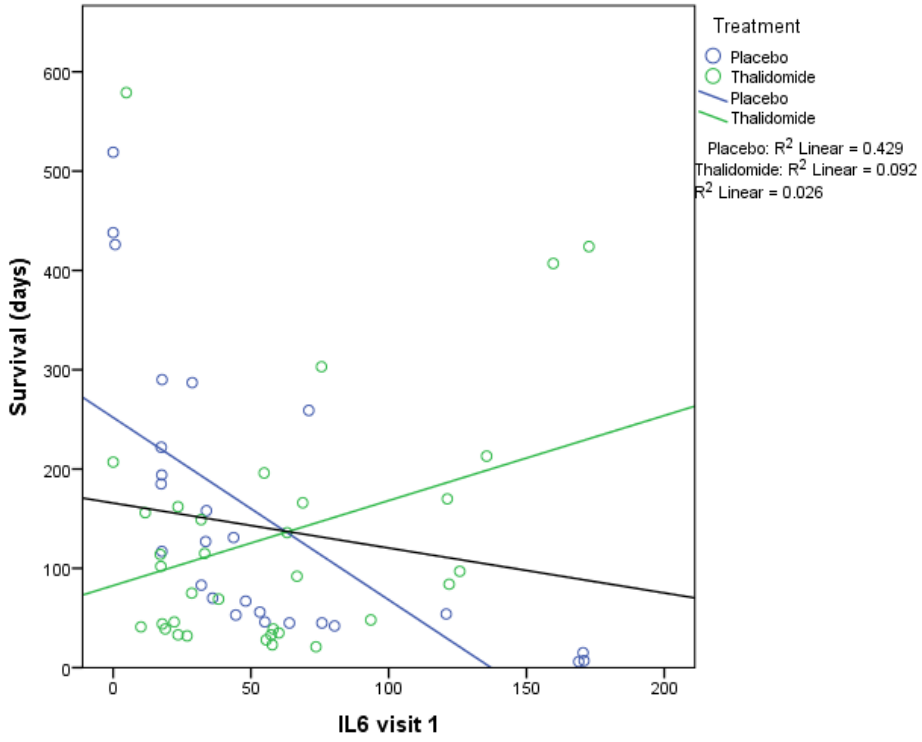
Thalidomide treatment did not have a measureable effect on peripheral levels of IL-1, IL-1ra, TNF-α, TNFR2 and VEGF levels at any time-point.

4.6 The significance of baseline IL-6 and CRP levels on the clinical response to thalidomide

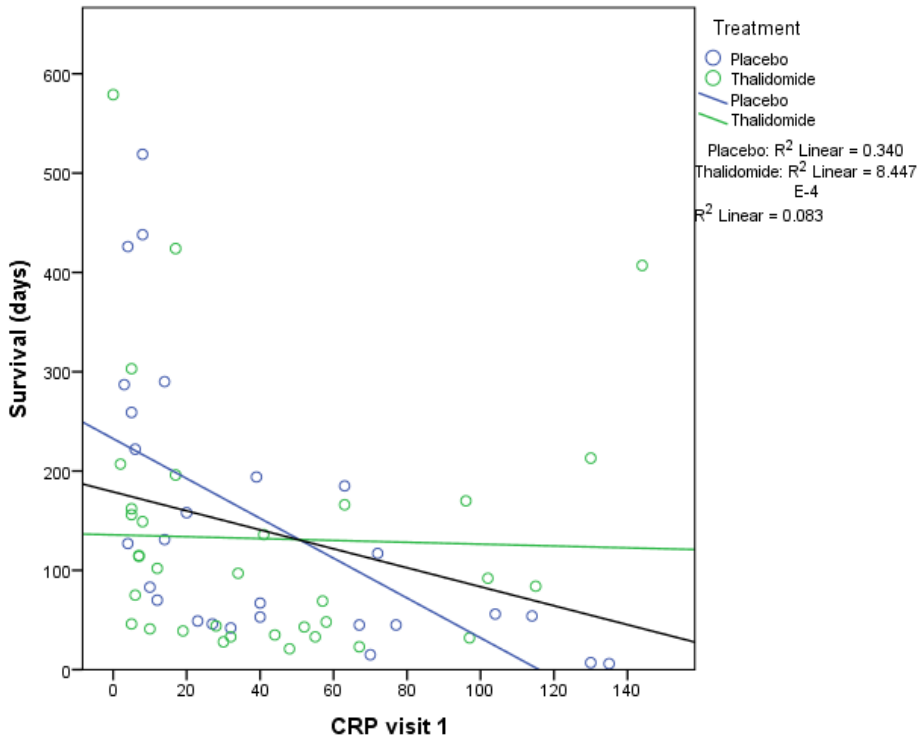
The results presented in this section suggest that the likelihood of responding to thalidomide treatment may be dependent upon the level of the peripheral inflammation at presentation, as evidenced by plasma IL-6 and CRP levels.

4.6.1 Baseline IL-6 and CRP with survival

In the placebo group there was a significant negative correlation between both baseline IL-6 and baseline CRP with survival (r=-0.655, p<0.001 and r=-0.583, p=0.001 respectively). In the thalidomide group this correlation was absent, perhaps even reversed (IL-6 r=0.303, p=0.08; CRP r=-0.029, p=0.870).

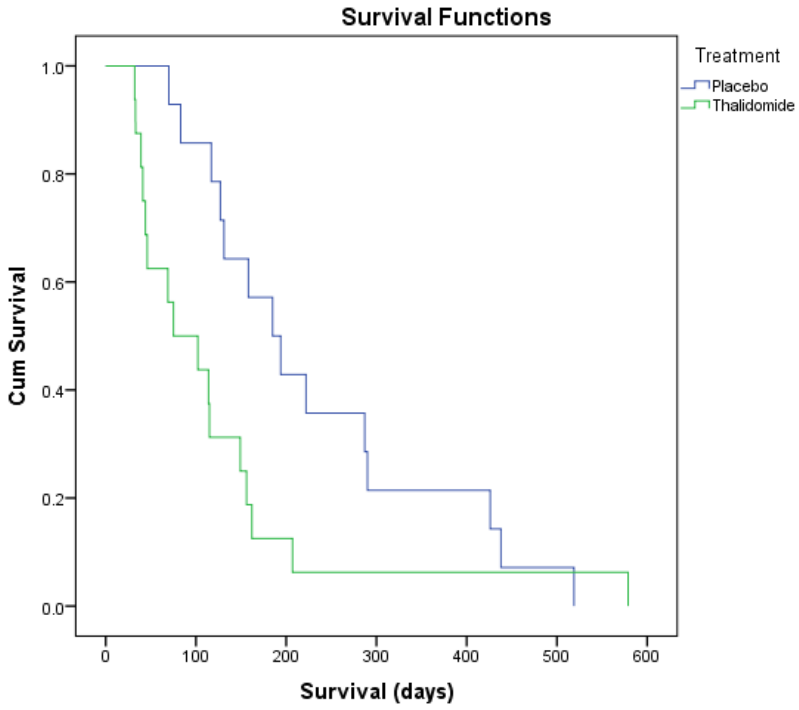


Graph 6 Showing baseline IL-6 levels and survival. In the placebo group higher baseline IL-6 is associated with a shorter survival but in the thalidomide group there is a trend in the opposite direction

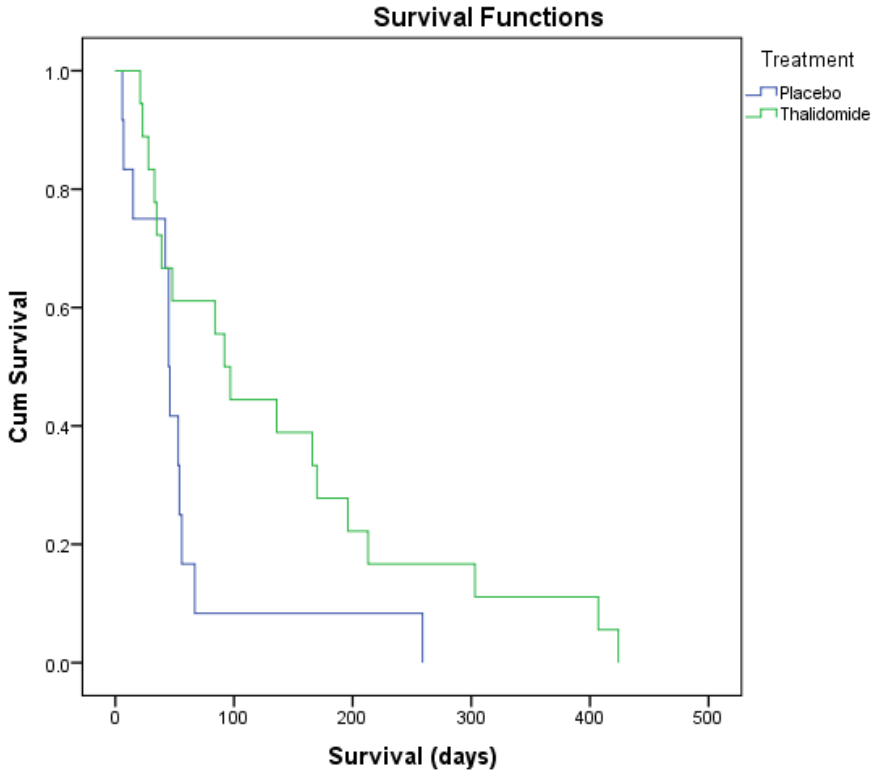


Graph 7 Showing baseline CRP levels and survival. In the placebo group higher baseline CRP is associated with a shorter survival. This association is absent in the thalidomide group

In those participants presenting with IL-6 below the median (</= 44pg/ml), survival was significantly (p=0.04) longer in the placebo group than in the thalidomide group (mean 232 (SD 142) days vs 123 (SD 133) days) whereas for those participants presenting with IL-6 above the median (> 44pg/ml), survival was significantly longer in the thalidomide group than the placebo group (p=0.03) (mean 140 (SD 128) days vs 58 (SD 66) days).



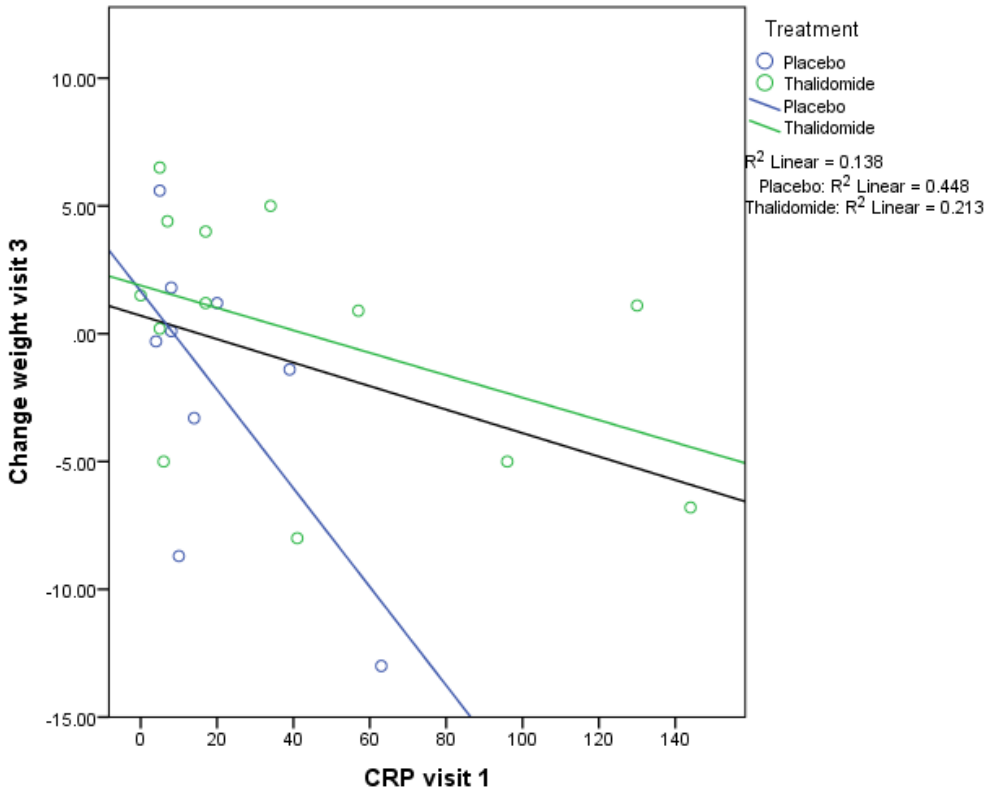
Graph 8 Kaplan Mier survival curve for those presenting with IL-6≤44pg/ml showing a significantly longer survival in the placebo group than the thalidomide group



Graph 9 Kaplan Mier survival curve for those presenting with IL-6>44pg/ml showing a significantly longer survival in the thalidomide group than the placebo group

4.6.2 Baseline CRP and IL-6 with weight

In the placebo group there was a significant correlation between baseline CRP (but not IL-6) and weight loss from baseline at every time point in the trial. There was no correlation between baseline CPR and weight loss in the thalidomide group. There was no association seen with IL-6 level in either group.



