THE AETIOLOGY OF SYSTEMIC INFLAMMATION AND ITS LINK WITH PROGNOSIS IN GASTRO-OESOPHAGEAL CANCER

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January 2008

DECLARATION

I declare that the work described in this thesis was undertaken by myself and the thesis was composed and referenced by me personally.

Some of the work described was performed in collaboration:

Analysis of food diaries was performed by Kerry Yuill, Senior Dietitian, Edinburgh Royal Infirmary.

Histological sections were cut and prepared by Frances Rae and Michael Herriot, Department of Pathology, Edinburgh University.

Cytokine gene polymorphism genotyping was performed by Matthew Rose-Zerilli, Martin Howell, and Professor Bob Grimble, Histocompatibility and Immunogenetics Laboratory, Human Genetics Division, University of Southampton.

Identification of urinary Proteolysis-inducing factor (PIF) was also performed by Bill Field and Professor Michael Tisdale at Aston University, Birmingham.

Mass spectrometry was performed by Andrew Cronshaw of the Institute of Structural and Molecular Biology, Edinburgh University.

The thesis was principally supervised by Professor Kenneth C.H. Fearon, University Department of Surgery, Edinburgh Royal Infirmary. Additional supervision was provided by Dr James A. Ross and Professor Stephen J. Wigmore, Tissue Injury and Repair Group, University of Edinburgh and also by Mr Simon Paterson-Brown, University Department of Surgery, Edinburgh Royal Infirmary.

Chris Deans

ACKNOWLEDGEMENTS

Many people were essential for the completion of this thesis and I am grateful to them all.

To the Royal College of Surgeons of England and the Melville Trust for funding my salary and research costs for two years. Also to the Royal College of Surgeons of Edinburgh and Chugai Pharmaceuticals who donated additional funds towards ever expanding research costs.

To Mr Simon Paterson-Brown and Mr Andrew de Beaux for granting me full access to the Department and to their patients. To the Pathology team, Dr Hugh Gilmour and Dr Hasan Kamel, who would cut samples of fresh tissue often at short notice and usually during their lunch break. To Kerry Yuill who provided invaluable dietetic advice. To Louise Graham and Michelle Gibson for their assistance in the identification and location of patients and who provided essential organisation in otherwise total chaos.

To the team in the Lister/Tissue Injury and Repair Group Laboratory. Ian, Jim, Carrie, and, of course, Kathryn who managed to teach me to use a pipette and other objects in the lab. To my research colleagues with whom we all shared the highs and lows of research and even more coffee breaks, and who 'volunteered' samples of blood and urine.

To all those in other Laboratories who provided valuable advice and technical assistance. Charlie Gourlay for expert advice on real-time PCR, Andy Cronshaw who introduced me to the new world of mass spectrometry and proteomics, and the genomics team in Southampton; Professor Bob Grimble, Martin Howell and Mat Rose-Zerilli, for providing rapid and high quality genotyping and analysis. To Professor Vicki Baracos and Barbara Wieland in Canada for performing independent PIF analysis and enabling exchange of thoughts regarding all things PIF. And finally Professor Tisdale and his team in Aston who performed sample analysis and provided world-leading expertise in PIF biology.

To the patients, who without exception, agreed to take part in this research. Who provided regular samples of blood and urine on demand despite confronting a devastating disease and often equally challenging treatments.

To my team of supervisors: Simon Paterson-Brown who devised the original concept and identified the clinical problem; who continually encouraged and kept my momentum going. To Steve Wigmore for his research expertise and problem solving when I (frequently) became stuck. To Jim Ross for helping to devise the project, assisting with continual grant applications and manuscript re-drafting, and day-to-day laboratory advice. And to Professor Ken Fearon without whom none of this would have been possible. His vision shaped this project into what it is today; at times gently guiding and at other times just pointing out the obvious. Always supportive and never flagging, even though the rest of us may be.

And finally to my wife and the boys. For their patience and support. For tolerating my moaning and always encouraging.

Thank you all.

CONTENTS

LIST OF ABBREVIATIONS

AMC	Arm muscle circumference
APPR	Acute phase protein response
BMI	Body mass index
CT	Computerised tomography
COX	Cyclo-oxygenase (enzyme)
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
EUS	Endoscopic ultrasound
IL	Interleukin
LUS	Laparoscopy/laparoscopic ultrasound
MAC	Mid-arm circumference
MALDI-TOF	Matrix-assisted laser desorption/ionisation, time-of-flight mass spectroscopy
MDT	Multi-disciplinary team
NSAID	Non-steroidal anti-inflammatory drugs
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PIF	Proteolysis-inducing factor
PTHrP	Parathyroid hormone-related peptide
REE	Resting energy expenditure
SIMS	Systemic immune metabolic syndrome
SIRS	Systemic inflammatory response syndrome
SNP	Single nucleotide polymorphism
TAAg	Tumour-associated antigen
TAM	Tumour-associated macrophage
TIL	Tumour-infiltrating lymphocyte
TNFα	Tumour necrosis factor-α
sTNF-R	Soluble tumour necrosis factor receptor
TNM	Tumour node metastases (staging)
UICC	International Union Against Cancer
VEGF	Vascular endothelial growth factor

ABSTRACT

Introduction

As the incidence of gastro-oesophageal cancer continues to increase accurate staging remains challenging and the general outlook for these patients is poor. As well as improving prognostic accuracy, investigation of systemic inflammation and cachexia in these patients may enable the identification of much needed novel therapeutic targets.

Aims

The aims of this thesis were to describe the genesis, mediators and clinical sequelae of systemic inflammation in patients with gastro-oesophageal cancer. The usefulness of systemic inflammation as a prognostic indicator and the role of cachexia as a factor in the adverse prognosis associated with systemic inflammation were expanded in detail. The key hypothesis being that tumour cells produce mediators (eg cytokines), which can either directly or indirectly (via systemic inflammation) induce a catabolic state in the peripheral tissues of the host. Such wasting may be one of the mechanisms linking systemic inflammation with adverse prognosis in patients with cancer.

Materials and Methods

A consecutive series of 220 patients with gastric or oesophageal cancer were studied. Data were collected prospectively and a nutritional assessment and performance status were determined for each patient and survival duration was recorded. Samples of blood, urine and tumour tissue were collected for determination of cytokine and acute phase protein concentrations. The expression of other potential tumour-derived mediators, parathyroid hormone-related peptide (PTHrP) and proteolysis-inducing factor (PIF), were also studied.

Results

Systemic inflammation (CRP>10 mg/l) was present in 43% of patients with gastro-oesophageal cancer. Serum acute phase protein concentrations (especially CRP), but not serum cytokine concentrations, were robust measures of systemic inflammatory activity.

However, concentrations of pro-inflammatory cytokines within tumour tissue were significantly elevated and were linked with markers of systemic inflammation. IL-1β in particular was over-expressed in tumour tissue and may be a key determinant of systemic inflammation in patients with gastro-oesophageal malignancy. A chronic inflammatory cell infiltrate into the tumour tissue was present in 75% of tumours and was also linked with markers of systemic inflammation. Tumour cells or host immune cells or a combination of the two may be the main source of these mediators. The presence of systemic inflammation was also influenced by host cytokine genotype. Other potential tumour-derived mediators, such as PIF and PTHrP, may also play a (minor) role in the generation of systemic inflammation. These factors may also have additional effects on the host, such as potentiating weight loss. CRP concentrations were identified as the best marker of prognosis and the magnitude of serum CRP concentrations were negatively linked with survival duration.

83% of patients had lost weight at the time of diagnosis and within 3 months this had increased to 92%. Increasing weight loss was positively associated with serum markers of systemic inflammation. Weight loss among patients with gastro-oesophageal cancer was not accounted for entirely by reduced food intake or mechanical obstruction secondary to the tumour. Alternatively, the presence of systemic inflammation contributed to nutritional decline (estimate of effect 34%). Weight loss was associated with adverse outcome and cachexia may be an aetiological factor involved in the link between systemic inflammation and adverse prognosis.

Conclusions

Systemic inflammation, weight loss, performance status, and stage of disease were the main determinants of outcome in patients with gastro-oesophageal cancer. These factors were used to devise a novel model to improve prognostic accuracy to aid clinical decision-making for these patients. These studies identify systemic inflammation as both an important prognostic indicator and a potential therapeutic target for patients with gastro-oesophageal malignancy.

SUMMARY OF THESIS

Introduction

The incidence of gastro-oesophageal cancer continues to increase at an alarming rate in the UK. Accurate clinical staging remains challenging and the general outlook for these patients is poor. Surgery offers the only real prospect of cure but carries significant risks in terms of morbidity and mortality. A number of alternative palliative treatment options are available, but are generally limited in their effectiveness. Current staging practices are failing to identify prospectively which patients will truly benefit from surgical resection in terms of survival benefit and quality of life. In addition, novel therapeutic strategies, both with curative and palliative intent, are much needed for these patients. Elevated markers of systemic inflammation are commonly found in patients with cancer and have been linked with survival duration, cachexia and outcome. As well as improving prognostic accuracy, investigation of systemic inflammation and cachexia in these patients may enable the identification of much needed novel therapeutic targets. Therefore, the aims of this thesis were to describe the genesis, mediators and clinical sequelae of systemic inflammation in patients with gastro-oesophageal cancer. The usefulness of systemic inflammation as a prognostic indicator and the role of cachexia as a factor in the adverse prognosis associated with systemic inflammation was also examined. The key hypothesis being that tumour cells produce mediators (for example, cytokines), which can either directly or indirectly (via systemic inflammation) induce a catabolic state in the peripheral tissues of the host. Such wasting may be one of the mechanisms linking systemic inflammation with adverse prognosis in patients with cancer.

In order to address the aetiology of systemic inflammation and the mechanisms linking it to adverse prognosis, a consecutive series of patients with newly diagnosed gastric or oesophageal cancer in the Lothian and Borders regions between March 2002 and June 2004 were studied. Following diagnosis patients were staged with a combination of computerised tomography (CT), endoscopic ultrasound (EUS) and laparoscopy/laparoscopic ultrasound

(LUS) and treatments were decided following discussion at the multi-disciplinary team meeting (MDT). Clinical and pathological data were collected prospectively and a nutritional assessment and performance status were determined for each patient and survival duration was recorded. Samples of blood, urine and tumour tissue were collected for determination of cytokine and acute phase protein concentrations. The expression of other potential tumour-derived mediators, parathyroid hormone-related peptide (PTHrP) and proteolysis-inducing factor (PIF), were also studied.

Prevalence of systemic inflammation in patients with gastro-oesophageal cancer

Serum concentrations of positive acute phase proteins were elevated significantly in patients with gastro-oesophageal cancer (Chapter IV). Systemic inflammation (serum CRP concentration >10 mg/l) was present in 43% of patients at the time of diagnosis. In contrast, serum cytokine concentrations (with the exception of sTNF-R concentrations) were not significantly different to those measured in healthy controls. Subsequent CRP measurements were influenced by treatment modality: surgical resection was associated with a transitory increase in CRP concentrations. In contrast, pre-operative chemotherapy was associated with a reduction in serial serum CRP concentrations. These findings suggest that at the time of diagnosis a substantial majority of patients have tumour-associated systemic inflammation, which may be reduced by chemotherapy or enhanced by surgical intervention.

Role of cytokines in the genesis of systemic inflammation in patients with gastro-oesophageal cancer

Cytokine concentrations were measured in tumour tissue and levels of expression compared with concentrations measured in tissue from healthy controls and with markers of systemic inflammation (Chapter V). Pro-inflammatory cytokine concentrations (mRNA and protein) were significantly elevated in tumour tissue when compared with concentrations measured in tissue samples from healthy controls. Moreover, tumour tissue IL-1β was highly expressed and concentrations correlated with markers of systemic inflammation (CRP). The degree of chronic inflammatory cell infiltrate into the tumour tissue was also assessed and compared with tissue cytokine concentrations and with markers of systemic inflammation (also Chapter

V). A chronic inflammatory cell infiltrate was identified in 75% of tumours and was associated with markers of systemic inflammation (CRP), but not with elevated tissue cytokine concentrations. Although circulating levels of cytokines did not correlate with the presence of systemic inflammation, tumour tissue cytokine concentrations were related to markers of systemic inflammation. These findings suggest that the tumour tissue itself may be an important primary source of the pro-inflammatory response. The latter may be generated both by tumour cell cytokine production and host inflammatory cell infiltrate or a combination of the two.