Graph 10 Showing a strong correlation between a higher baseline CRP with increased weight loss in the placebo group but no correlation in the thalidomide group. This example is taken at visit 3

Weight change from baseline		Month 1	Month 2	Month 3	Month 6
Placebo group	R	-0.570	-0.669	-0.903	-0.927
	Sig (2 tailed)	0.013	0.049	0.014	0.023
Thalidomide group	R	-0.159	-0.461	-0.760	-0.932
	Sig (2 tailed)	0.458	0.113	0.29	0.068
All patients	R	-0.260	-0.371	-0.633	-0.598
	Sig (2 tailed)	0.096	0.089	0.015	0.089

Table 5 Showing a strong and consistent correlation between baseline CRP and weight loss at every time point in the placebo group but no association in the thalidomide group

4.6.3 Baseline CRP and IL-6 with grip strength and arm muscle area

In the placebo group there was a correlation between a higher baseline CRP and a greater reduction of grip strength at 3 months (r= -0.879, p=021) and 6 months (r= -0.904, p=0.35). There was also a correlation between a higher baseline CRP and greater reduction in arm muscle area at 2 months (r= -0.804, p=0.009), 3 months (r= -0.879, P=0.21) and 6 months (r= -0.894, p=0.41).

In the placebo group there was also a correlation between a higher baseline IL-6 and a greater reduction in both grip strength (r= -0.995, p<0.001) and arm muscle area (r= -0.937, p=0.19) at 6 months.

None of these correlations were present in the thalidomide group.

4.6.4 Baseline IL-6 with QOL and functionality

In those presenting with baseline IL-6 lower than the median (≤44pg/ml), the thalidomide group lost less lean body mass as determined by bioimpedance than the placebo group (significant at 3 months both in absolute terms (p=0.002), and by percentage (p=0.015)). Despite this the thalidomide group showed a non-significant trend (p=0.062) towards greater deterioration in physical functioning QOL scores at 1 month as well as a greater deterioration in functional QOL scores (p=0.059) and a trend towards a greater reduction in grip strength (p=0.06) at 3 months.

In those presenting with IL-6 above the median (>44pg/ml), there was a significantly greater reduction in grip strength in the thalidomide group than the placebo group at 3 months (p=0.027) and a tendency to a greater reduction in functional QOL (p=0.075) at the same time point despite the improved survival in this group. There was no difference in change of LBM between groups.

4.6.5 Summary effects of thalidomide in high and low IL-6 groups

The following graphs demonstrate that thalidomide reduced QOL and functionality as well as reducing survival time in those with low IL-6 at presentation. In those presenting with a high IL-6 it was associated with increased survival time and a reduction in weight loss but still caused reduced QOL and functionality.

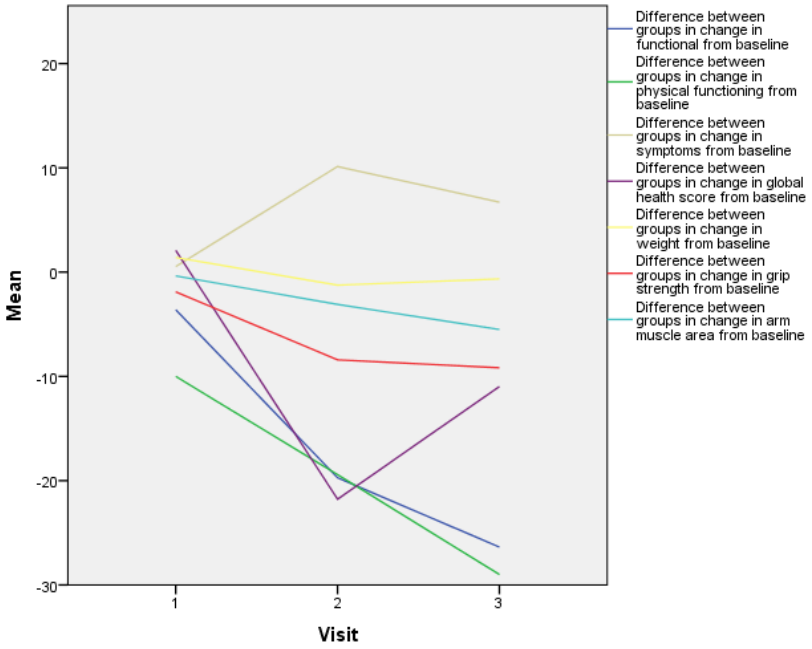


Figure 22 <u>All patients</u>. Showing the difference between groups in the change in a variety of key QOL and functionality scores. Every measured outcome deteriorates more in the thalidomide than in the placebo group (a higher symptom score indicates a worsening of symptoms)

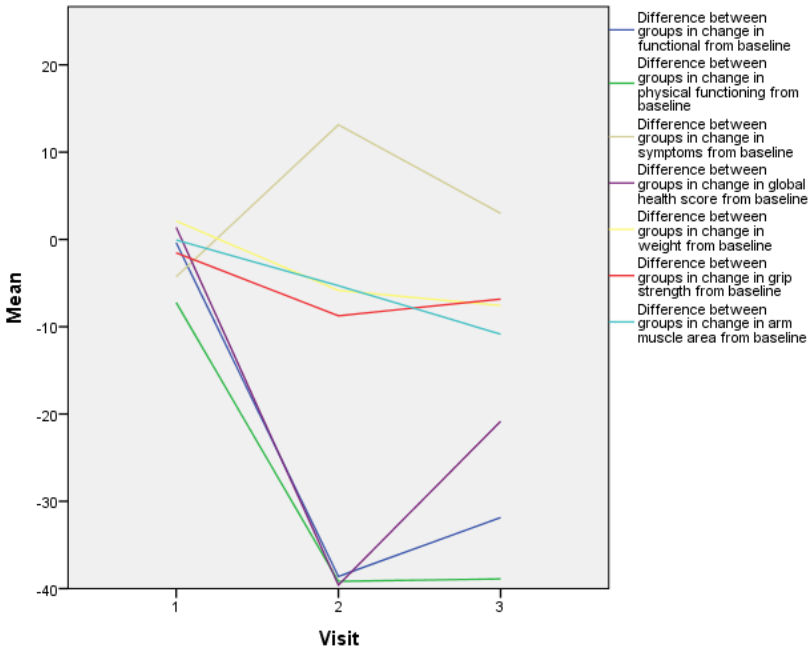


Figure 23 <u>Low IL-6 (<=44pg/ml) patients only.</u> The difference between groups in the change in a variety of key QOL and functionality scores. Every measured outcome deteriorates more in the thalidomide than in the placebo group (a higher symptom score indicates a worsening of symptoms). There is a similar pattern of great deterioration in all measured outcomes in those taking thalidomide but the effect is more dramatic in those presenting with a less inflammatory picture

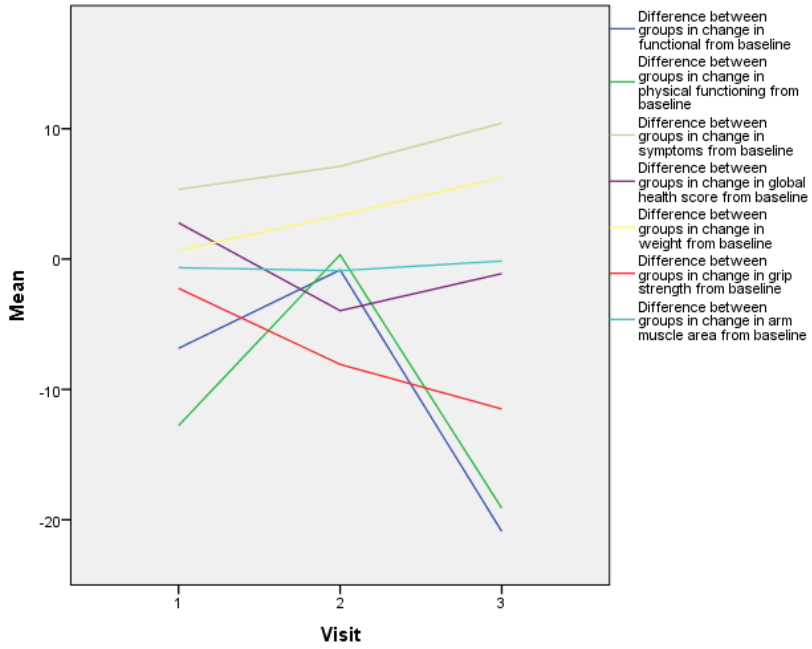
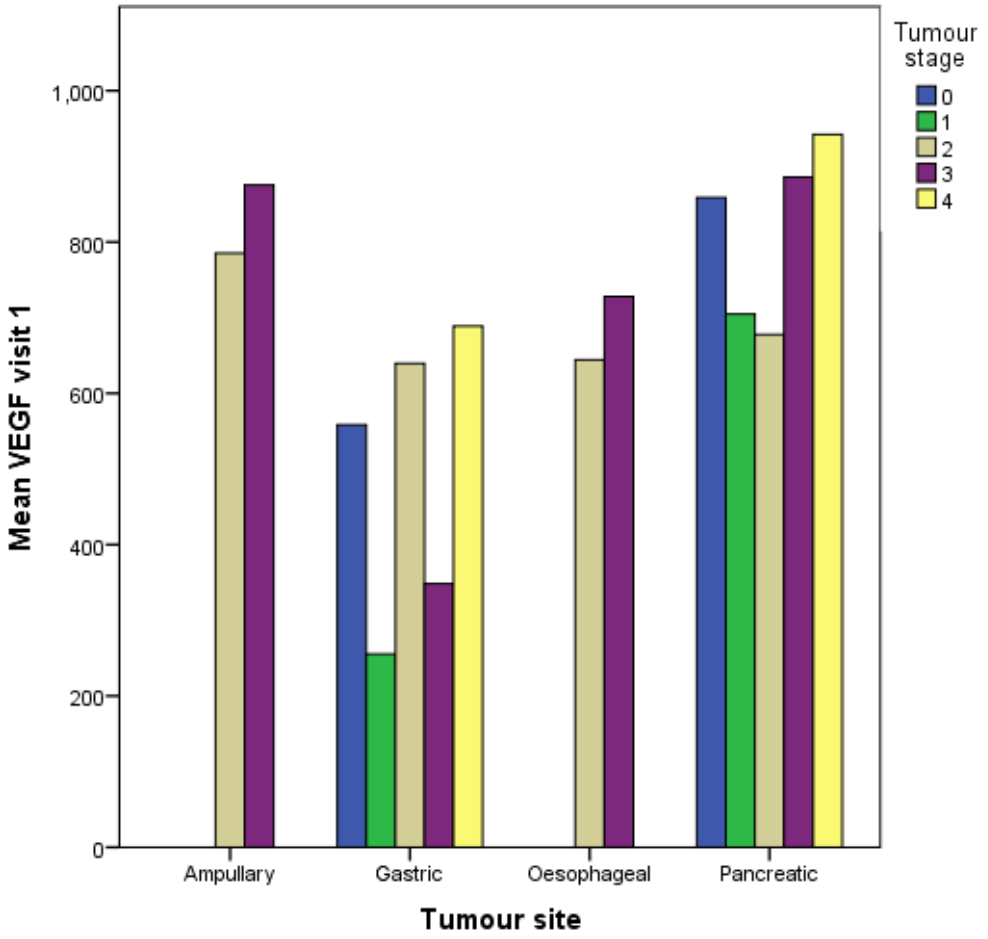


Figure 24 <u>High baseline IL-6 (>44pg/ml) patients only.</u> The difference between groups in the change in a variety of key QOL and functionality scores. Every measured outcome deteriorates more in the thalidomide than in the placebo group (a higher symptom score indicates a worsening of symptoms). The difference between groups is smaller in this subgroup presenting with a more inflammatory picture and there is less weight loss in the thalidomide group than the placebo group, overall the group taking thalidomide have a greater deterioration in all other measured outcomes

4.7 VEGF

Baseline VEGF levels were significantly (p=0.39) higher in those with a pancreatic than in those with a gastric primary and were significantly increased with more advanced tumour stage (p=0.004), metastatic stage (p=0.015) and overall stage (p<0.001) (505). There was no difference in VEGF levels between treatment groups over time.