The influence of cytokine genotype on the ability of the host to mount a systemic inflammatory response is described in Chapter VI. Genotype polymorphism expression patterns were compared with markers of systemic inflammation, tissue inflammation and outcome. IL-6 *–174 CC* and IL-10 *–1082 GG* genotypes were associated with elevated markers of systemic inflammation (serum CRP and sTNF-R concentrations) and the IL-6 *– 174 CC* genotype was also associated with elevated tumour tissue cytokine concentrations. These two genotypes were also linked with adverse prognosis. In addition, the TNFα *-308 AA* genotype was linked with adverse outcome and was identified as an independent prognostic indicator, but was not associated with markers of systemic inflammation. These findings suggest that host cytokine genotype may modify the generation of systemic inflammation in patients with gastro-oesophageal cancer and may partly explain the link between genotype and adverse prognosis.

Role of other mediators in the genesis of systemic inflammation in patients with gastro-oesophageal cancer

The potential role of mediators other than pro-inflammatory cytokines in the aetiology of systemic inflammation in patients with cancer is described in Chapters VII and VIII. Serum concentrations of parathyroid hormone-related peptide (PTHrP) were compared with markers of systemic inflammation, adverse nutritional status, and survival duration (Chapter VII). At the time of diagnosis, elevated serum PTHrP concentrations were detected in 17% of patients with gastro-oesophageal malignancy without hypercalcaemia. An elevated serum PTHrP concentration was associated with markers of systemic inflammation and demonstrated a

trend toward adverse prognosis. Subsequently, an elevated serum PTHrP concentration was detected in 49% of patients approximately three months following diagnosis (again without evidence of hypercalcaemia). These follow-up measurements were not influenced by treatment modality and were positively linked with markers of adverse nutritional status.

Studies on proteolysis-inducing factor (PIF) expression are described in Chapter VIII. Tumour tissue mRNA expression and urinary protein levels were compared with markers of systemic inflammation, cachexia and prognosis. PIF protein was detected in 51% of patients' urine and was associated with adverse nutritional variables and there was a trend between PIF detected in patients' urine and elevated markers of systemic inflammation. PIF mRNA expression was detected in tissue from healthy controls and at significantly elevated concentrations in tumour tissue from patients with gastro-oesophageal malignancy. However, levels of PIF mRNA were not associated with systemic inflammation, nutritional status or the presence of urinary PIF. This may reflect the lack of correlation between measured mRNA concentrations and translated protein concentrations *in vivo*.

The link between systemic inflammation, nutritional status and prognosis in patients with gastro-oesophagea! cancer

The association between systemic inflammation and survival duration is described in Chapter IX. Concentrations of serum acute phase proteins and sTNF-R, but not concentrations of serum cytokines, were associated with survival duration. CRP concentrations were identified as the best marker of prognosis and the magnitude of serum CRP concentrations were negatively linked with survival duration. An increase in serum CRP concentration from a value of 1 mg/l to 10 mg/l was associated with an almost 50% increase in likelihood of death within two years of diagnosis. However, CRP lost its prognostic value after initiation of treatment. The effect of treatments and disease progression clouds the value of systemic inflammation (CRP) as a prognostic marker.

The changes in nutritional status and the association between nutritional variables and markers of systemic inflammation and prognosis for patients with gastro-oesophageal cancer are described in Chapter X. Eighty-three percent of patients had lost weight at the time of diagnosis (median weight loss 7%) and within three months this had increased to 92%

(median weight loss 10%). Increasing weight loss was associated with elevated serum markers of systemic inflammation (serum acute phase protein concentrations). Multiple regression analysis estimated that 38% of weight loss was due to level of dietary intake, 34% was due to the presence of systemic inflammation (serum CRP concentrations), and 28% due to stage of disease. These data suggest that processes in addition to reduced dietary intake and mechanical obstruction by the tumour are important in the generation of cachexia and the presence of systemic inflammation appears to play a key role.

Weight loss was linked with adverse prognosis and cachexia may be an aetiological factor involved in the link between systemic inflammation and adverse prognosis in patients with gastro-oesophageal cancer.

Finally, a novel risk prediction model for gastro-oesophageal cancer was constructed from various clinical and investigative factors, including markers of systemic inflammation and cachexia (Chapter XI). Systemic inflammation, weight loss, performance status, and stage of disease were the main determinants of outcome. These factors were used to devise a novel model with improved prognostic accuracy to aid clinical decision-making.

Conclusions

Systemic inflammation is common in patients with gastro-oesophageal cancer. Serum acute phase protein concentrations (especially CRP), but not serum cytokine concentrations, are robust measures of systemic inflammatory activity. However, concentrations of pro-inflammatory cytokines within tumour tissue are significantly elevated and linked with markers of systemic inflammation. IL-1β in particular is over-expressed in tumour tissue and may be a key determinant of systemic inflammation in patients with gastro-oesophageal malignancy. Tumour cells or host immune cells or a combination of the two may be the main source of these mediators. The presence of systemic inflammation is also influenced by host cytokine genotype. Other potential tumour-derived mediators, such as PIF and PTHrP, may play a role in the generation of systemic inflammation. These factors may have additional effects on the host, outwith the context of systemic inflammation, such as potentiating weight loss and cachexia.

Weight loss is common among patients with gastro-oesophageal cancer and is not entirely due to reduced food intake secondary to mechanical obstruction by the tumour. Indeed, the presence of systemic inflammation appears to contribute to nutritional decline. Weight loss and systemic inflammation are associated with adverse outcome and cachexia may be an aetiological factor involved in this association.

Systemic inflammation, weight loss, performance status, and stage of disease are the main determinants of outcome in patients with gastro-oesophageal cancer. These factors can be combined in a novel model to improve prognostic accuracy to aid clinical decision-making for these patients. Overall, this thesis identifies systemic inflammation as both an important prognostic indicator and a potential therapeutic target for patients with gastro-oesophageal malignancy.

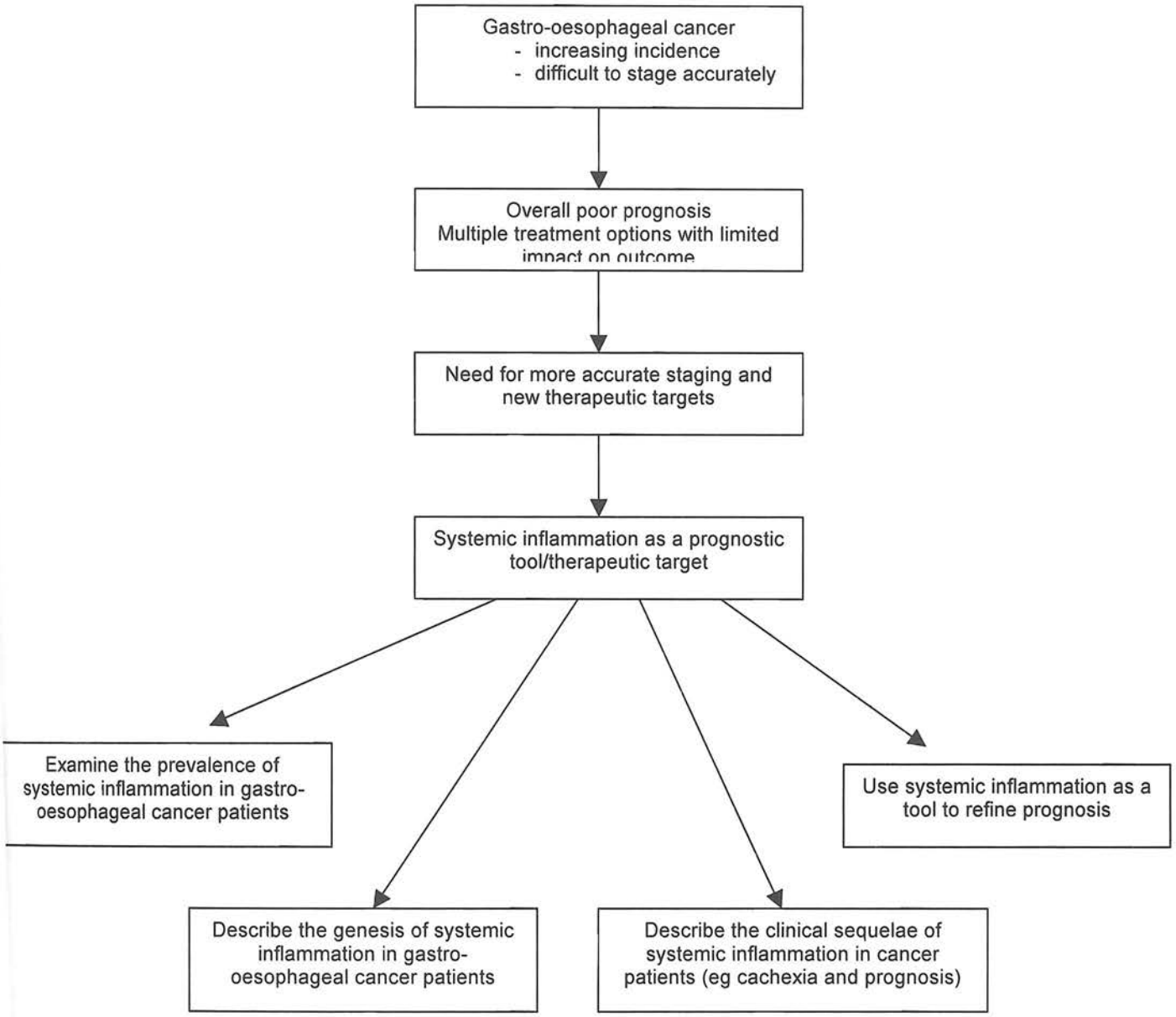
CHAPTER I

HYPOTHESES AND AIMS

The incidence of gastro-oesophageal cancer continues to increase at an alarming rate and is the third leading cause of cancer-related death in the UK. Accurate clinical staging remains challenging, particularly with regard to tumours located around the gastro-oesophageal junction. Surgery offers the only real prospect of cure but carries significant risks in terms of morbidity and mortality. A number of alternative palliative treatment options are available, but are generally disappointing. Current staging practices are unable to identify prospectively which patients will truly benefit from surgical resection in terms of survival benefit and quality of life. Many novel prognostic factors have been proposed including the presence of systemic inflammation. Previous work has also suggested an association between markers of systemic inflammation and cancer cachexia, which is commonly associated with gastro-oesophageal cancer and has also been linked with poor outcome. As well as improving prognostic accuracy, investigation of systemic inflammation and cachexia in these patients may enable the identification of much needed potential novel therapeutic targets.

The overall aim of this thesis is to describe the genesis, mediators and clinical sequelae of systemic inflammation in patients with gastro-oesophageal cancer (Figure 1.1). The usefulness of systemic inflammation as a prognostic indicator and the role of cachexia as a factor in the adverse prognosis associated with systemic inflammation are expanded in detail.

Figure 1.1 Overview of thesis aims and objectives



Hypotheses and Aims

1. **Markers of systemic inflammation are elevated in patients with gastro-oesophageal cancer and are associated with adverse prognosis.**

The prevalence of systemic inflammation at the time of diagnosis and following treatment is described in patients with gastro-oesophageal cancer. Markers of systemic inflammation (serum cytokine and serum acute phase protein concentrations) will be measured at the time of diagnosis and later in the course of the disease in a cohort of consecutive patients newly diagnosed with gastric or oesophageal cancer. The association between elevated serum markers of systemic inflammation and adverse prognosis was examined (Chapters IV and IX).

2. **Tumour cells produce mediators (such as pro-inflammatory cytokines, proteolysis-inducing factor, and parathyroid hormone-related peptide), which can stimulate a systemic inflammatory response in the host and may induce a catabolic state in the peripheral tissues. Such wasting (cachexia) may be one of the mechanisms linking systemic inflammation with adverse prognosis in patients with cancer.**

The role of tumour tissue in the generation of the systemic inflammatory response was investigated by measuring cytokine protein and mRNA concentrations within the tumour tissue and relating these values to systemic concentrations (Chapter V). Proteolysis-inducing factor (PIF) and parathyroid hormone-related peptide (PTHrP) are tumour-derived mediators that may contribute to the systemic inflammatory response and tissue wasting and may be related to adverse prognosis. The expression of these mediators in the host was investigated and their association with systemic inflammation, nutritional status and prognosis was examined (Chapters VII and VIII). The role of cachexia in explaining the link between

systemic inflammation and adverse prognosis was investigated by examining the relationship between markers of systemic inflammation and nutritional variables in the patient cohort (Chapter X).

3. **Cytokine genotype may influence the ability of the host to generate a systemic inflammatory response via alterations in tumour tissue cytokine concentrations which, in turn, may influence survival duration.**

Cytokine genotypes were determined for the study population and individual polymorphisms were studied with relation to tumour tissue cytokine concentrations, the magnitude of markers of systemic inflammation, and prognosis (Chapter VI).

4. **The development of a prognostic model for gastro-oesophageal cancer.**

The usefulness of systemic inflammation and cachexia as prognostic indicators was investigated in Chapter XI. Various clinical and investigative factors, including markers of systemic inflammation and cachexia, predictive of death from gastro-oesophageal cancer were determined with the aim of constructing a novel risk prediction model. This model may then be used to assist in the prospective prognostic evaluation of patients with gastro-oesophageal malignancy and assist in the clinical decision making for these patients.

It is hoped that this work will advance the understanding of tumour-associated inflammation with particular reference to the clinical sequelae and impact on nutritional status and prognosis. This may, in turn, lead to the identification of potential new therapeutic targets for this devastating disease and allow the development of individually tailored therapeutic options and selective interventions.

CHAPTER II

GENERAL INTRODUCTION

This chapter outlines the increasing incidence of gastric and oesophageal cancer in the UK and describes current staging modalities and therapeutic options available. The need for improved prognostic accuracy and therapeutic strategies for these patients is emphasised. Previous studies suggest that markers of systemic inflammation may have a prognostic role in gastro-oesophageal cancer and, as such, may also provide novel therapeutic targets. The overall aim of this thesis is to describe the genesis, mediators and sequelae of systemic inflammation in patients with gastro-oesophageal cancer. The mechanisms involved in the generation of acute and chronic systemic inflammation are described in detail in this chapter with particular emphasis on key cytokines and acute phase proteins. The clinical sequelae of these acute and chronic inflammatory syndromes are also described and their contribution to wasting and prognosis in cancer patients is explored. The usefulness of systemic inflammation as a prognostic indicator and the role of cachexia as a factor in the adverse prognosis associated with systemic inflammation are expanded in detail.

1. GASTRO-OESOPHAGEAL CANCER

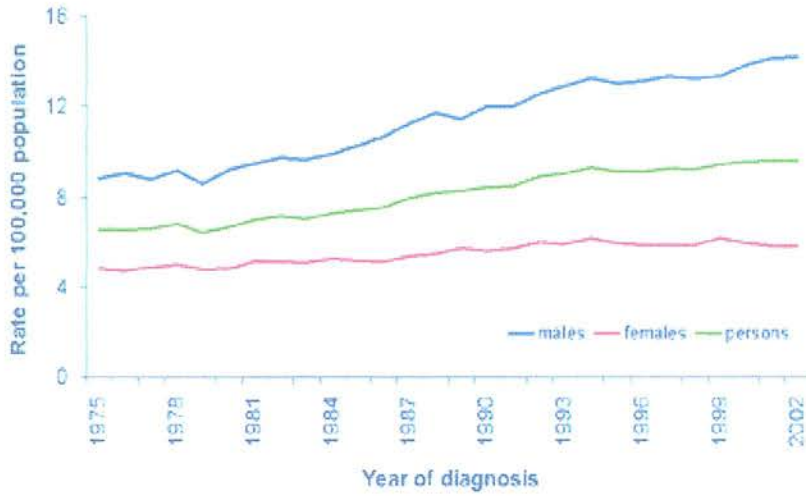
a) Epidemiology of gastric and oesophageal cancer

Oesophageal cancer

The incidence of oesophageal cancer has risen steadily in the UK and throughout Western Europe and North America over the past few decades (NHS Scotland, 2004; Office for National Statistics, 2004). The incidence of adenocarcinoma of the oesophagus is increasing at the fastest rate of any solid tumour in Europe and North America (Blot el al, 1991). The incidence rates for oesophageal cancer have increased substantially in Scotland which now has the highest incidence rates in the UK (Figure 2.1.1) (NHS Scotland, 2004). Scottish female adenocarcinoma rates have been reported as among the highest in the world (Parkin et al, 1997). In 2004 there were 7531 new diagnoses of oesophageal cancer in the UK (783 in

Scotland) making oesophageal cancer the 7th most common malignancy in men and the 11th most common cancer diagnosed in females in the UK (Cancer Research UK, 2004).

Figure 2.1.1 Trends in incidence rates of oesophageal cancer in the UK (1975-2002). [Adapted from Cancer Research UK cancer statistics online]

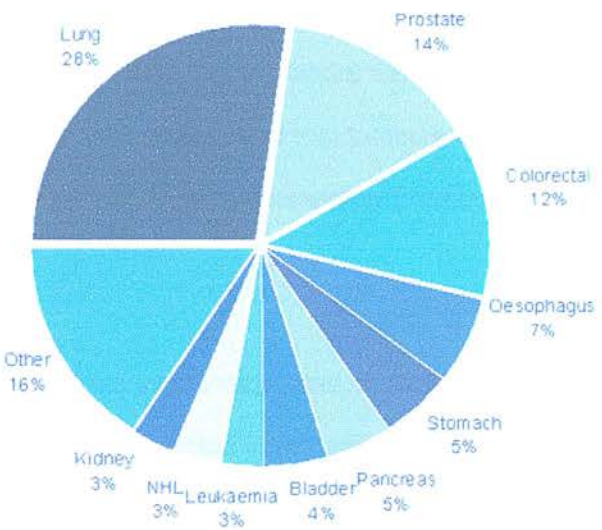


The increasing incidence of oesophageal malignancy has been attributed largely to an increase in adenocarcinoma arising in the distal oesophagus and at the oesophago-gastric junction [OGJ] (Jankowski et al, 2000). These are now the most common sites for tumour occurrence and the incidence of such tumours is increasing at 2% per year in Scotland (Reed, 1991; McKinney et al, 1995). These tumours may arise from areas of Barrett's metaplasia affecting the distal oesophagus and the increasing incidence of OGJ cancers has been matched by a similar increase in the rates of Barrett's oesophagus (Jankowski et al, 2000). Metaplastic changes predispose the oesophagus to dysplasia and malignant transformation and around 62% of oesophageal tumours have histological evidence of Barrett's metaplasia within the specimen (Jankowski et al, 1999; Cameron et al, 2000). In addition, while the total number of squamous cell carcinomas has remained relatively constant, the incidence of adenocarcinoma has risen considerably throughout the past three

decades and approximately 73% of oesophageal malignancies in the UK are now adenocarcinoma in type (AUGIS database report, 2004; Pera et al, 2001; Cameron, 1997).

Prognosis remains generally poor for patients with oesophageal cancer with most patients presenting late in the UK with locally advanced or metastatic disease at the time of diagnosis (NHS Scotland, 2004; Cancer Research UK, 2004). Oesophageal cancer is the 4th leading cause of cancer-related death in males in the UK (behind lung, prostate, and colorectal) (Figure 2.1.2).

Figure 2.1.2 Causes of male cancer deaths UK (2004). [Adapted from Cancer Research UK cancer statistics online]



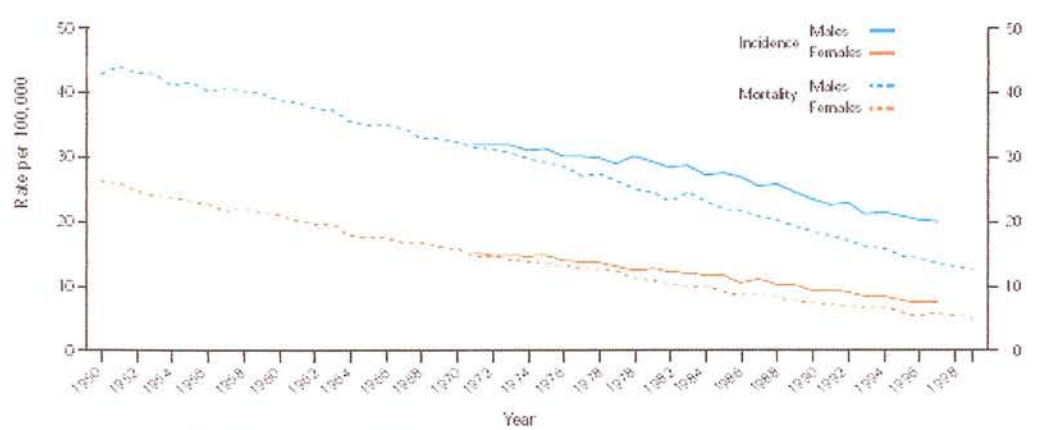
The trends in mortality follow closely those for incidence due to the very poor survival rates from this disease. As such, mortality rates have risen despite advances in staging, chemo/radiotherapeutics and surgical techniques, although there has been a modest improvement in median survival (Office for National Statistics, 2004). Overall 5-year survival remains extremely poor at 6-8%.

Gastric cancer

Fifty years ago stomach cancer was the leading cause of cancer-related death in the UK. Since then the incidence has declined, but gastric cancer still accounts for almost 9000 new cases per year in the UK and the surgical workload in Lothian has not declined (NHS Scotland, 2004; Office for National Statistics, 2004; Cancer Research UK, 2004; Sedgwick et al, 1991) (Figure 2.1.3). In 2004 the incidence of gastric cancer in males in Scotland was 19.5 per 100,000 population and 8.6 per 100,000 for females, making stomach cancer the 5th commonest cancer diagnosed in UK men and the 9th most common cancer in UK women (Cancer Research UK, 2004).

Despite an overall decrease in the incidence of stomach cancer, there is evidence to suggest increasing numbers of tumours arising in the proximal stomach and gastric cardia (Dolan et al, 1999; Wayman et al, 2001). These tumours have been associated with more advanced disease stage at the time of presentation and with overall stage-related poorer long-term survival (Rohde et al, 1991). Some authors have suggested that these tumours have a different biological behaviour compared with tumours located more distally in the stomach and should therefore be addressed separately (Webb etal, 1978).

Figure 2.1.3 Age standardised incidence of, and mortality from, stomach cancer in England and Wales (1950-1999). [Adapted from Cancer Research UK cancer statistics online]



* Figures for incidence for 1995-1997 and mortality for 1999 are provisional.

Gastric cancer is the 5th leading cause of cancer-related death in UK males. An overall decline in incidence has been matched by a fall in mortality rates (Figure 2.1.3) and survival rates have improved by 2% every five years from 1970 (Office for National Statistics, 2004). Some of these improvements in survival have resulted from better survival rates following surgical resection (Akoh and Macintyre, 1992). Despite these improvements in survival, many patients present with advanced stage disease in the UK and as such overall survival rates remain very disappointing. Current 1-year survival rates are 28% and 5-year survival rates are 10% (NHS Scotland, 2004; Cancer Research UK, 2004).

b) Current Staging Modalities and Treatment Options

Currently, treatment decisions for patients with gastro-oesophageal cancer are based on the clinical stage of disease, patient co-morbidity and patient preferences. Accurate pre-operative assessment and staging of patients with oesophageal and gastric cancer is essential in order to determine the most appropriate management option for each patient. With the introduction of new therapeutic strategies and as neoadjuvant regimens are designed and tested, accurate staging is essential not only in terms of patient selection, but also in assessing outcome. It is now clear that surgical resection is no longer the best form of palliation for the majority of patients with gastro-oesophageal cancer whose tumour is not curable by resection (Blazeby et al, 2000). These patients are better served by alternative palliative treatments directed at improving symptoms.

Clinical staging is based on estimating the anatomical extent and distribution of the disease, principally using imaging modalities such as computerised tomography (CT) and endoscopic ultrasound (EUS). The TNM staging systems for gastric and oesophageal cancer are used in the UK (Sobin et al, 2003). The TNM system is based on an anatomical classification of disease involvement, where T represents the extent of the primary tumour, N the presence or absence and extent of regional lymph node metastases, and M the presence or absence of distant metastases. The addition of numbers to these groups indicates the progression of the disease (Table 2.1.1).

Table 2.1.1 TNM stage classification for (A) oesophageal and (B) gastric cancer.

(A)

T stage	T1	Tumour invades lamina propria or submucosa
	T2	Tumour invades muscularis propria
	T3	Tumour invades adventitia
	T4	Tumour invades adjacent structures
N stage	N0	No regional lymph node metastasis
	N1	Regional lymph node metastasis
M stage	M0	No distant metastasis
	M1	Distant metastasis
For tumours of the lower thoracic oesophagus	M1a	Metastasis in coelic lymph nodes
	M1b	Other distant metastases
For tumours of the upper thoracic oesophagus	M1a	Metastasis in cervical lymph nodes
	M1b	Other distant metastases

(B)

T stage	T1	Tumour invades lamina propria or submucosa
	T2A	Tumour invades muscularis propria
	T2B	Tumour invades the subserosa
	T3	Tumour penetrates serosa (visceral peritoneum) without invasion of adjacent structures
	T4	Tumour invades adjacent structures
N stage	N0	No regional lymph node metastasis
	N1	Metastasis in 1 to 6 regional lymph nodes
	N2	Metastasis in 7 to 15 regional lymph nodes
	N3	Metastasis in more than 15 regional lymph nodes
M stage	M0	No distant metastasis
	M1	Distant metastasis

Once a TNM category has been decided, a stage grouping is assigned (Table 2.1.2). This simplifies the staging analysis allowing easier comparison of outcomes. The groupings are chosen to ensure that each group is similar in terms of prognosis, while at the same time maintaining differences between groups.