Graph 11 Showing baseline VEFG levels. Levels are significantly higher in pancreatic cancer than in gastric cancer and increased with more advanced tumour stage

There was no other detectable association between any of the other measured cytokines (TNF-α, sTNF RII, IL-1b, IL1RA) and measured clinical parameters.

4.8 Validating methods of LBM measurements

Three methods of measuring body composition (DEXA, bio-impedance and anthropometry) were used. DEXA is considered the gold standard. Bio-impedance and DEXA are different methods of measuring the same and results should be identical. Anthropometry assessed arm muscle area and will therefore produce different results but these should be proportionally similar to LBM measured by DEXA.

Measurements were taken at five different time points. Data from all time points were pooled for a single comparative analysis.

All methods of measurement of LBM correlated to a high significance level, both as an absolute value (grams) and by percentage.

Correlations

		Arm muscle Area	Bio LEAN (kg)	DEXA Lean (kg)
Arm muscle Area	Pearson Correlation	1	.537**	.546**
	Sig. (2-tailed)		.000	.000
	N	152	133	38
Bio LEAN (kg)	Pearson Correlation	.537**	1	.561**
	Sig. (2-tailed)	.000		.001
	N	133	135	33
DEXA Lean (kg)	Pearson Correlation	.546**	.561**	1
	Sig. (2-tailed)	.000	.001	
	N	38	33	39

**. Correlation is significant at the 0.01 level (2-tailed).

Table 6 Showing the highly significant correlation between results of all three methods of LBM per gram measurement

Correlations

		Arm muscle Area	Bio LEAN %	DEXA Lean %
Arm muscle Area	Pearson Correlation	1	-.096	-.599**
	Sig. (2-tailed)		.273	.000
	N	152	133	36
Bio LEAN %	Pearson Correlation	-.096	1	.622**
	Sig. (2-tailed)	.273		.000
	N	133	135	31
DEXA Lean %	Pearson Correlation	-.599**	.622**	1
	Sig. (2-tailed)	.000	.000	
	N	36	31	37

**. Correlation is significant at the 0.01 level (2-tailed).

Table 7 Showing the highly significant correlation between results of all three methods of LBM as a percentage of total body mass

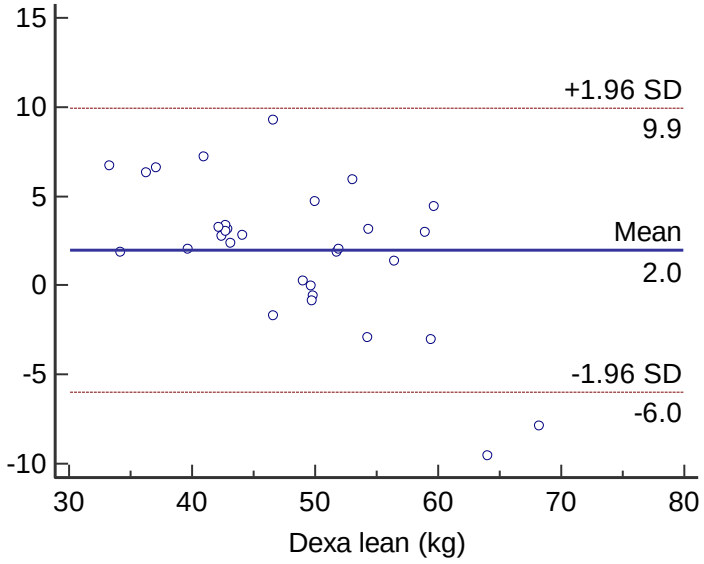
The paired t-test suggested that bioimpedance measured LBM measurements were on average 2kg less than those measured by DEXA (Mean -2.0kg, SD -3.4, -0.5kg, p=0.12).

Paired Samples Test

	Paired Differences			Sig. (2-tailed)
	Mean	95% Confidence Interval of the Difference		
		Lower	Upper	
Bio LEAN - DEXA Lean (kg)	-1.95458	-3.44636	-.46279	.012

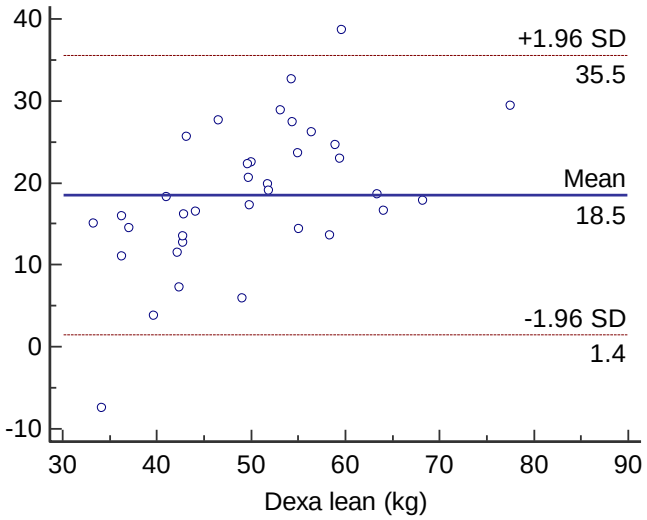
Bland Altman plots verify the 2 kg average underestimation of LBM by bio-impedance. Bland Altman limits of agreement are -9.9kg, 6.0kg. Bio-impedance tended to over-estimate LBM in those with high LBM and underestimate LBM in those with less LBM as measured by DEXA.

Figure 25 Bland Altman plot comparing DEXA and bio-impedance



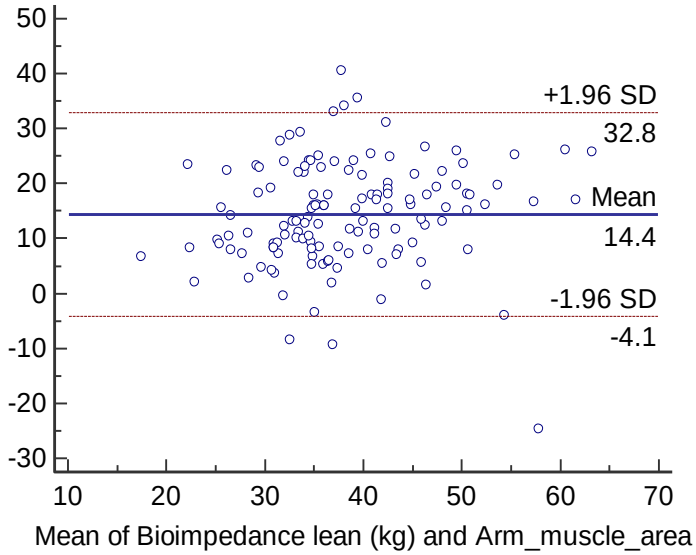
The mean difference between arm muscle area and DEXA was 18.5. This in itself is of little relevance, the two measures are not the same and were not expected to produce identical or even similar results. The wide range in the standard deviation (S.D. 1.4, 35.5) is more concerning with anthropometry tending to overwstimate more as the DEXA measured LBM increases.

Figure 26 Bland Altman plot comparing DEXA and arm muscle area



Comparing anthropometry with bio-impedance produces similar results to comparing it with DEXA. The mean difference is 14.4 with standard deviation -4.1-32.8.

Figure 27 Bland Altman plot comparing bioimpedance and arm muscle area



ICC values between 0.7 are generally taken to indicate reasonable agreement. Values above 0.8 are considered good and those above 0.9 are considered excellent. The values presented below (Table 8) suggest an excellent (but not perfect) agreement between the values obtained for measurement of LBM by bioimpedance and by DEXA. Anthropometry in our hands showed a statistically reasonable agreement with

LBM measured by both DEXA and by bioimpedance but we felt not to a clinically acceptable level in the context of this trial.

Table 8 ICC values for LBM measurement methods

ICC value	Bioimpedance (kg) vs DEXA (kg)	Anthropometry (cm^2) vs DEXA (kg)	Bioimpedance (kg) vs anthropometry (cm^2)
Single measures	0.92 (0.84, 9.61)	0.59 (0.33,0.77)	0.53 (4.0, 6.5)
Average measures	0.96 (0.92, 0.98)	0.74 (0.49, 0.87)	0.70 (0.57, 0.78)

ICC value (95% confidence interval)

Chapter 5 Discussion

5.1 Overall

We found thalidomide to be better tolerated than in a recently published study by Wilkes(506). This may have been because we suggested patients should increase the dose gradually as tolerance allowed rather than starting immediately at 200mg, although there was no measureable relationship of side effects to drug dose. The terminally ill patients in the trial often had multiple symptoms and it was inevitably a matter of judgement which were expected as part of their disease process and which could be a side effect of the drug. We therefore grouped relatively minor symptoms into categories to assess whether one group was experiencing a greater number than the other. The only possible difference was an increase in reported infections in the thalidomide group, not an expected finding. The difference in absolute number of infections between groups was significant using the Chi Squared test ($p=0.04$) but non-significant when analysed using the more appropriate Fischer's Exact Test ($p=0.071$) and it is likely that this was a chance finding. When time spent on the medication was taken into account, there was no difference in infections between groups. The overall number of new reported symptoms per patient per trial month were remarkably similar between groups – 0.924 in the placebo group and 1.04 in the thalidomide group. Of the 10 patients who completed the trial (4 active, 6 placebo) only two (both active) chose to stay on the study medication. These decisions will have been influenced by our counsel though and we generally advised people that the known long-term side effects may well outweigh any unknown but potential long-term benefits.

Thalidomide had little if any effect on survival, QOL or cachexia overall. All non-significant trends were suggestive of thalidomide conferring harm rather than benefit. For those patients with a low inflammatory burden at presentation thalidomide reduced survival, and increased the deterioration in QOL. For those presenting with a high IL-6 the effects were more mixed. In this group thalidomide treatment was associated with increased survival and a reduction in weight loss. Disappointingly this did not translate into clinical benefit. The reduction in weight loss was not echoed by

a reduction in LBM loss and QOL was worse in the thalidomide group, although to a lesser extent than it was in the patients with a lower inflammatory burden. We hypothesise that the anti-inflammatory effect of the drug had some benefit in those who had inflammation to treat but that the know side effect of somnolence reduced activity levels across the board leading to reduced QOL. Those without an inflammatory burden did badly in every measured outcome with thalidomide as they had nothing to gain from the anti-inflammatory effects of the drug. Although the improved survival and reductions in weight loss in the thalidomide treated group with high inflammatory burden are interesting from a scientific perspective, in reality few people with incurable cancer would feel that gains in survival are worth a decrease of QOL in the time they have remaining.

Thalidomide inhibits production of TNF-α but has no influence on IL-6 production from monocytes stimulated with lipopolysaccharide in vitro(449). Our study showed a significant reduction in IL-6 serum levels by 4 weeks after thalidomide treatment but no change in peripheral TNF-α levels. This could be a direct in vivo effect or could be the final result of a complex cytokine cascade. Equally, mononuclear cells taken from the blood of people with pancreatic cancer have been shown to produce larger amounts of TNF-α if taken from a patient with an acute phase response (as evidenced by elevated CRP)(227). Perhaps, by reducing this acute phase response, thalidomide is dampening down TNF-α production at a local level, not detectable in the serum.