Table 2.1.2 Internationally unified TNM stage groupings for (A) oesophageal cancer and (B) gastric cancer

(A)

Stage group	T stage	N stage	M stage
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T2, T3	N0	M0
Stage IIB	T1, T2	N1	M0
Stage III	T3	N1	M0
	T4	Any N	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

(B)

Stage group	T stage	N stage	M stage
Stage 0	Tis	N0	M0
Stage Ia	T1	N0	M0
Stage Ib	T1	N1	M0
	T2A or B	N0	M0
Stage II	T1	N2	M0
	T2A or B	N1	M0
	T3	N0	M0
Stage IIIa	T2A or B	N2	M0
	T3	N1	M0
	T4	N0	M0
Stage IIIb	T3	N2	M0
Stage IV	T4	N1, N2, N3	M0
	T1,T2,T3	N3	M0
	Any T	Any N	M1

Some authors have also proposed a separate additional staging method for tumours arising from the gastro-oesophageal junction (Hardwick and Williams, 2002). These tumours are rapidly increasing in incidence and are defined as those that are centred within 5cm proximal or distal of the anatomical cardia. The International Society for Diseases of the Oesophagus has endorsed a classification of junctional tumours which is based on the likely origin of the

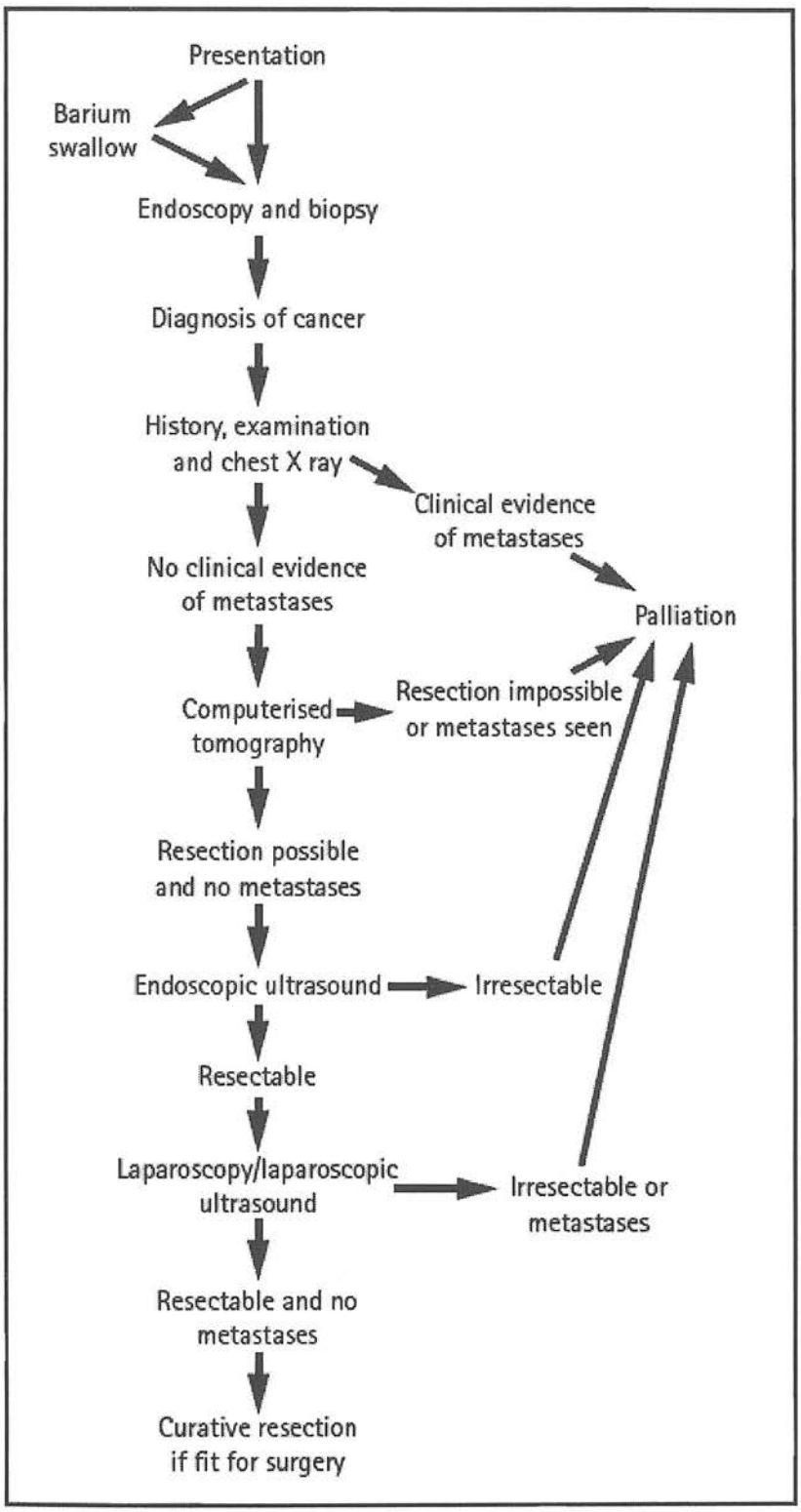
tumour (Siewert and Stein, 1998). Type I represents adenocarcinoma of the distal oesophagus with the centre of the tumour lying 2-5cm above the anatomical cardia. Type III cancer is a gastric carcinoma with its centre 2-5 cm below the anatomical cardia. Type II lesions are true junctional tumours with centres 2cm above or below the anatomical cardia.

Adenocarcinoma arising in the region of the oesophago-gastric junction pose a problem for staging with the main difficulty lying in the identification of regional lymph nodes. Due to their anatomical location, these tumours may metastasise to lymph nodes above or below the diaphragm. Involvement of these nodes may therefore be classified as regional nodal involvement (N) or metastatic (M) both of which may have repercusions on management decisions and prognosis. At the present time, Type I tumours are staged as oesophageal cancers and Type III lesions are staged as gastric tumours but debate still exists as to the best staging system for Type II tumours and some authors have proposed the need for a separate staging system in order to address these particular problems (Hardwick and Williams, 2002; Wijnhoven et al, 1999).

Staging algorithm

Clinical staging includes physical examination, laboratory studies and imaging. An algorithm for the investigation and clinical staging of a patient presenting with gastro-oesophageal cancer in the Lothian and Borders regions is shown in Figure 2.1.4. Initially an endoscopy and biopsy are performed on any patient in whom the disease is suspected. Once the disease is confirmed histologically, the patient undergoes clinical staging to assess the extent of disease. In our region, patients are generally assessed by computerised tomography (CT) and endoscopic ultrasound (EUS). A laparoscopy is also undertaken in patients who are thought to be suitable for surgery to exclude peritoneal disease. The result of each investigation is documented and patients are discussed at regular multi-disciplinary team (MDT) meetings. Following this meeting an overall clinical stage is agreed for each patient, individual patient characteristics and preferences are considered, and treatment options are decided. The clinical stage also provides information on prognosis. Estimations of prognosis as well as management decisions are, therefore, largely based on the clinical (anatomical) stage of disease.

Figure 2.1.4 An algorithm for the investigation and clinical staging of a patient presenting with gastro-oesophageal cancer.



Therapeutic options

The only real prospect of cure lies in surgical resection, with or without pre-operative chemo/radiation, although comparable survival rates among patients with squamous cell carcinoma of the oesophagus treated with radical chemoradiotherapy have been documented (Toh et al, 2006). Patients without evidence of metastatic disease and who are medically fit are offered surgery. It is practice in our region for patients with local disease (T1/2 N0) to go directly for surgery. Patients with more advanced disease (T2 N1 or T3 N0 or T3 N1) are considered for pre-operative (neo-adjuvant) chemotherapy. Surgery carries significant risks both in terms of morbidity (40-50%) and mortality, which is in the region of 5-10% (Stephens et al, 2006).

Patients with metastatic disease may receive a number of palliative treatments, such as palliative chemotherapy, radiotherapy, or endoscopic procedures, such as placement of an oesophageal stent or endoscopic laser therapy to improve swallowing or reduce tumour bleeding. These procedures attempt to alleviate symptoms and improve quality of life, but are overall disappointing.

c) The clinical problem – accurate prognosis and novel therapeutic targets

Despite advances in surgical techniques, critical care and chemo-therapeutics overall survival rates remain dismal as most patients present with locally advanced disease in the UK (NHS Scotland, 2004; Cancer Research UK, 2004). Treatment decisions are based on clinical stage and overall patient fitness. Improvements in staging techniques have lead to improved staging accuracy and better patient selection for surgery, which has resulted in improvements in survival duration (Hofstetter et al, 2002). However, despite advances in these staging techniques, 5-year-survival rarely exceeds 30% (Dresner and Griffin, 2000). This figure, however, represents all patients undergoing resection and those patients with locally advanced disease (stage IIB and III) achieve 5-year-survival post-resection survival rates of less than 10% (Dresner and Griffin, 2000). Indeed in this group, which represents

approximately two-thirds of all patients who are suitable for curative resection, around 50% do not survive more than 18 months, even though these patients have undergone 'curative' (R0) resection. Data from Bristol has shown that those patients who failed to live 2 years following surgery do not regain pre-operative quality of life (QOL) levels compared with those who live greater than 2 years, whose QOL measurements returned to normal by 9 months (Blazeby et al, 2000). These results provide strong support for the increasingly held view that patients in whom a potentially curative resection is not possible should not undergo surgery for oesophageal cancer. The problem lies in identifying prospectively such patients. Clearly improvements in pre-operative staging techniques (for example, CT and EUS) are likely to help, but additional factors which influence survival and can be used to refine prognosis are required. Tumours located at the OGJ are particularly difficult to stage accurately (Department of Health, 2001).

If clinicians could identify prospectively patients unlikely to live more than 12-18 months following radical oesophagectomy/gastrectomy then these patients could be offered more appropriate non-surgical palliative therapy. More rigorous selection of patients would avoid surgery in those who gain no survival benefit, may suffer considerable morbidity, a chance of procedure related death, and greatly reduced quality of life.

In addition to better prognostic accuracy, improved understanding of tumour biology may provide much needed novel therapeutic targets for this devastating disease (such as targeting mediators involved in the generation of systemic inflammation) and also novel palliative therapy targets, such as anti-cachexia treatments. A better knowledge of these factors may also offer individually tailored therapeutic options and selective interventions.

d) Potential novel prognostic markers (and therapeutic targets) for gastro-oesophageal cancer

Biological staging attempts to analyse tumour behaviour, assessing the likelihood for metastatic spread, disease recurrence and responsiveness to various chemotherapeutic

agents. There have been many different molecules that have been investigated as potential prognostic markers in gastric and oesophageal cancer, some of these are novel molecules whereas others have demonstrated previous prognostic value in other diseases. Most of these potential prognostic markers may be grouped as tumour markers, tumour suppressor genes/proto-oncogenes, adhesion molecules, heat shock proteins, growth factors, and markers of systemic inflammation (Table 2.1.3).

Tumour markers do not offer any diagnostic aid to gastro-oesophageal cancer and their use as prognostic markers remains unclear. Some studies have reported a correlation between carcino-embryonic antigen (CEA), CA19-9 and CA72-4 and increased stage of gastric cancer (Gaspar et al, 2001; Lai et al, 2002; Kim et al, 2000; Lundin et al, 1995; Kodera et al, 1996). They also found that CA72-4 was an independent prognostic indicator on multivariate analysis and elevated levels were associated with over four times increased chance of recurrence. However, other researchers have failed to identify any association between tumour markers and stage or survival (Duraker and Celik, 2001).

The important consequences of mutations of the tumour suppressor gene p53 in tumour genesis is well known and up to 90% of adenocarcinomas of the oesophagus possess a p53 mutation (Younes et al, 1993). There is some evidence that p53 expression is associated with an adverse prognosis in gastric and oesophageal cancers (Liu et al, 2001; Ogawa et al, 2001; Diez et al, 2000) and elevated levels have been associated with a poorer outcome in node negative oesophageal cancer patients treated by surgical resection (Aloia et al, 2001; Shimada et al, 2000). In contrast, the frequency of proto-oncogene activation is very low in gastric and oesophageal cancers.