High CRP and IL-6 levels have previously been associated with a variety of negative outcomes in a variety of circumstances (507;508) including degree of cachexia(509) and shortened survival in pancreatic cancer patients(510). In our placebo group, higher CRP and IL-6 levels were associated with reduced survival, greater loss of grip strength, weight and arm muscle area. In our thalidomide group these correlations were not present suggesting a possible protective effect for those patients with a high inflammatory burden who would otherwise be expected to have a poor prognosis. IL-6 is also known to be a potent tumour growth factor (511-513), so it is possible that by reducing IL-6 levels the thalidomide produced benefits through an anti-tumour action instead or as well as an anti-cachexia action. It seems less likely that this would have had a large influence as the survival times for most patients on this trial are were

probably too short to allow a substantial benefit and previous murine trials of anti-IL-6 antibodies have been unsuccessful in slowing tumour growth(190)

The median baseline IL-6 of 44.1pg/ml was identified as a cut off, under which survival seemed to be shortened in those taking thalidomide but above which it seemed to be lengthened. In this trial, those who seemed to benefit from thalidomide were not therefore those with a more obviously cachexic picture (as evidenced by recent weight loss or reduced strength, lean body mass or quality of life at presentation) but those with higher inflammatory cytokines. Perhaps the benefits of thalidomide in cachexia suggested by previous trials (514-517), have been mediated by a reduction of the inflammatory drivers of this condition. Cachexia is a complex, multi-factorial condition only partly driven by inflammation. If there is not a large inflammatory component to an individual's cancer related weight loss there may be nothing to gain, or even something to lose, from taking an anti-inflammatory drug with potentially harmful side effects.

5.2 Validating methods of LBM measurements

Anthropometry, bio-impedance and DEXA scanning were used with DEXA being considered the gold standard but not available for each trial visit. Results were highly correlated for all methods ($p \leq 0.001$). Bioimpedance overestimated the LBM measurement by an average of 2kg. ICC values suggested excellent, although not perfect, agreement between bioimpedance and DEXA which we felt was clinically acceptable. ICC values comparing anthropometry with DEXA (or with bioimpedance) were only just reasonable. Bland-Altman limits of agreement comparing bioimpedance to DEXA were not narrow, suggesting that the two methods are not interchangeable. Bioimpedance was therefore taken as the primary method of LBM measurement for the purposes of the trial.

Bioimpedance works on the principle that impedance of current flow through the body is dependent on internal structures such as cell membranes and different tissues. Different current frequencies will be impeded differently so by applying impedance at varying mHz and comparing results, equations can be developed to provide an estimate of body composition and cell membrane permeability, Bodystat®'s 'Illness

Marker'(518). Equations developed on the results for healthy volunteers with not be directly transferable to an unwell population and no specific equations have yet been developed in terminal cancer patients. Previous investigators have also found bioimpedance to be an imperfect measure of LBM in this patient population (519). Accuracy of bioimpedance measurements can be improved by taking the reading prior to food consumption(520) but we felt it would be unethical to in any way restrict food intake in our patient group so did not take this into account. Equations specific to this group are under development and will increase accuracy in the future (see 5.5).

5.3 Recruitment difficulties

The overwhelming difficulty in running this trial was slow recruitment. It is well documented that there are particular challenges in recruiting to palliative care trials including the nature of the patient population, the high prevalence of cognitive problems and the unstable nature of the disease process(521;522). Gatekeeping, that is clinical staff preventing access to potential candidates, is also a well known problem(523). Many medical staff consider it unethical to include palliative patients in clinical trials(524). It is difficult to exactly quantify the influence of these factors on our difficulties but we certainly found that, despite using a number of methods to locate eligible patients, the number we were able to find was far less than that our predictions suggested. A number of patients mentioned during the consent process that although their consultant had suggested they consider the trial, some members of the nursing staff had advised them against it, presumably in an understandable attempt to protect their patient from unnecessary interference. There were times during the trial when the front line investigators felt uncomfortable, usually when taking clinical measurements from people who very obviously had a short time to live and would generally be having any unnecessary tests or treatments withdrawn. A bigger problem was the introduction of gemcitabine. The trial was conceived at a time when a large number of people with a new diagnosis of upper gastrointestinal adenocarcinoma had no real palliative options available. The beginning of the trial coincided with the introduction of gemcitabine as a palliative chemotherapy. The population of potential participants therefore reduced but it also changed the dynamic of the recruitment process. During our previous trial people diagnosed with inoperable pancreatic cancer had no other treatment options and were often given information on the trial at initial

diagnosis. The introduction of gemcitabine meant there were other options available. This meant that only those who were very sure they did not want to consider this could be recruited immediately and by their nature people refusing palliative chemotherapy are also likely to refuse a clinical trial. Most people went into the multidisciplinary team (MDT) meeting process whereby their treatment options are discussed at a weekly meeting. After that meeting, if gemcitabine was felt appropriate they would require a pancreatic biopsy for histological proof of the diagnosis, usually taking 1-3 weeks for the procedure to be completed and results to be available. Gemcitabine could then be started. A number of people would then stop the gemcitabine due to side effects and become eligible for our trial. However, by that time their already short prognosis would be substantially shorter and often they would have deteriorated significantly or died; often they would have simply had had enough of medical interventions and be unwilling to even consider the trial and by that stage they will have made a definite decision to stop all active interventions, meaning that they wouldn't have any need for further medical outpatient appointments and so become inaccessible. It is noticeable that another group running a similar trial using thalidomide in terminal oesophageal cancer(525) at around the same time were having similar recruitment difficulties(526).

The trial recruited slower than predicted and there were a number of repercussions, mainly that we planned to recruit 180 patients but pragmatically stopped at 63 rather than allow it to continue with slow progress.

During the trial, the British drug company that agreed to supply our trial medications was bought out by another much larger American company. Although contracts were honoured, communication was much more difficult with the new company and mistakes were made when replenishing drug, namely that blocks of four should have been re-supplied until all trial numbers in that block were used but in the event new blocks of four were supplied. This wouldn't have caused a problem if the trial had fully recruited as blocks would subsequently been filled but because it was stopped early caused an unequal allocation of patients to active and placebo groups. Had we been able to recruit faster, the trial would have been complete before the takeover and this would have been avoided.

Thanks to support from the Moulton Charitable Trust and the National Cancer Research Network (NCRN), there was a generous amount of investigator and research nurse time available for the trial but only for a limited period of time. The drawn out recruitment process meant that the PI had to return to part-time clinical work and the research nurses had to dedicate more of their time to other trials. This probably resulted in potential recruits being missed.

The lower number of participants recruited led to an underpowered trial. Trials stopped early due to interim analyses showing overt efficacy are prone to over-estimation of beneficial effect(527). As this trial was stopped early simply due to slow recruitment it is unlikely that positive findings are over estimated but negative findings may be false.

5.4 Funding

This trial was funded by a generous research grant from the Moulton Charitable Trust (registered charity number 1109891). The National Cancer Research Network supported the time of the research nurses. Thalidomide and placebo were supplied free of charge from Pharmion Plc.

5.5 Future directions

Bodystat® are currently using our data to develop software equations to provide more accurate LBM measurements in this patient population.

Further clinical trials using thalidomide in this patient population are unlikely to be conducted, partly because the marketing strategy of the drug company is towards Lenalidomide, a newer thalidomide analogue with a more favourable side effect profile, and towards haematological disease, meaning that they would be reluctant to supply thalidomide as a trial medication for this purpose. Our results suggest that those patients with a high inflammatory burden benefit from anti-inflammatory intervention and that a medication with a wide range of anti-inflammatory effects has a benefit not previously delineated in treatments with more specific influences. This lends support to the recent tendency to use a combination of therapies to control cancer cachexia.

Future trials in cancer cachexia should measure inflammatory cytokines when possible and clarify whether this effect is reproducible. If so it may be possible in the future to predict those patients likely to benefit from the wide range of anti-inflammatory therapies available.

Any future trials need to be carefully planned with particular thought given to likely extreme recruitment difficulties in this patient population and the ethical dilemmas involved in running any sort of clinical trial with participants at the very end of their lives.

Chapter 6 Conclusions

In incurable upper gastrointestinal cancer patients, presenting with an acute inflammatory response predicts a poor prognosis in terms of survival, functional quality of life, grip strength and symptomatology.

Thalidomide treatment of this population leads to a reduction in serum IL-6 levels, particularly marked in those presenting with high IL-6 levels at baseline. Overall thalidomide does not affect survival but leads to a significant deterioration in functional quality of life, grip strength and an increase in negative symptoms.

Thalidomide treatment in those presenting with a low inflammatory burden is associated with worse outcomes for survival, weight loss and QOL. In those with a high inflammatory burden it is associated with a survival advantage and a reduction in weight loss but still a reduction in QOL which to most patients with terminal cancer is their primary concern.

Chapter 7 Reference list

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Chapter 8 Appendices

8.1 Establishing the IL-6 ELISA SOP

8.1.1 Il-6 Checkerboard 11.10.2006

Manufacturers suggested ranges:

Capture antibody: 2-8µg

Detection antibody 100-400ng

These suggested ranges assume use of a 96 well plate. We found we often required different doses for the 384 well plate. The following checkerboard was set up to ascertain the optimal antibody pair using 2 or 4µg of capture antibody and between 16-1000ng of detection antibody.

Figure 28 Layout for IL-6 checkerboard 11.10.2006

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Detection
A																									1000ng/ml
B																									500ng/ml
C																									250ng/ml
D																									125ng/ml
E																									62.5ng/ml
F																									31.25ng/ml
G																									16ng/ml
H																									0
I																									0
J																									16ng/ml
K																									31.25ng/ml
L																									62.5ng/ml
M																									125ng/ml
N																									250ng/ml
O																									500ng/ml
P																									1000ng/ml
	4ug/ml			2ug/ml			4ug/ml			2ug/ml			Capture concentration												

100pg/ml

50pg/ml
10pg/ml
0pg/ml

All standards in pure undiluted plasma

Table 9 IL-6 checkerboard results

												standard concentration pg/ml:	100	50
All samples in my pure undiluted plasma													10	0
RLUs			1	2	3	4	5	6	7	8	9	10	11	12
	detection antibody		capture antibody 4ug/ml			capture antibody 2ug/ml			capture antibody 4ug/ml			capture antibody 2ug/ml		
A	100%	1000	953780	1000780	955918	555266	704869	681228	446806	457974	429416	313565	328100	278126
B	50%	500	956665	974490	883084	558488	619479	609549	409012	431814	418417	277220	295182	248982
C	25%	250	866072	917136	848880	434973	628191	552558	339084	383969	396114	256130	269024	237450
D	12.50%	125	784290	830970	787925	392971	589083	550378	331392	367785	370620	244432	251842	222642
E	6.25%	63	678099	784769	763078	409523	537802	534337	300651	343601	333861	228530	208196	165607
F	3.13%	31	558515	705004	658536	395604	475012	471889	261416	313506	267380	183415	161966	126058
G	1.56%	16	2303	2206	1717	1113	1418	1840	1351	1390	998	1123	1330	1641
H	0.00%	0	1950	1350	1166	768	893	1103	960	948	841	891	982	1218
I	100%	1000	124479	128888	120770	70667	85850	82143	27083	32759	26134	20483	19196	16532
J	50%	500	96120	91831	90769	46273	53970	58247	8585	9938	7625	5568	7076	6041
K	25%	250	82758	80399	76075	38300	46797	48799	4678	6077	4588	3130	3178	3609
L	12.50%	125	66205	74228	73540	40521	51276	45037	4039	5268	2817	2216	2101	3382
M	6.25%	63	64254	67148	67452	36792	42510	39409	2486	2928	2103	1672	1395	2118
N	3.13%	31	55922	56154	51984	30762	34536	32924	1564	2256	1650	1500	1465	1998
O	1.56%	16	1557	1593	1038	1097	1218	986	837	1117	1003	870	1194	2453
P	0.00%	0	1968	1713	1039	1019	1235	1036	794	1138	1030	952	1250	2474
			capture antibody 4ug/ml			capture antibody 2ug/ml			capture antibody 4ug/ml			capture antibody 2ug/ml		
averages			average	st error	% error	average	st error	% error	average	st error	% error	average	st error	% error
	100%	1000	970159.3333	18766.48184	1.934371107	647121	56867.08182	8.78770459	444732	10176.34384	2.288196901	306597	18176.42618	5.928442279
	50%	500	938079.6667	34262.40775	3.652398509	595838.6667	23140.39164	3.88366733	419747.6667	8102.80218	1.930398385	273794.6667	16468.29805	6.014835223
	25%	250	877362.6667	25103.08496	2.861198216	538574	68847.52382	12.78329883	373055.6667	21241.83206	5.694011365	254201.3333	11225.39944	4.415948294
	12.50%	125	801061.6667	18360.07364	2.291967573	510810.6667	73447.80302	14.37867449	356599	15468.58014	4.337808053	239638.6667	10732.94073	4.478801721
	6.25%	63	741982	39864.79109	5.37274369	493887.3333	51676.91511	10.46330036	326037.6667	15922.92425	4.883768312	200777.6667	22705.72322	11.30888888
	3.13%	31	640685	52932.74238	8.261898183	447501.6667	31799.87529	7.106090916	280767.3333	20158.83857	7.179908836	157146.3333	20492.41631	13.04034009
	1.56%	16	2075.333333	222.0972009	10.70176041	1457	258.1404656	17.71725914	1246.333333	152.6963217	12.25164389	1364.666667	184.3669349	13.51003431
	0.00%	0	1488.666667	289.9011326	19.47387814	921.3333333	119.7044973	12.99252865	916.3333333	46.32673814	5.055664402	1030.333333	119.3405491	11.58271263
	100%	1000	124712.3333	2873.700953	2.304263641	79553.33333	5597.349924	7.035971579	28658.66667	2533.248935	8.839381694	18737	1424.88789	7.604674656
	50%	500	92906.66667	2003.259136	2.156206016	52830	4290.621692	8.121562922	8716	821.6942862	9.427424119	6228.333333	545.3605841	8.756123908
	25%	250	79744	2396.600822	3.005368206	44632	3941.615722	8.831367006	5114.333333	590.3686701	11.543414	3305.666667	186.5265843	5.642631371
	12.50%	125	71324.33333	3144.361329	4.408539389	45611.33333	3818.697444	8.372255676	4041.333333	866.5605384	21.44244156	2566.333333	501.1438583	19.52762144
	6.25%	63	66284.66667	1248.160513	1.883030535	39570.33333	2024.03092	5.115021153	2505.666667	291.9300715	11.65079439	1728.333333	257.9363617	14.92399392
	3.13%	31	54686.66667	1657.069904	3.030116855	32740.66667	1339.025267	4.089792309	1823.333333	266.6920821	14.62662242	1654.333333	210.8154801	12.7432287
	1.56%	16	1396	219.5984973	15.73055138	1100.333333	82.04978164	7.456811418	985.6666667	99.56237576	10.10101885	1505.666667	591.3223881	39.27312739
	0.00%	0	1573.333333	339.4041347	21.5722967	1096.666667	84.92447625	7.743873214	987.3333333	124.3972133	12.59931262	1558.666667	570.3408338	36.59158472