Impaired adhesion molecule expression is thought to favour cancer invasion and metastases. Abnormal expression of E-cadherin has been associated with advanced stage in gastric cancer and has been related with early disease recurrence (Chan et al, 2001; Chan et al, 2003; Liu et al, 2002; Tanaka et al, 2003). Matrix metalloproteinases (MMPs) are a family of peptidases capable of degrading the extracellular matrix and play important roles in allowing tumor invasion. MMP expression has also been associated with adverse outcome in early stage oesophageal cancer (Gu et al, 2005).

Table 2.1.3 Value of novel prognostic factors for gastro-oesophageal cancer

Marker	Negative survival effect	Prognostic impact
Tumour markers		
CEA	Elevated expression	Weak
CA19-9	Elevated expression	Weak
CA72-4	Elevated expression	Weak
Tumour suppressor genes		
p53	Mutation	Strong
Adhesion molecules		
E-cadherin	Reduced expression	Strong
MMP-1	Elevated expression	Moderate
Heat shock proteins		
HSP 27	Reduced expression	Weak
Growth factors		
VEGF	Elevated expression	Weak
FGF	Elevated expression	Weak
EGF	Elevated expression	Weak
Systemic inflammation		
CRP	Elevated expression	Strong
IL-1β	Elevated expression	Weak
IL-6	Elevated expression	Weak
IL-10	Elevated expression	Weak
TNF-α	Elevated expression	Weak

Heat shock proteins are a family of protective proteins produced in response to stress. Recent work has associated some of these proteins with prognosis in gastric cancer and reduced HSP 27 tumour expression has been associated with adverse outcome on mutivariate analysis among patients with oesophageal cancer who had undergone surgical resection (Kapranos et al, 2002; Kawanishi et al, 1999).

Growth factors, such as the angiogenic factor vascular endothelial growth factor (VEGF), have been linked with adverse out come in gastric cancer, but it's prognostic value in oesophageal cancer is less clear (Shida et al, 2005; Kleespies et al, 2004). Fibroblast growth factor (FGF) has been associated with early recurrence and poorer outcome in surgically treated oesophageal cancer (Barclay et al, 2005).

The role of systemic inflammation as a prognostic aid to cancer patients will be described separately in the following Section (e). Many other potential prognostic markers have been investigated in relation to gastro-oesophageal cancer and novel candidates are continually proposed, especially following the introduction of DNA microarray systems (Yamabuki et al, 2006). The prognostic role of many of these remains unclear and require prospective evaluation (see Lagarde et al, 2007 for a current review).

e) Role of systemic inflammation in cancer prognosis

Elevated serum pro-inflammatory cytokine concentrations have been found in association with most epithelial malignancies, but the relationship between serum cytokine concentrations and prognosis in cancer patients remains unclear (Table 2.1.3) (Ashizawa et al, 2005; Galizia et al, 2002; Forones et al, 2001; De Vita et al, 1999, De Vita et al, 2001; Wang et al, 1999; Wu et al, 1998). Elevated serum cytokine concentrations are generally regarded as markers of poor prognosis, but conflicting results have been found in pancreatic cancer, lung cancer and many other malignancies (Tas et al, 2005; Songur et al, 2004; Martignoni et al, 2005; Falconer et al, 1994). The prognostic value of serum cytokine concentrations in gastric and oesophageal cancer remains equally contradictory. Elevated serum concentrations of interleukin-6 (IL-6), IL-10 and tumour necrosis factor-α (TNFα) have been associated with poor and favourable outcomes in equal measure. These inconsistent findings may be partly explained by the difficulty in reliably measuring serum cytokine concentrations. Short half-lives, molecular lability and fluctuating levels of circulating cytokines contribute to the difficulties in measuring circulating cytokine concentrations (Gabay and Kushner, 1999). In addition, the relevance of circulating cytokine concentrations may not be as functionally important as the local concentrations within target tissues and those produced by peripheral blood mononuclear cells (PBMC's) (see later Sections).

In contrast to circulating cytokine concentrations, serum acute phase protein concentrations demonstrate more stability within the systemic compartment and may be a more reliable measure of systemic inflammatory activity (Gabay and Kushner, 1999). Serum

acute phase protein concentrations have been linked with outcome in benign disease, for example ischaemic heart disease and acute pancreatitis, as well as in malignant disease (Shah, 2000; Puolakkainen et al, 1987). An acute phase protein response (APPR) has been shown to be an independent adverse prognostic indicator in the majority of types of cancer, including pancreatic, lung, breast, melanoma, lymphoma, ovarian, renal, and gastrointestinal tumours (Rashid et al, 1982; Nozoe et al, 2001; Shimada et al, 2003; Crumley et al, 2006; Alexandrakis et al, 2003; Falconer et al, 1995; Cooper, 1988; Caspers et al, 1984; Forrest et al, 2003; McMillan et al, 2001; Masuda et al, 1998; Kodama et al, 1999; Elahi et al, 2005; McMillan et al, 2003; Fujita et al, 1999; Jamieson et al, 2005). In patients with gastric cancer the presence of an APPR has been associated with a markedly reduced median survival (9 versus 53 weeks, $p<0.001$) (Rashid et al, 1982). Similarly, two Japanese studies have identified a shortened survival in oesophageal cancer patients with an elevated serum C-reactive protein (CRP) at the time of diagnosis (Nozoe et al, 2001; Shimada et al, 2003). More recently a group from the UK has similarly identified elevated serum CRP and reduced serum albumin concentrations as independent prognostic indicators among patients with inoperable gastro-oesophageal cancer (Crumley et al, 2006). In addition, a large study of patients with colorectal, breast, gastric and bronchogenic cancers demonstrated a negative relationship between the magnitude of the systemic inflammatory response and survival duration (McMillan et al, 2001). Despite this apparent strong association little is known about the mechanism initiating systemic inflammation and the factors that link systemic inflammation to adverse outcome.

Serum concentrations of acute phase proteins have also been linked with disease progression and the development of recurrent disease. Patients with irresectable pancreatic cancer demonstrate increasing serum CRP concentrations as the disease progresses and the proportion of patients with an elevated serum CRP concentration increases from 43% at the time of diagnosis to 78% as patients approach the final stages of their disease (Falconer et al, 1995; Barber et al, 1999). An elevated serum CRP concentration following potentially curative surgery for colorectal, pancreatic and gastric cancers has also been associated with earlier disease recurrence (McMillan et al, 2003; Fujita et al, 1999; Jamieson et al, 2005).

Therefore, serum markers of systemic inflammation, such as acute phase protein concentrations, appear to be linked with survival duration in patients with cancer. Moreover, these concentrations may alter as the disease progresses and responds to treatment. The association between systemic inflammation and prognosis in patients with gastro-oesophageal cancer will be explored in this thesis along with the origin of the systemic inflammatory response. The potential mechanisms involved in the generation of such inflammation will now be described in the following Section.

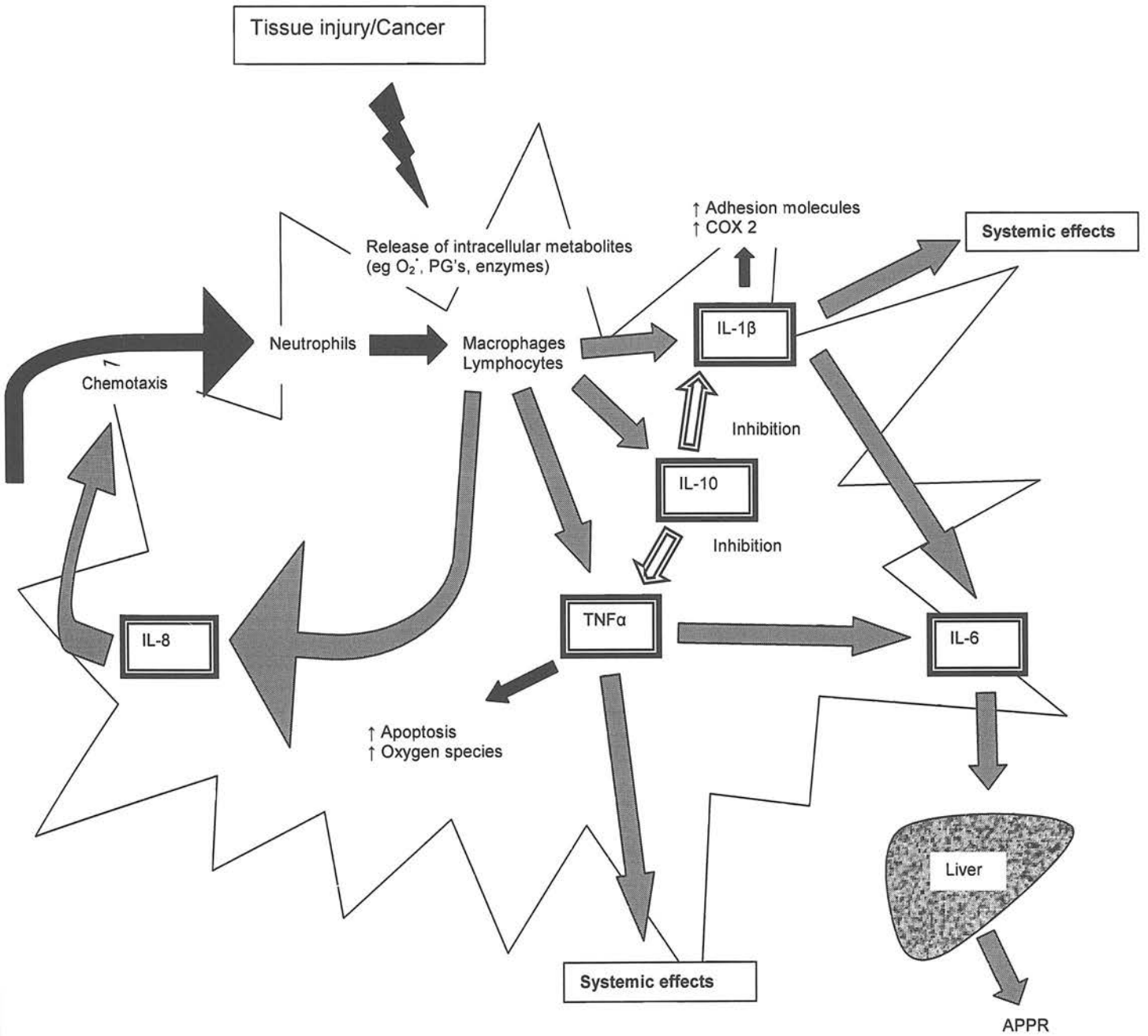
2. THE GENERATION OF AN INFLAMMATORY RESPONSE – AN OVERVIEW

The inflammatory response is a reaction of the body to tissue injury and immune system activation. It functions principally as a defense mechanism designed to destroy or reduce an injurous agent, limit tissue damage, and promote tissue healing. Initiation and propagation of the inflammatory response is mediated through a complex interaction of chemical mediators derived from host tissues and immune cells as well as the injurous agent itself. Such chemical mediators, acting together or in sequence, then influence the evolution and magnitude of the inflammatory response. The inflammatory response is not limited to the site of tissue injury and inflammatory cells and chemical mediators are free to circulate in the plasma and tissue fluid exerting effects on distant target tissues (see Figure 2.2.1). The effect of such systemic inflammation has consequences on the host distant from the site of injury. These systemic effects, such as tissue wasting and altered metabolism (cachexia), will be explored later in this chapter. The mechanisms underlying the generation of the inflammatory response will now be described.

a) Cellular events

Initial cellular events in the generation of inflammation involve the influx (emigration) of neutrophils to the site of injury. Neutrophils function primarily to aid the destruction of the injurous agent (Figure 2.2.1). This may be achieved by phagocytosis or release of enzymes and metabolites (degranulation). These metabolites, for example, oxygen free radicals, may serve to amplify the inflammatory stimulus and further recruitment of inflammatory cells may occur. Peripheral blood mononuclear cells (PBMC's), macrophages and lymphocytes (T and B cell) are the main cell types attracted to the site of inflammation. These cells also fight the injurous agent through mechanisms such as phagocytosis, degranulation and activation of adaptive immunity. A key role is also in the release of the chemical mediators of inflammation.

Figure 2.2.1 A schematic overview of the processes involved in the generation of an inflammatory response. Tissue injury leads to the release of local metabolites, such as free radicals, which recruit inflammatory cells to the site of injury. Initially neutrophils are attracted, then macrophages and lymphocytes. These cells secrete cytokines and other chemical mediators, which interact to control the inflammatory process. These mediators act locally at the site of tissue inflammation, but are also free to circulate throughout the body and exert systemic effects (eg IL-6 stimulates acute phase protein production in the liver).



b) Chemical mediators of inflammation

Cytokines

Cytokines are a group of polypeptides produced predominantly by macrophages and lymphocytes in response to inflammation. They modulate the function of other cell types through autocrine, paracrine and endocrine pathways. Cytokines may be grouped into interleukins, tumour necrosis factors and interferons. There are many cytokines described, however, the key mediators and their principal actions are listed in Table 2.2.1. Interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor-α (TNFα) are generally regarded as key pro-inflammatory cytokines. Interleukin-10 (IL-10) is thought to act predominantly as a counter-inflammatory cytokine.