Table 10 IL-6 checkerboard with relevant background subtracted

		4 ug/ml	2 ug/ml	4 ug/ml	2 ug/ml
100%	1000	941500.6667	628384	416073.3333	287860
50%	500	929363.6667	589610.3333	411031.6667	267566.3333
25%	250	872248.3333	535268.3333	367941.3333	250895.6667
12.50%	125	797020.3333	508244.3333	352557.6667	237072.3333
6.25%	63	739476.3333	492159	323532	199049.3333
3.13%	31	638861.6667	445847.3333	278944	155492
1.56%	16	1089.666667	-48.66666667	260.6666667	-141
0.00%	0	501.3333333	-637.3333333	-71	-528.3333333
100%	1000	96053.66667	60816.33333		
50%	500	84190.66667	46601.66667		
25%	250	74629.66667	41326.33333		
12.50%	125	67283	43045		
6.25%	63	63779	37842		
3.13%	31	52863.33333	31086.33333		
1.56%	16	410.3333333	-405.3333333		
0.00%	0	586	-462		

Table 11 IL-6 checkerboard signal : noise ratio

100%	1000	4 ug/ml	2 ug/ml	4 ug/ml	2 ug/ml
50%	500	33.85221457	34.5370657	15.51823765	16.36318514
25%	250	107.6273138	95.6658282	48.15829127	43.95953974
12.50%	125	171.5497621	162.9244731	72.94316626	76.89865887
6.25%	63	198.2171726	199.0429926	88.23795777	93.37784128
3.13%	31	296.1215911	285.7593057	130.1201277	116.1683703
1.56%	16	351.38117	270.5027201	153.9857404	94.99073141
0.00%	0	2.105512344	0.967677662	1.26445722	0.906353775
100%	1000	1.507765024	0.591103507	0.928089129	0.661035073
50%	500	4.351644645	4.245788191		
25%	250	10.65932385	8.482204977		
12.50%	125	15.59225706	13.50166381		
6.25%	63	17.6487133	17.77295753		
3.13%	31	26.45390448	22.89508197		
1.56%	16	29.99268739	19.79085231		
0.00%	0	1.416300304	0.730794775		
		1.593517893	0.703592814		

Figure 29 RLUs emitted by detection antibody concentration, standard concentration 100pg/ml

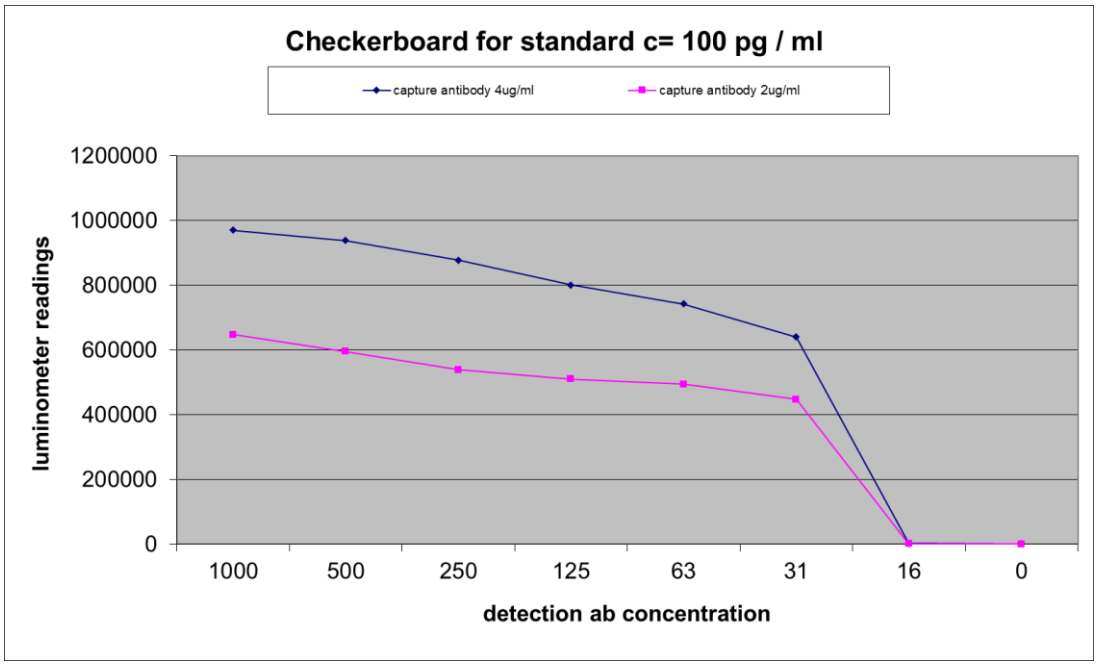


Figure 30 RLUs emitted by detection antibody concentration, standard concentration 50pg/ml

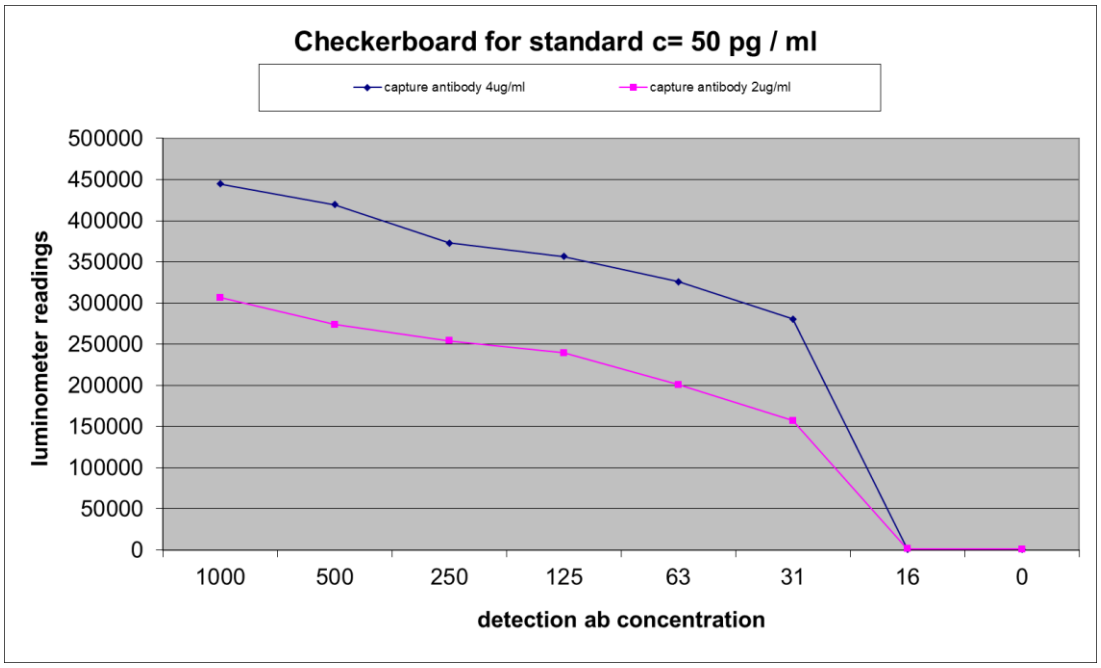


Figure 31 RLUs emitted by detection antibody concentration, standard concentration 10pg/ml

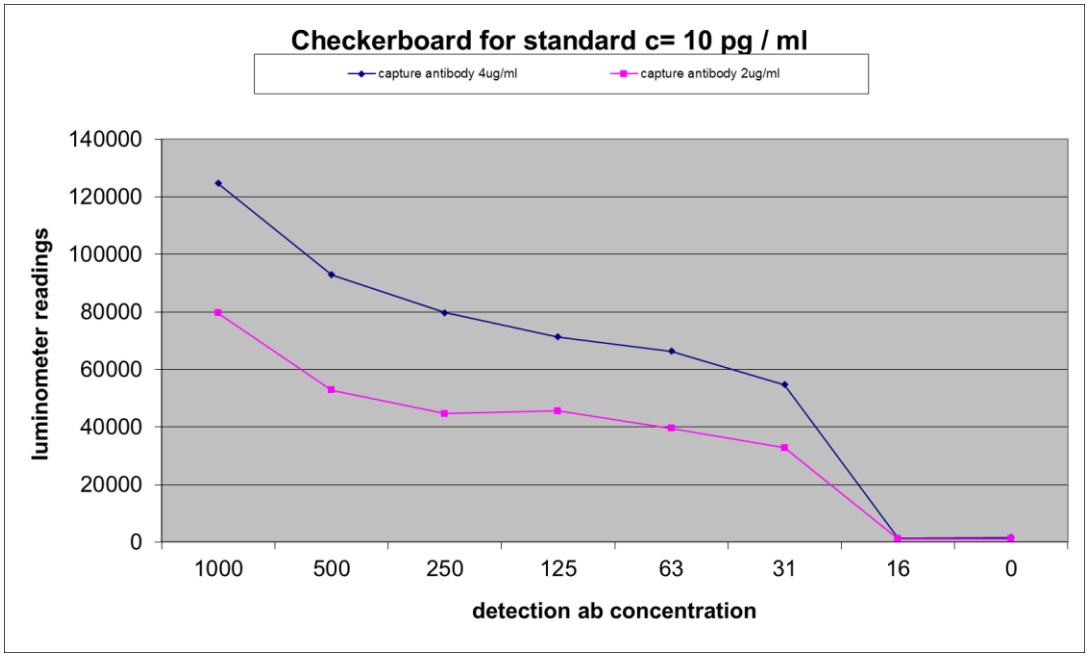


Figure 32 RLUs emitted by detection antibody concentration, standard concentration 0pg/ml

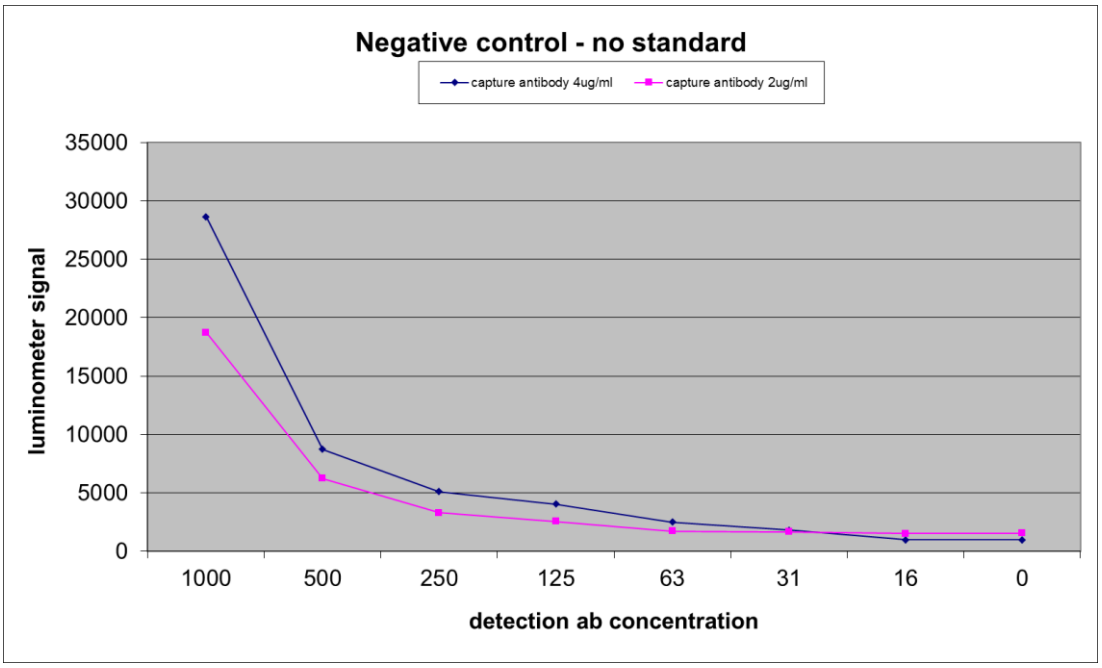


Figure 33 RLUs emitted with relevant background level subtracted by detection antibody concentration, standard concentration 100pg/ml

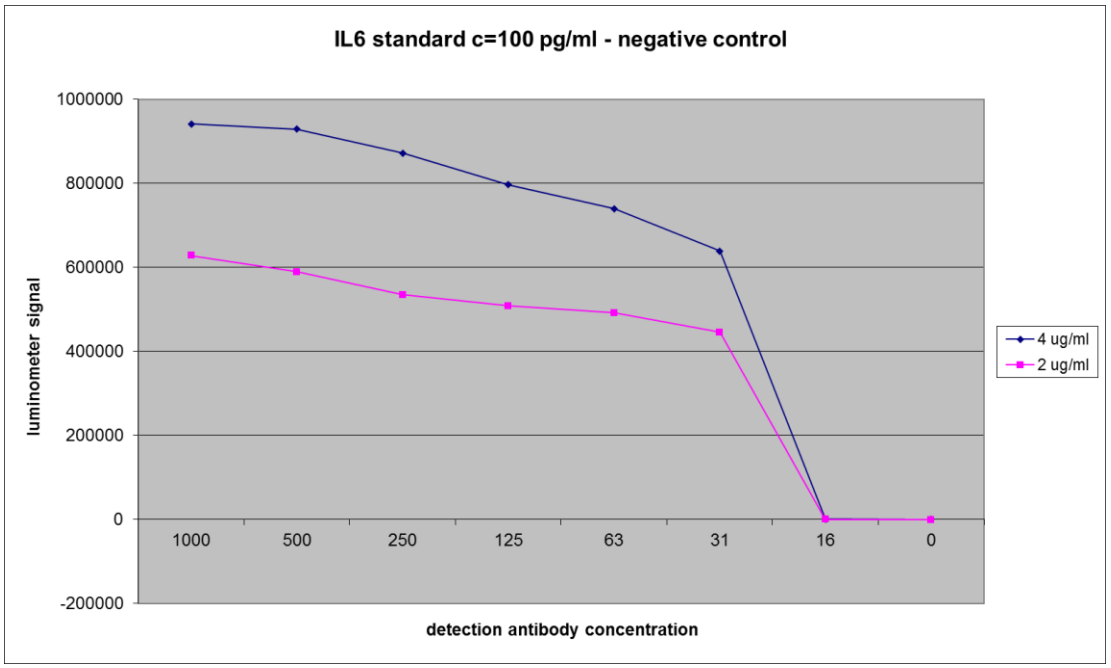


Figure 34 RLUs emitted with relevant background level subtracted by detection antibody concentration, standard concentration 50pg/ml

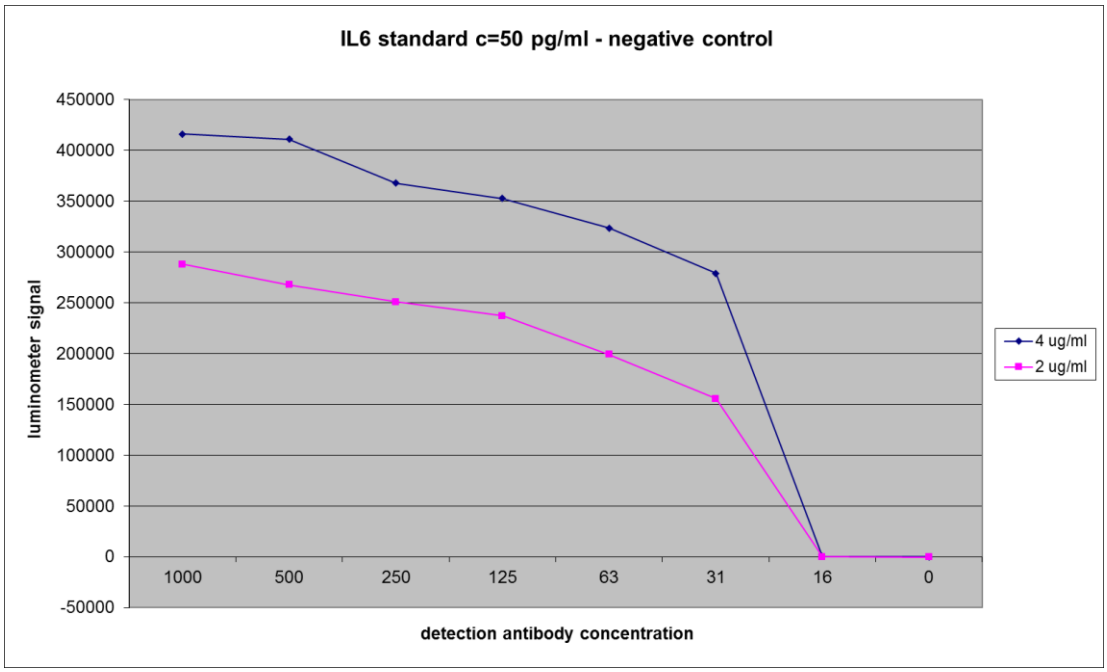


Figure 35 RLUs emitted with relevant background level subtracted by detection antibody concentration, standard concentration 10pg/ml

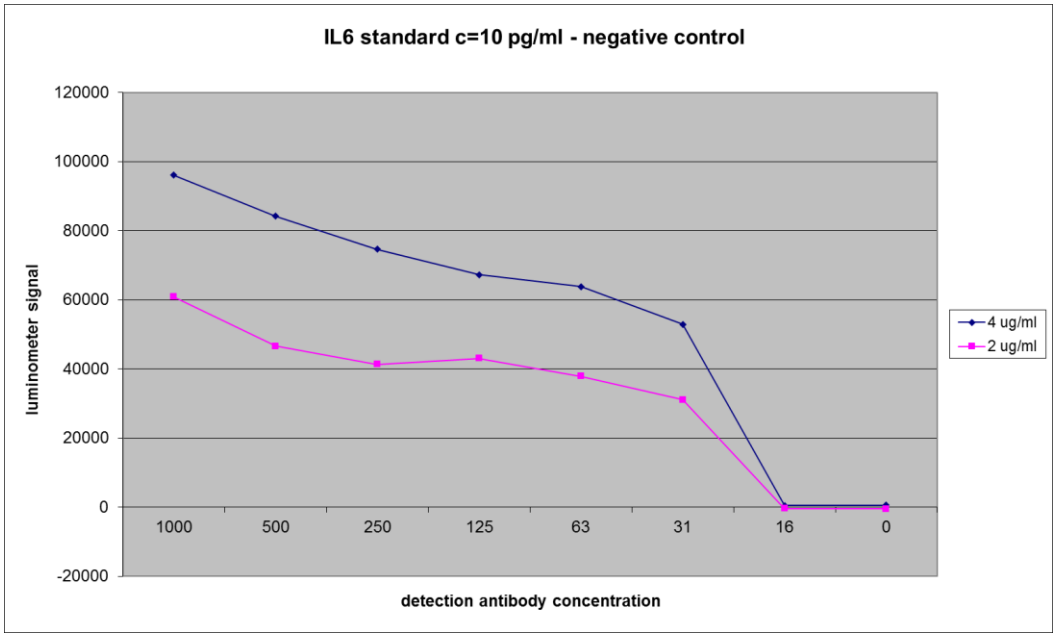


Figure 36 Il-6 signal : noise ratio by detection antibody concentration, standard concentration 100pg/ml

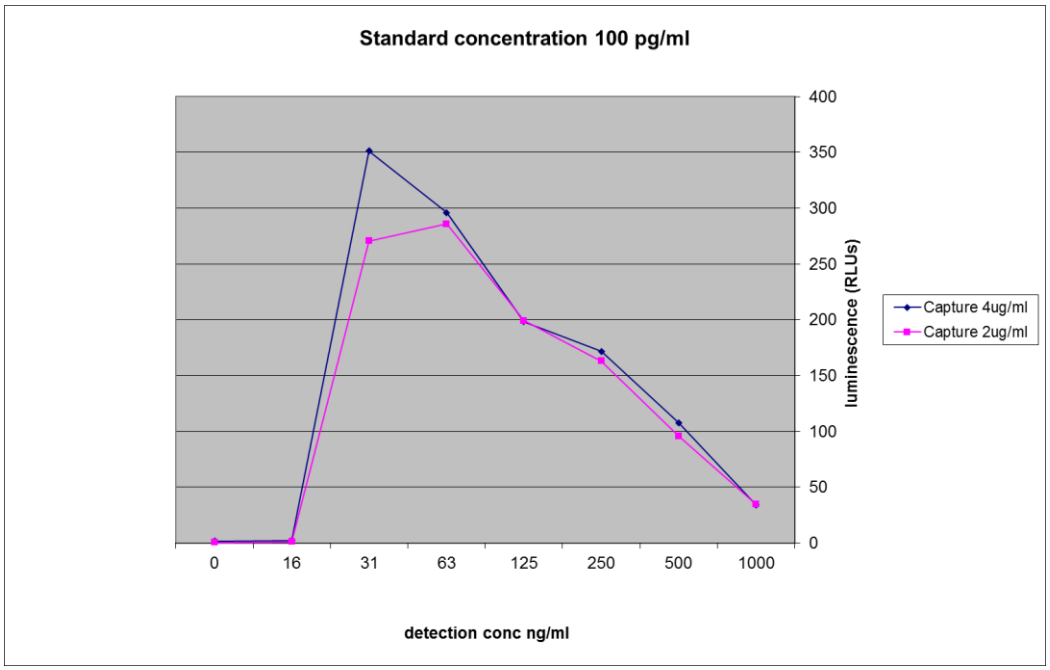


Figure 37 Il-6 signal : noise ratio by detection antibody concentration, standard concentration 50pg/ml