Table 2.2.1 Key cytokines involved in the regulation of inflammation and their systemic effects.

Cytokine	Main sources	Inflammatory effects	Systemic effects
IL-1β	Macrophages Endothelial cells	Pro-inflammatory Induces IL-1β, TNFα, and IL-6 production Up-regulates adhesion molecule expression Induces COX 2 expression	Fever Muscle protein loss Elevated blood glucose Changes in trace element concentrations
IL-6	Macrophages T lymphocytes	Pro-inflammatory Up-regulated by IL-1β and TNFα Stimulates acute phase protein production in the liver	Fever Weight loss Increased energy expenditure Increased cortisol/glucagon
IL-8	Macrophages	Pro-inflammatory Induced by IL-1β and TNFα Principally a chemokine	Unknown direct effect Contributes via maintenance of inflammatory response
TNFα	Macrophages	Pro-inflammatory Induces IL-1β and TNFα production Stimulates apoptosis Promotes release of oxygen species	Fever Muscle protein loss Anorexia Altered serum lipid and trace element concentrations
IL-10	Macrophages T lymphocytes	Inhibits pro-inflammatory cytokine production Sheds TNF receptors into the circulation	

IL-1β is a key pro-inflammatory cytokine in humans. It is released principally from macrophages during the inflammatory process, but may also be produced by a number of other cell types, such as endothelial cells. IL-1β up-regulates the inflammatory response by inducing its own synthesis and also by increasing TNFα, IL-6, and IL-8 production (Yasumoto et al, 1995). IL-1β can act directly by increasing adhesion molecule expression, especially intercellular adhesion molecule (ICAM), which promotes leucocyte sticking to endothelial cells to aid chemotaxis. IL-1β also stimulates the production of acute phase proteins by the liver indirectly through increased stimulation of IL-6 and promotes leucocyte recruitment via IL-8 (Gershenwald et al, 1990; Hellerstein et al, 1989). The systemic effects of IL-1β include fever, anorexia, weight loss, alterations in glucose metabolism, and loss of muscle protein (Moldawer et al, 1988). Loss of muscle mass is due to increased rates of skeletal muscle breakdown and decreased rates of muscle synthesis, principally through activation of the cyclo-oxygenase pathway (Baracos et al, 1983).

TNFα is also an important pro-inflammatory cytokine, capable of stimulating its own synthesis as well as IL-1β, among others. TNFα and IL-1β work together as the key conductors of the generation of the inflammatory response. TNFα is produced principally by macrophages and promotes macrophage apoptosis and release of oxygen free radicals at the site of inflammation. TNFα also has widespread metabolic effects upon the body, some of which are similar to IL-1β and include fever, anorexia, weight loss, and muscle protein loss (Mahony and Tisdale, 1988; Charters and Grimble, 1989; Starnes et al, 1988). In addition, TNFα is associated with increased lipolysis, insulin resistance and elevated energy expenditure in both animals and humans (Selbey et al, 1987). Circulating TNFα concentrations appear to exhibit circadian changes and concentrations are not reliably detected in the circulation (Muc-Wierzgon et al, 1996). TNFα binds to two receptors of differing molecular weight: 55 kDa and 75 kDa (Brockhaus et al, 1990) and these two receptors are shed from cells into the circulation in response to TNFα release and may therefore provide an indirect measure of TNFα activity (Spinas et al, 1992). Plasma soluble TNF receptor (sTNF-R) levels are, therefore, a more robust indirect index of TNFα production in tissue compartments and are not subject to the diurnal variation of cytokine release (Muc-Wierzgon et al, 2003).

IL-6 is released from macrophages and activated T lymphocytes mainly in response to stimulus by TNFα and IL-1β. The main inflammatory function of IL-6 is the induction of the acute phase response by the liver (O'Riordain et al, 1999). The acute phase response is a component of innate immunity and will be described in detail in the following section (Section 2 (c)). Administration of IL-6 to human subjects produces weight loss, fever, increased energy expenditure, and increased serum cortisol and glucagon concentrations (Stouthard et al, 1995). IL-6 is generally regarded as a pro-inflammatory cytokine, however, infusion of IL-6 into human subjects has been shown to increase the release of anti-inflammatory mediators (IL-1 receptor antagonist and soluble TNF receptors) leading some groups to postulate that IL-6 may also function as an anti-inflammatory cytokine (Tilg et al, 1994). It is equally possible that the release of these anti-inflammatory mediators is simply a counter-response to the pro-inflammatory activity of IL-6.

IL-8 is also stimulated by TNFα and IL-1β and is released by macrophages. IL-8 acts locally at the site of inflammation promoting neutrophil degranulation and recruitment of leucocytes (chemotaxis) (Ward and Westwick, 1998). The direct systemic effects of IL-8 remain unclear, however, IL-8 contributes to the overall effects of systemic inflammation through maintenance of the inflammatory response.

In contrast to the previously described cytokines, IL-10 acts as an anti-inflammatory cytokine via inhibition of TNFα and IL-1β production. IL-10 suppresses synthesis of TNFα and IL-1β mainly through accelerated degradation of TNFα and IL-1β mRNA (Adib-Conquy et al, 1999). IL-10 also promotes the shedding of soluble TNF receptors into the circulation thus binding biologically active TNFα. As such, IL-10 acts as a powerful counter-inflammatory cytokine.

There are many other cytokines as well as other chemical mediators (such as prostaglandins, leukotrienes, complement factors, and kinin products) that contribute to the inflammatory process. Their interactions are complex and poorly understood. The inflammatory process will be influenced not only by the interaction of these multiple mediators, but also by the concentrations of specific binding proteins and the levels of receptor expression and affinity. This overview has highlighted some of the key mediators involved in the regulation of

systemic inflammation and it is these mediators – namely IL-1β, IL-6, IL-8, TNFα and IL-10 – that will be studied in this thesis.

c) The acute phase protein response

The acute phase protein response (APPR) is a component of innate immunity and is also a systemic manifestation of tissue inflammation. It is characterised by re-prioritisation of liver protein synthesis leading to alterations in serum protein concentrations. There is a fall in the concentration of negative acute phase proteins, such as albumin and transferrin, and a rise in the concentrations of positive acute phase proteins, such as C-reactive protein (CRP), haptoglobin, α1-antichymotripsin, and fibrinogen (Table 2.2.2) (Morley and Kushner, 1982). The function of the APPR is to destroy harmful agents, prevent on-going tissue injury and promote tissue healing (Figure 2.2.2).

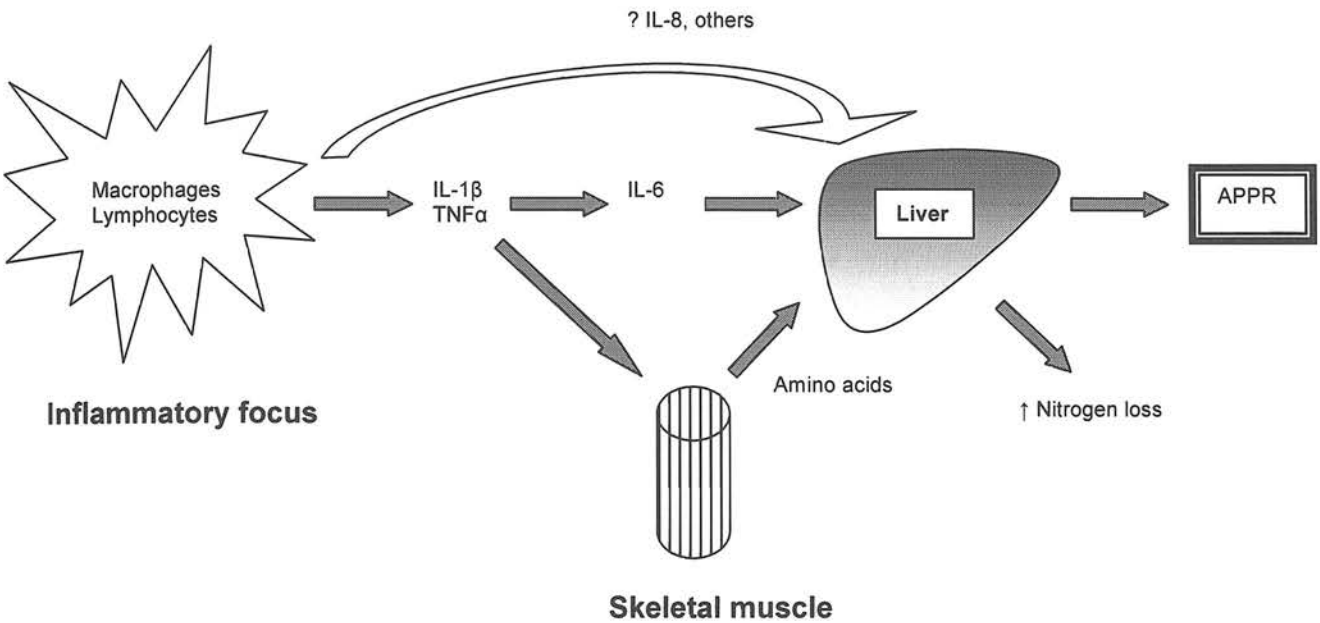
Table 2.2.2 Some examples of acute phase proteins produced by the liver, their functions and changes in serum concentrations during an acute phase response.

Protein	Principal Function	Serum concentrations during acute phase response
C-reactive protein	Pro-inflammatory (eg activation of complement)	Increase
Haptoglobin	Binding of free haemoglobin Antioxidant	Increase
α1-antichymotripsin	Antiprotease during inflammation Antioxidant	Increase
Albumin	Binding and carrier protein Osmotic regulator	Decrease
Transferrin	Transport of iron	Decrease

IL-6 is the chief inducer of the APPR (Gauldie et al, 1987). IL-6 is stimulated principally by IL-1β and administration of anti-IL-1β antibodies attenuates IL-6 production and the magnitude of the APPR (Gershenwald et al, 1990; Yasumoto et al, 1995). However, an APPR may still be mounted in IL-6 knockout mice, suggesting that other cytokines are capable of inducing the APPR via alternative pathways (Fattori et al, 1994). There is some evidence that IL-8 can also induce an APPR in the liver and novel tumour-derived mediators, such as proteolysis-inducing factor and parathyroid hormone-related peptide, may similarly be capable of activating the APPR (Wigmore et al, 1997; Ogata, 2000; Takahashi et al, 2003; Watchorn et al, 2001).

The re-prioritisation of proteins that characterises the APPR has systemic consequences on the host. There are altered demands for amino acids that may not be met through dietary intake in the anorexic cancer patient and alternative amino acid stores in the body may be sacrificed in preference for the APPR. Such stores include skeletal muscle and increased rates of skeletal muscle proteolysis and reduced rates of protein synthesis have been associated with pro-inflammatory cytokines involved in the generation of the APPR (Baracos et al, 1983). In addition, the alteration in circulating protein concentrations may lead to impaired function, especially for the negative acute phase proteins whose concentrations decline during the APPR. For example, reduced concentrations of transferrin may lead to anaemia that is associated with chronic (inflammatory) disease. The systemic effects of inflammation will be discussed in detail in Section 3.

Figure 2.2.2 Diagramatic representation of the mechanisms involved in the generation of the acute phase protein response and associated changes in protein metabolism.



The APPR occurs in association with inflammation at any site in the body and may occur secondary to any stimulus, for example, infection or trauma. An APPR may also be found in a significant proportion of patients with cancer (Rashid et al, 1982; Nozoe et al, 2001; Shimada et al, 2003; Crumley et al, 2006; Alexandrakis et al, 2003; Falconer et al, 1995; Cooper, 1988; Caspers et al, 1984; Forrest et al, 2003; McMillan et al, 2001; Masuda et al, 1998; Kodama et al, 1999; Elahi et al, 2005; McMillan et al, 2003; Fujita et al, 1999; Jamieson et al, 2005). The pattern of acute phase protein expression varies depending on the underlying stimulus for the inflammatory response and may also demonstrate individual variation (Gabay and Kushner, 1999). This thesis will, therefore, investigate the expression of a range of positive and negative acute phase proteins in order to determine the most relevant among patients with gastro-oesophageal cancer. The acute phase proteins listed in Table 2.2.2 will be investigated; namely CRP, haptoglobin, α1-antichymotripsin, albumin, and transferrin.