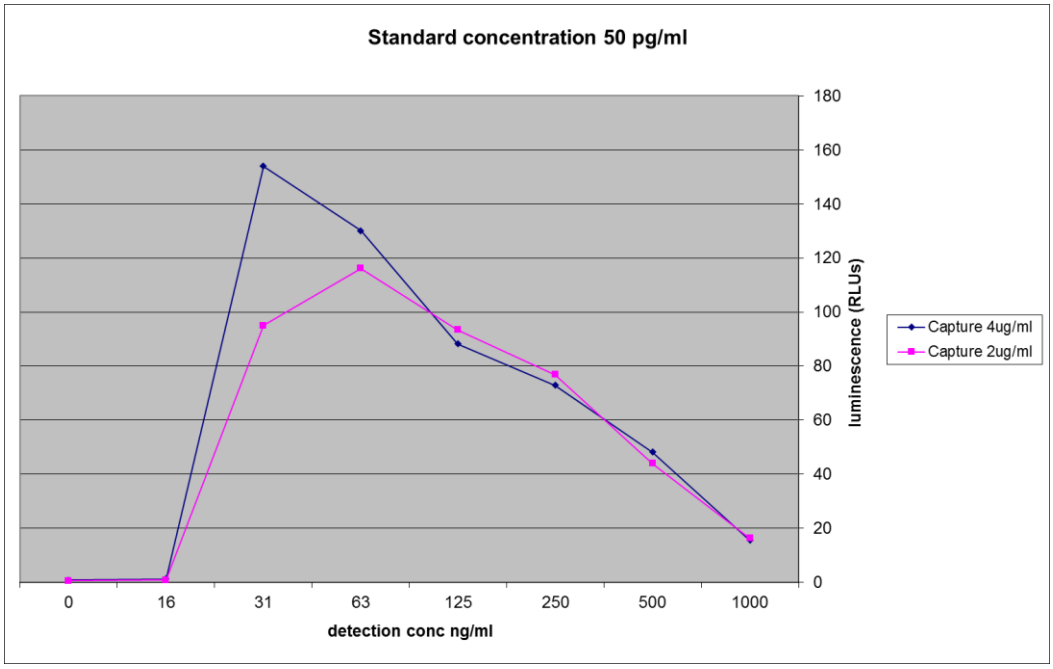
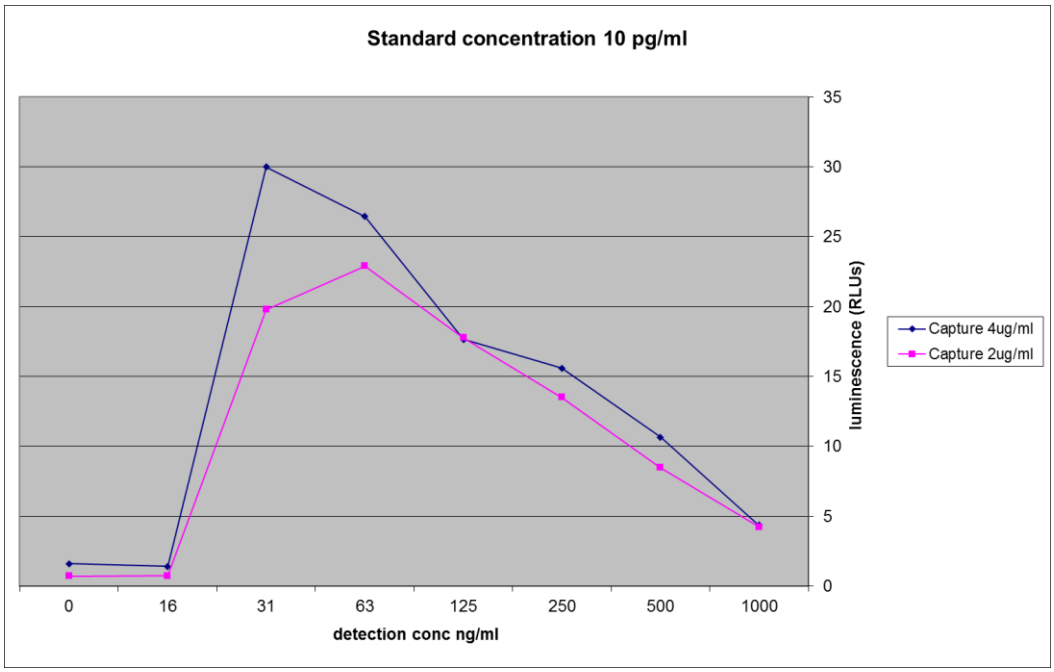


Figure 38 Il-6 signal : noise ratio by detection antibody concentration, standard concentration 10pg/ml



Interpretation:

An increase in the capture antibody from 2 to 4µg results in an increase in RLUs emitted at all levels of standard tested with no real increase in the background (noise) level. Signal : noise ratios therefore favoured using 4µg. As the concentration of detection antibody increased the absolute level of RLUs increased slightly for each level of standard concentration. Background (noise) levels increased proportionally more though resulting in a more favourable ratio at lower detection levels

All subsequent ELISAs for IL-6 used 4µg/ml capture antibody and 50ng/ml detection antibody.

8.2 ELISA to establish the optimum serum dilution for IL-6 detection

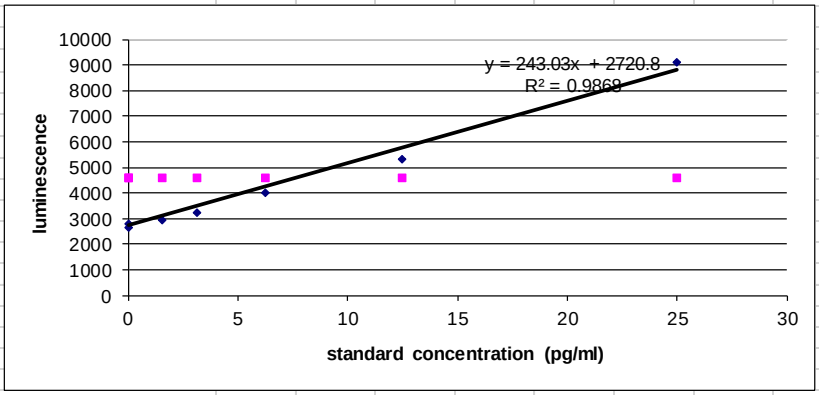
Capture 4µg/ml throughout

Detection 50ng/ml throughout

Serum was spiked with concentrations of recombinant standard at low levels to establish the optimum dilution at the lower end of the range. After spiking serum was serially diluted with standard buffer.

Figure 39 IL-6 serum dilutions. Pure serum

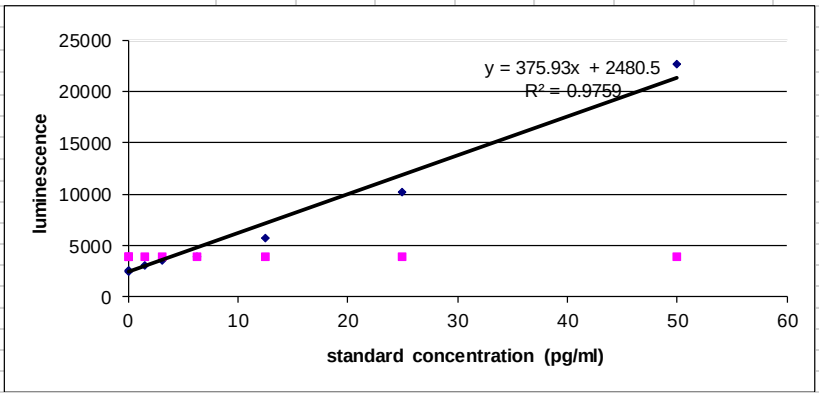
Standard curve	IL-6		Date	12.10.06		Serum dilution	100% serum			
standard concentration [pg/ml]	25	12.5	6.25	3.125	1.5625	0	0	Background		
	10584	6716	4868	4270	3750	3747	3203	3747	2454	
	8640	5169	3884	2922	2849	2454	2709	3203	2709	
	8105	4110	3238	2532	2267	1777	2435	1777	2435	
Average reading	9109.6667	5331.6667	3996.6667	3241.3333	2955.3333	2659.3333	2782.3333	Average	2720.833	
standard deviation	1304.5307	1310.5931	820.81992	911.94371	747.19632	1000.9227	389.21631	St dev	623.07903	
coefficient of variation	14.32%	24.58%	20.54%	28.13%	25.28%	37.64%	13.99%	CV	22.90%	
Average - average background	6388.8333	2610.8333	1275.8333	520.5	234.5	-61.5	61.5			
detection level	4590.0704	4590.0704	4590.0704	4590.0704	4590.0704	4590.0704	4590.0704			
(average background + 3 SD)										



IL-6	
detection level	7.691521
background CV	22.90%
max signal CV	28.13%

Figure 40 IL-6 serum dilutions. 50% serum, 50% standard buffer

Standard curve	IL-6		Date	12.10.06		Plasma dilution	50% serum, 50% PBS			
standard concentration [pg/ml]	50	25	12.5	6.25	3.125	1.5625	0	0	Background	
	23645	10194	5818	4264	3951	3993	3116	3144	3116	3144
	23220	11243	5687	3863	3233	2706	2425	2150	2425	2150
	21070	9023	5522	3513	3393	2299	2169	1879	2169	1879
Average reading	22645	10153.333	5675.6667	3880	3525.6667	2999.3333	2570	2391	Average	2480.5
standard deviation	1380.4438	1110.5586	148.32509	375.7885	376.93678	884.27503	489.86835	666.04579	St dev	485.6675
coefficient of variation	6.10%	10.94%	2.61%	9.69%	10.69%	29.48%	19.06%	27.86%	CV	19.58%
Average - average background	20164.5	7672.8333	3195.1667	1399.5	1045.1667	518.83333	89.5	-89.5		
detection level	3937.5025	3937.5025	3937.5025	3937.5025	3937.5025	3937.5025	3937.5025	3937.5025		
(average background + 3 SD)										



IL-6	
detection level	3.875728
adjusted detection	7.751456
background CV	19.58%
max signal CV	29.48%

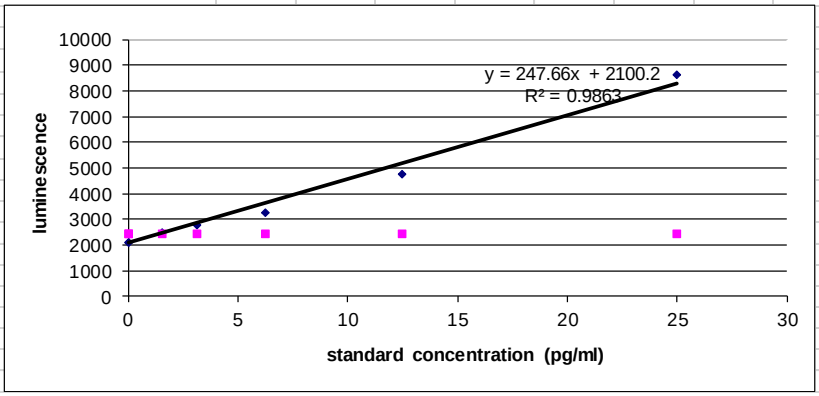
Figure 41 IL-6 serum dilutions. 25% serum, 75% standard buffer

Standard curve	IL-6		Date	12.10.06		Serum dilution	25% serum, 75% PBS

standard concentration [pg/ml]	25	12.5	6.25	3.125	1.5625	0	0
	9066	4630	3085	2756	2482	1977	1988
	8363	4406	3483	2599	2125	2075	2065
	8425	5235	3263	2909	2886	2241	2255
Average reading	8618	4757	3277	2754.6667	2497.6667	2097.6667	2102.6667
standard deviation	389.21588	428.84379	199.369	155.0043	380.74182	133.45161	137.42756
coefficient of variation	4.52%	9.02%	6.08%	5.63%	15.24%	6.36%	6.54%
Average - average background	6517.8333	2656.8333	1176.8333	654.5	397.5	-2.5	2.5

detection level	2432.045	2432.045	2432.045	2432.045	2432.045	2432.045	2432.045
(average background + 3 SD)							

Background	
1977	1988
2075	2065
2241	2255
Average	2100.167
St dev	110.62612
CV	5.27%



IL-6	
detection level	1.339922
adjusted detection	5.359687
background CV	5.27%
max signal CV	15.24%

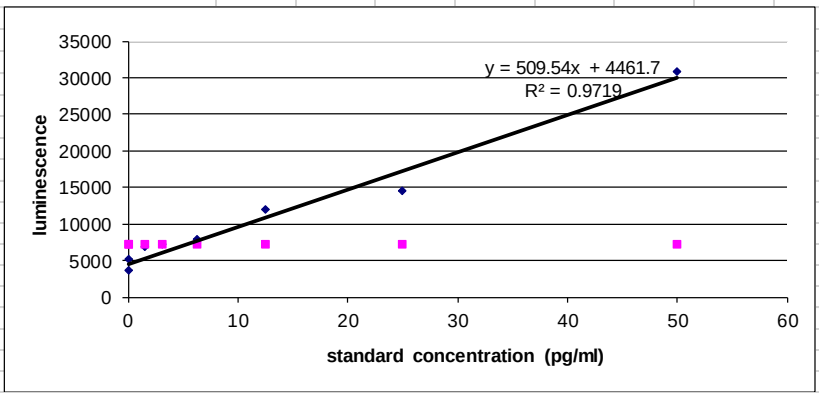
Figure 42 IL-6 serum dilutions. 100% standard buffer

Standard curve	IL-6		Date	12.10.06		Serum dilution	100% PBS

standard concentration [pg/ml]	50	25	12.5	6.25	3.125	1.5625	0	0
	30250	14932	12613	8050	7647	7204	5019	4325
	32383	12623	11926	8170	6804	5891	4826	3455
	29863	15970	11508	7448		7744	5945	3200
Average reading	30832	14508.333	12015.667	7889.3333	7225.5	6946.3333	5263.3333	3660
standard deviation	1357.0715	1713.2491	557.9304	386.88672	596.09102	952.99335	598.17584	589.85168
coefficient of variation	4.40%	11.81%	4.64%	4.90%	8.25%	13.72%	11.36%	16.12%
Average - average background	26370.333	10046.667	7554	3427.6667	2763.8333	2484.6667	801.66667	-801.66667

detection level	7272.5816	7272.5816	7272.5816	7272.5816	7272.5816	7272.5816	7272.5816	7272.5816
(average background + 3 SD)								

Background	
5019	4325
4826	3455
5945	3200
Average	4461.667
St dev	936.97166
CV	21.00%



IL-6	
detection level	5.516508
background CV	21.00%
max signal CV	13.72%

Interpretation:

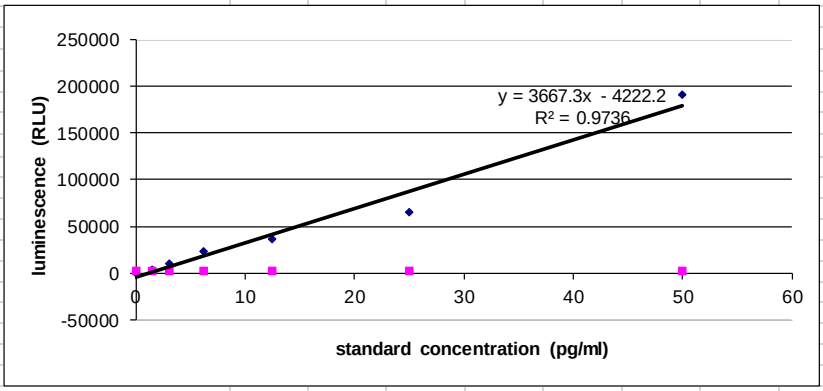
This run was disappointing in that CVs of both signal and background were unacceptably high and absolute values were lower than on other runs. Perhaps the

standard was inadvertently diluted by a factor of 10. It does demonstrate though that there is no obvious advantage in diluting the serum as detection levels and CVs remained similar.

8.3 Final SOP ELISA - Trial run, healthy volunteers

After a few days of refining the technique, an SOP was established producing low signal and background CVs and low detection levels which was successful in analysing both spiked serum and raw serum from healthy human volunteers

Lab peoples blood	IL-6		Date	16.10.06		Serum dilution	100% Serum	Detection	50ng/ml
Standard curve									
standard concentration [pg/ml]	50	25	12.5	6.25	3.125	1.5625	0	0	Background	
	195504	69050	33782	29904	13503	6311	2001	1935	2001	1935
	189199	65372	42626	21861	11827	5954	1679	1723	1679	1723
Average reading	192351.5	67211	38204	25882.5	12665	6132.5	1840	1829	Average	1834.5
standard deviation	4458.3083	2600.7387	6253.6524	5687.2598	1185.111	252.43712	227.68838	149.90664	St dev	136.41389
coefficient of variation	2.32%	3.87%	16.37%	21.97%	9.36%	4.12%	12.37%	8.20%	CV	7.44%
Average - average background	190517	65376.5	36369.5	24048	10830.5	4298	5.5	-5.5		
detection level	2243.7417	2243.7417	2243.7417	2243.7417	2243.7417	2243.7417	2243.7417	2243.7417		
(average background + 3 SD)										



IL-6	
detection level	1.763134
background CV	7.44%
average signal CV	9.82%
max signal CV	21.97%

Calculated IL-6 levels				Average RLU	Standard deviation	CV	Calculated IL-6 pg/ml x=(y+4222.2)/3667.3
Subject 1	23762	23606	23362	23442	1072.0985	4.57%	7.543478854
	23546	24842	21534				
Subject 2	3359	3300	2771	3159.5	270.79346	8.57%	2.012843236
	3098	3485	2944				
Subject 3	40574	44220	47717	44921.16667	3881.4563	8.64%	13.40042175
	41871	44054	51091				
Subject 4	14779	17257	15310	15782	1304.6873	8.27%	5.454748725

8.4 Final IL-6 ELISA SOP

Luminescence immunoassay 384 well plate

Reagents

Matched pair antibodies (capture and detection) (R&D)

Recombinant standard protein (R&D)

Horse peroxidase reagent (HPR) (Sigma)

Luminol: Supersignal West Pico Chemiluminescence Substrate (Pierce)

Sodium azide ($NaN_{3)}$ comes as powder – 100%. Standardly make up to 8% in fridge (8g in 100ml PBS)

Tween 20 solution 10% (Sigma)

Bovine serum albumin (BSA) (7.5% Sigma)

Buffers

Capture buffer:

Need 14ml: (0.03ml per well x 384 wells = 11.52ml + allow extra for wastage)

PBS +/- 0.08% $NaN_{3,}$ filtered + 0.05% Tween

14ml PBS + 0.14ml 0.8% NaN_3 + 70µl 10% Tween

Blocking buffer:

Need 60ml: (0.127ml per well x 384 wells = 48.77ml + allow extra for wastage)

PBS + 1% BSA (7.5%) + 3% lactose + 0.08% NaN_3

51.5ml PBS + 8ml 7.5% BSA + 1.8g lactose (measure on fine scales) + 0.6ml 8% NaN_3

Standard buffer:

Need 14ml: (0.03ml per well x 384 wells = 11.52ml + allow extra for wastage)

PBS + 1% BSA + 0.05% Tween + 0.08% NaN_3, filtered

9.43ml PBS + 1.87ml 7.5% BSA + 70µl 10% Tween + 140µl NaN_3

Detection buffer:

Need 14ml: (0.03ml per well x 384 wells = 11.52ml + allow extra for wastage)

PBS + 1% BSA + 0.05% Tween + 0.08% NaN_3

9.43ml PBS + 1.87ml 7.5% BSA + 70µl 10% Tween + 140µl NaN_3

HPR buffer:

Need 14ml: (0.03ml per well x 384 wells = 11.52ml + allow extra for wastage)

PBS + 0.05% Tween

14ml PBS + 70µl 10% Tween

Wash buffer:

Pre-detection

PBS + 0.05% Tween + 0.08% NaN_3, filtered

1l PBS + 5ml 10% Tween + 10ml 8% NaN_3

Post-detection

PBS + 0.05% Tween **no NaN_3**

1l PBS + 5ml 10% Tween **no NaN_3**

Requirements

Plasma dilution: None

Capture 4ug/ml

Standard start from about 1000pg/ml

Detection 50ng/ml

Calculations

$\frac{\text{Concentration needed}}{\text{Stock concentration}}$ x volume needed = stock volume

Capture

Capture stock = 500µg/ml

Need 14ml of 4µg/ml

$\frac{4}{500}$ x 14 = 0.112ml (112µl) capture stock in 14ml capture buffer

Standard

Standard stock = 10µg/ml (10,000,000pg/ml)

Start at 800pg/ml (even the first row gets diluted 50:50)

Need 90µl (30µl per well x 3 wells = 90µl) of 800pg/ml

Tiny amounts – too small to accurately pipette therefore:

Dilute standard stock 10µl into 10ml standard buffer to produce 10,000pg/ml solution

Then:

$\frac{800}{10{,}000}$ x 10 = 0.8ml of diluted standard stock in 10ml standard buffer

Detection

Detection stock = 200µg/ml (200ng/µl).

Need 14ml (0.03ml per well x 384 wells = 11.52ml – allow extra for wastage) of 50ng/ml

$\frac{50}{200}$ x 14 = 3.5µl detection stock in 14ml detection buffer

Method

Prior to incubating at each step remove the vial required for the next stage from the freezer. When defrosted, Vortex the solution and prepare during the incubation time for the next step

Microplate

1. Label the 384 Nunc maxisorp plate (340372) with tested antigen date and draw lines dividing it into subsets of 3 wells each, leaving the final 3 cells of the last 13 rows free for control samples
2. Set up a template on Microsoft office Word mapping the 384 with location of each patient sample and control run

Capture

3. Use 4ug/ml capture
4. Use a multi-channel pipette (green top) to put 30ul into each cell. Try to get the tip of the pipette to the bottom of each well to avoid precipitation on the sides
5. Cover the plate with plastic sticky foil to avoid evaporation, put the lid on and wrap in and plastic bag
6. Leave overnight at 4°c
7. Put 1 litre sterile water with 5 tablets PBS onto stirrer for 20mins
8. Add 5ml Tween and 10ml 8% NaN_3
9. Set up the plate washer using wash buffer
    a. Wash joins to fill nozzle on washer
    b. Vac on pump joins to top bung on waste bottle
    c. Side port on waste bottle connects to vac nozzle on pump

d. Pressure nozzle on pump hangs into sink

10. Wash plate 5 times using the plate washer
    a. Fill each cell with buffer
    b. Shake with vigorousness adjuster set at a half or more for 30sec.
    c. Suck out buffer from each cell
11. Tap several times hard onto paper towel to ensure any excess fluid removed.
12. Remove one vial of serum for each patient from the -80^{0}c freezer to defrost

Blocking buffer

13. Add 127ul blocking buffer into each well – needs to be all the way to the top to block whole well otherwise HRP / luminol will stick to unblocked sites
14. Cover the plate with plastic sticky foil, put the lid on
15. Shake gently for 1hr at room temperature (vigorous setting at about a third)
16. Wash 5 times using plate washer (with NaN_3 and Tween) as above
17. Make up a 2nd litre of wash buffer as above

Serum

18. Take the first vial of serum (trial number 1, first visit)
19. Vortex the serum
20. Pipette 30ul into each of the first 3 cells (A1-A3)
21. Repeat for each patient vial, following the template already set up

Standard

22. Use 800pg/ml (even first row gets diluted)
23. Use cells D22-P24 for standards
24. Add 30ul control serum to all cells
25. Add 30ul x 800pg/ml standard to cells P22-P24
26. Pipette up and down several times to mix
27. Take 30ul from each cell in row P
28. Transfer to cells in row O
29. Continue to double dilute across 3 cells up to row F
30. Discard 30ul from final row (F) to leave 30ul
31. Leave 3 cells in 2 rows (D&E) with control serum only to measure background
32. Cover the plate with plastic sticky foil, put the lid on
33. Shake gently for 10mins
34. Incubate for 1hr at 37°c

35. Wash 5 times using wash buffer as above
36. Make up a third litre of wash buffer as above but without NaN_3

Detection

37. Use 50ng/ml detection
38. Pipette 30ul x detection into each well
39. Cover the plate with plastic sticky foil and put the lid on
40. Shake gently for 10mins
41. Incubate at 45min at 37°c
42. Wash plate 5 times using wash buffer **without NaN_3**

HRP

43. Switch on the Berthold Luminometer to preheat the lasers
44. Dilute HRP (Sigma) (streptovirin in blue box in freezer) 1:10,000 with buffer
45. i.e. 14ml buffer with 1.4ul HRP (tiny amount but can be done in one step)
46. Use multichannel pipette to add 30ul HRP to each well
47. Cover the plate with plastic sticky foil and put the lid on.
48. Shake gently for 30mins at room temperature

Luminol

49. Prepare luminol : H_2O_2 dilution 1:1 in a tube covered with tin foil. Mix it gently and leave to incubate for 30mins at room temperature
50. Wash plate five times using wash buffer **without NaN_3**
51. Add 30ul luminol mix to each well using the multi-channel pipette
52. Cover the plate with silver foil and put in a draw
53. Incubate for 10min at room temperature
54. Put the plate in the luminometer (A1 at top right)
55. Push down central plate, pull gripping frame down over the top
56. Close machine
57. On laptop chose simplistic 2.1 programme
58. Chose correct plate size
59. Can highlight only some cells and press the symbol with a spot inside a rectangle to read only those
60. Press green spot symbol to read plate
61. Transfers automatically to excel
62. Save in my documents / susi & use memory stick to transfer to personal laptop

8.5 Ethics committee approval letter

8.6 Form UPR16

8.7 Trial protocol