This section has outlined an overview of the key processes involved in the generation of an inflammatory response. The main cell types and the key cytokines involved have been described along with the important role of the acute phase protein response. The systemic effects of tissue inflammation on the host ('systemic inflammation') will now be described in the following section.

3. Systemic Effects of Inflammation

The consequences of the inflammatory response are not confined to the site of tissue inflammation, but the activated inflammatory cells and mediators may circulate and act on target tissues distinct from the site of tissue injury resulting in systemic sequelae of inflammation. As such, systemic inflammation may affect the host in a number of ways, including alterations in protein and fat metabolism, changes in endocrine function, and increased energy expenditure. These clinical sequelae may be broadly classified into acute and chronic systemic inflammatory syndromes.

a) Acute systemic inflammatory response syndrome (SIRS)

The early consequences of systemic inflammation are better known as systemic inflammatory response syndrome (SIRS). The definition of SIRS was outlined by the American Society of Critical Care Medicine and is shown in Table 2.3.1 (Bone et al, 1992). SIRS may be induced by any insult to the body, including infection, trauma, hypoxia, burns and surgery and is associated with poor outcomes. SIRS is generated, in part, by the complex network of cytokines involved in the generation of inflammation as previously described. Circulating concentrations of several cytokines, including TNFα, IL-1β, IL-6, IL-8 and IL-10 have been

linked with morbidity and mortality in patients with SIRS (Casey et al, 1993; Pinsky et al, 1993; Hack et al, 1997).

Table 2.3.1 Definition of systemic inflammatory response syndrome (SIRS).

Two or more of	
Temperature	<36°C or >38°C
Pulse	> 90 beats/minute
Respiratory rate	> 20 breaths/minute or $PaCO_2$ < 4.3 kPa
White blood count	> 12 x 10^9/l or < 4 x 10^9/l or > 10% immature (band) forms

The circulating inflammatory mediators affect the function of several organ systems, which may lead to organ dysfunction and even organ failure. Increased endothelial permeability in response to pro-inflammatory cytokines leads to interstitial and alveolar oedema that may progress to acute lung injury and acute respiratory distress syndrome (ARDS) and concentrations of IL-8 in lung broncheoalveolar lavage fluid in patients with ARDS has been shown to correlate with mortality (Donnelly et al, 1993). Nitric oxide is synthesised in vascular endothelium in response to cytokine release. Nitric oxide is a powerful vasodilator and consequently there is a fall in peripheral vascular resistance, which in turn leads to reduced cardiac output, hypotension and shock (Rees et al, 1995). Cytokines can also induce myocardial depression directly. Renal dysfunction is common affecting up to 50% of patients with SIRS. This may be partly as a consequence of the hypoperfusion of the kidney accompanying the relative hypotension, but also may be linked with direct tissue injury secondary to the release of free radicals and other cell-mediated inflammatory toxins. SIRS may also be associated with coagulation disorders secondary to the cytokine-mediated activation of the coagulation pathways. IL-6 and IL-8 induce tissue factor expression and

activation by monocytes and endothelial cells leading to activation of the clotting cascade (Neumann et al, 1997). Dysfunction in the control of the clotting cascade may lead to disseminated intravascular coagulation (DIC), characterised by both bleeding and micro-thrombi within the circulation. This in turn may contribute to further organ dysfunction.

In contrast, over-expression of counter-inflammatory cytokines, such as IL-10, may lead to a loss of balance between pro-inflammatory and anti-inflammatory mediators. This may lead to relative immunosuppression, which has been linked with organ failure following major surgery (Bone, 1996). This condition has been termed compensatory anti-inflammatory response syndrome (CARS) and elevated serum anti-inflammatory cytokine concentrations have been linked with increased complications following major surgery (Mokart et al, 2002).

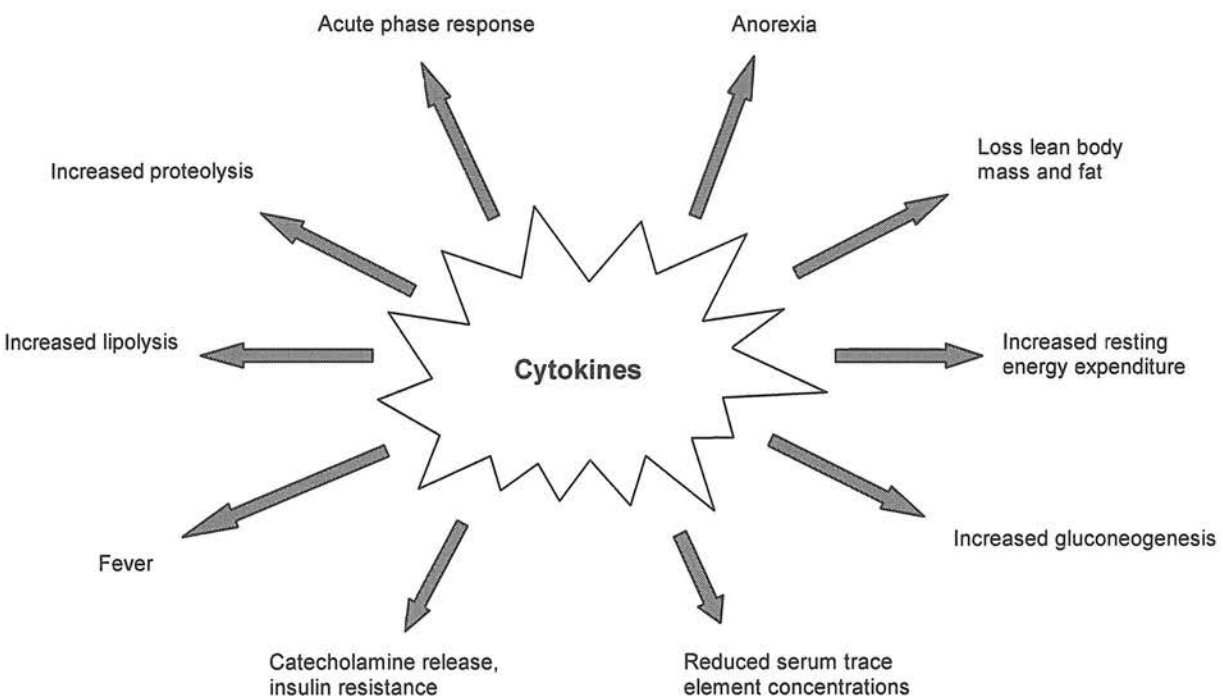
Localised tissue inflammation, therefore, results in the systemic release of inflammatory mediators, which act on target organs throughout the body. In the acute setting, an imbalance between pro-inflammatory and anti-inflammatory cytokines may lead to serious and life-threatening sequelae.

b) Chronic systemic inflammatory response syndrome

The chronic sequelae of systemic inflammation occur more insidiously and are more relevant to the cancer patient, but may accompany any chronic inflammatory condition. The effects are characterised by changes in metabolism, including alterations in lipid and protein synthesis, elevated resting energy expenditure, and changes in patterns of endocrine secretion (Table 2.2.1 and Figure 2.3.1). These metabolic changes result in a number of physiological changes in the host and patients may experience several symptoms as a consequence. The symptoms may include fatigue, weakness, nausea, anorexia, early satiety, and weight loss and these symptoms are associated with reduced performance scores and prognosis in cancer patients (Deans and Wigmore, 2005). The symptoms share a similar pathophysiologic background and some authors have labelled the cluster of symptoms as 'systemic immune-metabolic syndrome' (SIMS) (Cerchetti et al, 2004). The widespread metabolic effects of systemic inflammation that may lead to SIMS are mediated through the direct and indirect effects of

pro-inflammatory cytokines and changes in the neuro-endocrine network. The systemic effects of individual cytokines have already been described in Section 2 (b). This section will outline the metabolic effects of chronic systemic inflammation on the host.

Figure 2.3.1 Systemic effects of pro-inflammatory cytokines on host metabolism during inflammation.



Changes in protein metabolism

Muscle protein is catabolised to provide the amino acids required to synthesise the proteins and cells necessary for the immune response. This is largely mediated through IL-1β and TNFα acting via the cyco-oxygenase pathway to increase the rate of skeletal muscle proteolysis and reduce the rate of muscle synthesis (Baracos et al, 1983). More recently, TNFα (and γ-interferon) have been shown to selectively down-regulate myosin heavy chain

expression through an RNA-dependent mechanism (Acharyya et al, 2004). This group also demonstrated the selective breakdown of myosin heavy chain protein in a mouse model of cachexia via up-regulation of the ubiquitin-proteolytic pathway. Cytokines may, therefore, stimulate skeletal muscle proteolysis through a more targeted approach that is as yet unclear.

The acute phase protein response stimulated by IL-6 and generated by the liver is a main consumer of many of these amino acids (see Figure 2.2.2). Fibrinogen (a positive acute phase reactant) synthesis rates are significantly elevated among cancer patients demonstrating a systemic inflammatory response when compared with controls (Barber et al, 2000). In addition, amino acids are converted to glucose as a preferential energy source to help fuel the inflammatory process and there is increased urinary nitrogen loss.

Other groups have also suggested that muscle protein may be lost through cytokine-generated muscle apoptosis (Carbo et al, 2002). Mice bearing a cachexia-inducing tumour demonstrated marked DNA fragmentation in skeletal muscle (suggesting muscle apoptosis) compared with mice bearing the same tumour but gene-deficient for TNF receptor protein 1. These findings suggest that TNFα may play a role in skeletal muscle apoptosis.

As a consequence of these changes in protein metabolism there is a net loss of lean body mass, which may contribute to weakness, fatigue, weight loss and reduced activity levels, commonly experienced in patients with SIMS.

Changes in fat metabolism

Fat catabolism is increased to provide the additional energy sources required by the immune response. This is driven mainly by TNFα acting on the liver to stimulate lipolysis (Selby et al, 1987). Rates of lipogenesis are also reduced and this may be due to reduced levels of lipoprotein lipase expression (Jeevanandam et al, 1986; Vlassara et al, 1986). These changes in fat metabolism may also contribute to the weight loss commonly identified in these patients.

Changes in energy expenditure

Resting energy expenditure (REE) is elevated among patients demonstrating a systemic inflammatory response, particularly in those patients with burns, sepsis or trauma. In patients

with cancer the pattern is less clear. Longitudinal animal studies suggest that there may be an initial hypermetabolic period, followed by a slowing in energy comsumption as subjects approach death (Zylicz et al, 1990). Elevated resting energy expenditure has been documented in pancreatic cancer patients when compared with healthy controls and, moreover, levels of resting energy consumption were significantly higher in those cancer patients with elevated markers of systemic inflammation (Falconer et al, 1994). Both IL-6 and TNFα have been implicated in the pathophysiology of elevated REE, possibly mediated by induction of mitochondrial uncoupling proteins, however, changes in patterns of neuroendocrine activation are also important determinants of this catabolic syndrome (Selby et al, 1987; Stouthard et al, 1995).

Altered patterns of endocrine expression

As previously described, pro-inflammatory cytokines may affect concentrations of insulin, cortisol and glucagon leading to metabolic consequences for the patient. Elevated concentrations of cortisol and glucagon result in increased rates of gluconeogeneis and raised blood glucose concentrations to help fuel the energy demands of the immune response. In addition, these elevated hormone concentrations may amplify the magnitude of the acute phase response (Baumann and Gauldie, 1994). The insulin resistance associated with systemic inflammation also contributes to elevated serum glucose concentrations with an associated reduction in anabolic growth in the tissues. Alterations in serum insulin-cortisol ratios may also contribute to tissue catabolism (Fearon et al, 1998). Therefore, pro-inflammatory cytokines may indirectly exert a wide range of metabolic effects through alterations in hormonal secretion patterns.

Other metabolic consequences of systemic inflammation include reduced plasma trace element concentrations (for example, zinc and iron). These changes are mediated through IL-1β and TNFα and are partly due to redistribution of the trace elements in the body to aid immune cellular function and also due to depletion through tissue healing. Anorexia is also commonly associated with SIMS. Reduction in food intake is common among patients with

cancer and TNFα concentrations have been linked with reduced rates of food intake in animal studies (Darling et al, 1990).

The chronic sequelae of systemic inflammation are characterised by changes in protein and fat metabolism, elevated energy expenditure and other metabolic changes, mediated by pro-inflammatory cytokines and changes in hormonal expression. These changes exert a high metabolic and nutritional cost upon the body and induce a catabolic state. Such metabolic alterations result in a cluster of common symptoms, such as anorexia, fatigue and weakness, collectively known as systemic immune-metabolic syndrome (SIMS). These symptoms and the underlying metabolic consequences are commonly identified in patients with cancer. They are important due to their negative impact on quality of life, performance status, and overall prognosis among these patients (Deans and Wigmore, 2005).

The next section (Section 4) will describe the incidence of systemic inflammation among patients with cancer, outline possible mechanisms involved in its genesis, and describe the clinical sequelae of systemic inflammation in cancer patients, with particular emphasis on changes in nutritional status (cachexia).

4. TUMOUR-ASSOCIATED TISSUE INFLAMMATION

Tumour-associated inflammation has been implicated in carcinogenesis, tumour growth and progression, and the development and spread of metastatic disease. Tumour tissue inflammation may also be associated with the development of systemic inflammation and cancer patients are therefore exposed to the systemic sequelae of inflammation. Previous studies have also suggested that the presence of tumour-associated inflammation may also have a role in improving prognostic accuracy. Increased understanding of the mechanisms underlying the aetiology of tumour-associated inflammation may, therefore, provide insights

into the origins of systemic inflammation in cancer patients, improve prognostic accuracy, and, in turn, may lead to the identification of novel inflammation-based therapeutic targets.

a) Innate and adaptive immunity

The immune system may be considered to have two components; innate and adaptive immunity. The innate immune response is a non-specific, stereotyped, rapid response to the presence of a foreign antigen or tissue injury. Innate defences include mechanical (for example, skin), chemical (for example, gastric acid) and the acute phase protein response. The key cells of the innate response are macrophages and natural killer (NK) cells. Macrophages are a mobile population of cells that circulate throughout the body compartments and tissues ready to respond to environmental stimuli. Macrophages become activated in response to local stimulation from cytokines (such as interleukins and γ-interferon), tissue injury or complement receptors (Duffield, 2003). Once activated, macrophages fulfil their immunological role; namely release antimicrobial molecules and enzymes, produce cytokines, and undertake phagocytosis. Exposure of macrophages to different environmental stimuli, in particular to cytokines such as IL-10 and IL-4, results in a different macrophage response characterised by tissue repair and growth rather than the tissue destruction that typifies the classical activation pathway (Duffield, 2003). This creates a local environment that favours angiogenesis and growth and this may actually contribute to carcinogenesis and tumour progression (see later section) (O'Byrne and Dalgleish, 2001; Sharma et al, 2003).

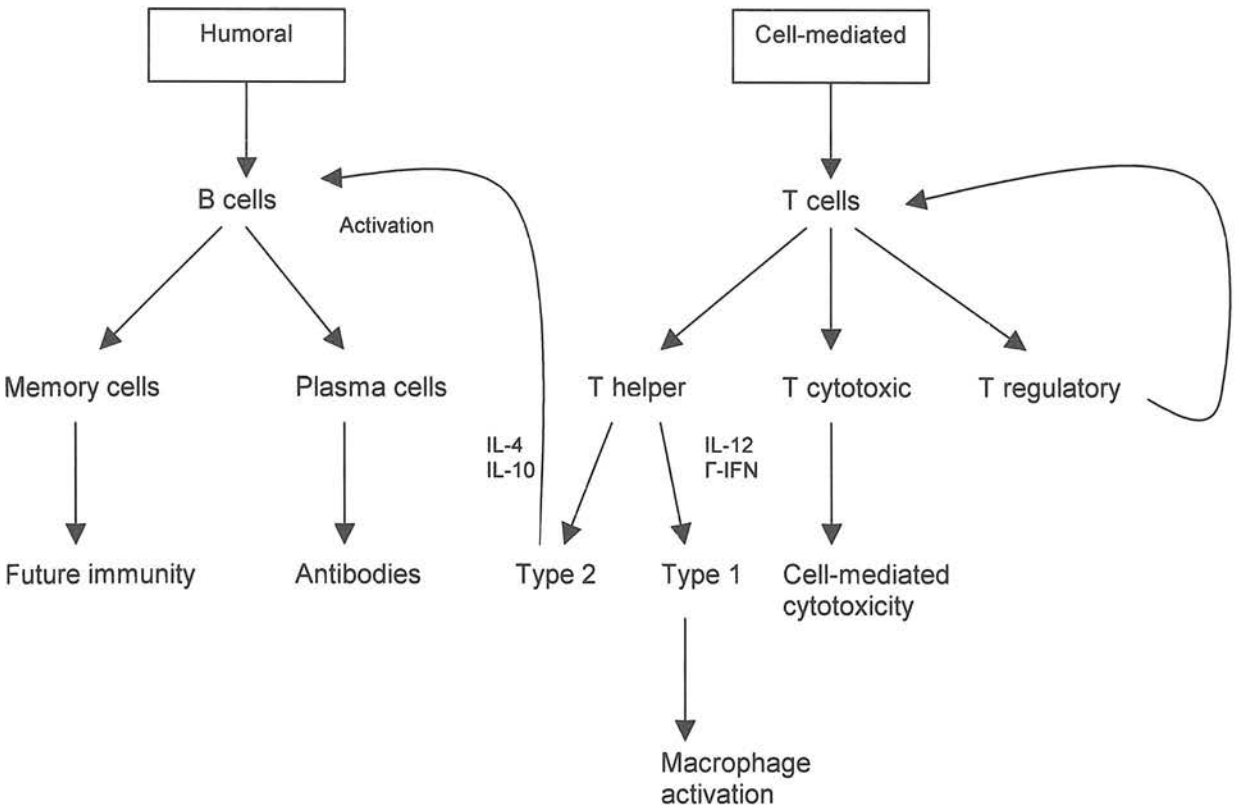
Natural killer (NK) cells are lymphocytes lacking receptors typical of T and B cells. The main role of NK cells is in the recognition and destruction of 'non-self' cells, including cells that do not express HLA antigens correctly on the cell surface, such as some cancer cells, and, therefore, NK cells play an important role in tumour immunosurveillance (also see following section) (Moretta et al, 1996).

The adaptive immune response, in contrast, is a delayed response that is specific for an antigen, characterised by antibody formation and demonstrates memory. The adaptive

response consists of humoral and cell-mediated components (Figure 2.4.1). B cells produce antibodies and develop into memory cells, conferring future immunity to a particular antigen. B cells depend upon T cell subsets (T helper cells, CD4 +ve) to develop and function correctly. T helper cells are responsible for the regulation of the adaptive immune response and macrophage response. T helper cells may differentiate into type 1 or type 2 cells and the path of differentiation is principally controlled by local cytokines. Type 1 cell differentiation is stimulated by IL-12 and γ-IFN and favours macrophage cell activation. Type 2 maturation is controlled by IL-4 and IL-10 and promotes B cell activation. Another T cell subset, cytotoxic T cells (CD8 +ve) undertake the cell-mediated immune response. Cytotoxic T cells are the effector cells and are key orchestrators of the immune reponse. They are directly involved in targetted cell death through TNFα and Fas ligand receptor binding resulting in triggering of apoptosis and are the main secretors of chemical mediators of inflammation, such as pro-inflammatory cytokines. The T cells of the adaptive immune response can only recognise antigens expressed in the correct form on cell surfaces. So called antigen presenting cells, such as macrophages and dendritic cells, endocytose antigen molecules and express the antigen on their cell surface in the correct form in association with a major histocompatability complex (MHC) molecule. This allows T cell receptor binding and correct activation of the T cell (Steinmann, 2003). Another subset of T cells, regulatory T cells, regulate the adaptive immune response by exerting inhibition on T cell effector mechanisms.

Both the innate and adaptive immune responses are inter-related and dependent upon each other for an effective immune response. Each component has also been implicated in the generation of tumour-associated inflammation.

Figure 2.4.1 Key cells involved in the adaptive immune response and their interactions.



b) Tumour immunosurveillance

Host antigens are recognised as 'self' by the immune system and in normal circumstances no immune reaction is generated. Such immune tolerance is attained early in development. 'Non-self' or foreign antigens are detected by immune surveillance and destroyed by the host immune response. In addition to recognising foreign antigens, the immune system may react to abnormally expressed 'self' antigens. Abnormal expression of host antigens may arise due to over-expression, point mutation or re-arrangement of tumour chromosomal transcripts, or following re-expression of developmental or embryonic genes as may occur in certain tumour phenotypes. These abnormally expressed host antigens are termed tumour-associated

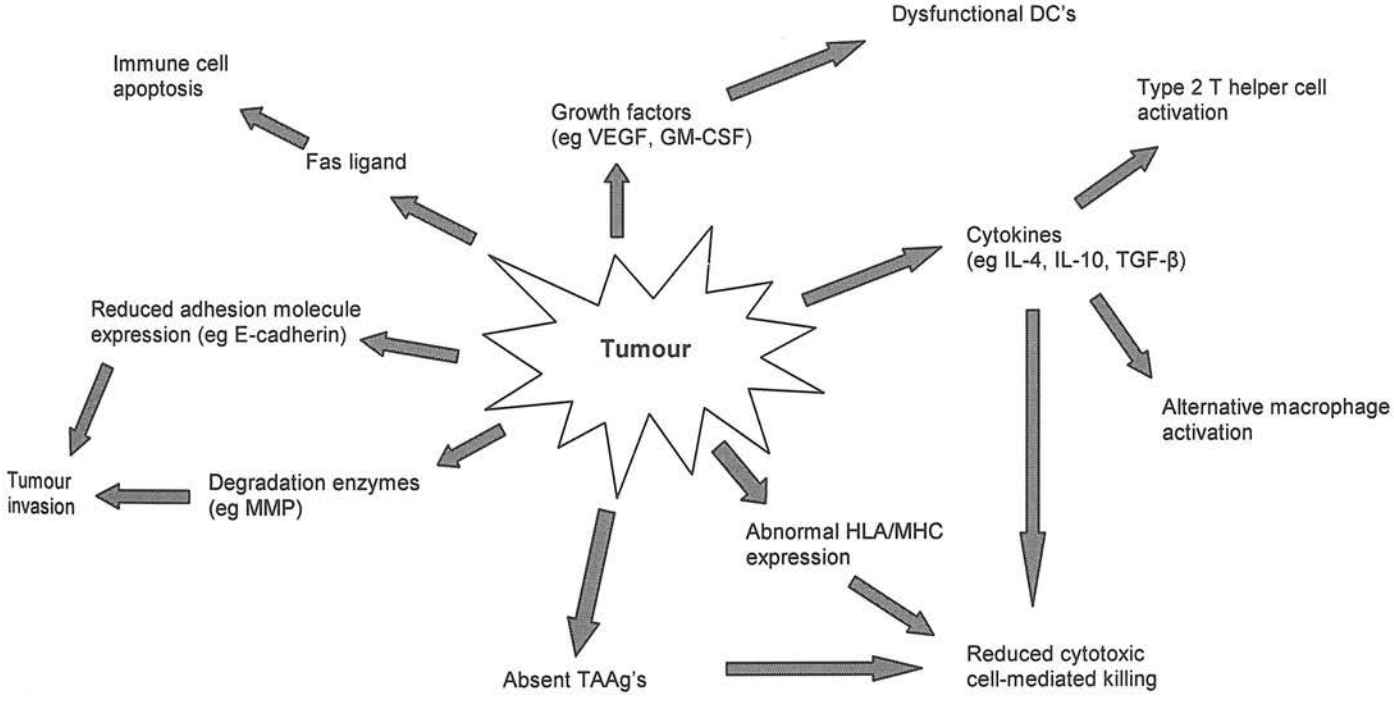
antigens (TAAg's) (Lagarkova et al, 2000; Akervall et al, 2002; Benchimol et al, 1989). Anti-tumour immunity directed towards these TAAg's may be mediated through several processes, including humoral (B cell mediated), cell-mediated T cell cytotoxic killing, and innate cell responses (macrophage and NK cell activation). These processes serve to recognise abnormal host cells which are then destroyed by the immune response and consequently reduce the likelihood of tumour development.

c) Tumour escape of immunosurveillance

Immunosurveillance may fail and tumour development may occur. Failure of immunosurveillance may occur due to tumour escape mechanisms or through host immunosuppression (Figure 2.4.2). The tumour may fail to produce TAAg's, which is more common among poorly differentiated tumours, or abnormal HLA/MHC expression may prevent effective T cell receptor binding resulting in failure of T cell activation (Seliger et al, 2001; Garrido and Algarra, 2001). Such lack of immunogenecity may account for the little inflammatory infiltrate noted in some tumours. Tumours may also produce mediators, including cytokines and growth factors, which act locally within the tumour mass and may influence immune cell function. Variations in cytokine concentrations and receptor expression within the tumour tissue may create a local environment which favours the tumour and dampens down the local immune response. IL-10 and transforming growth factor-beta (TGF-β) are produced by some tumour types and promote activation of type 2 T helper cell activation (Logullo et al, 2003). This favours humoral rather than cell-mediated activity and reduces tumour cell destruction. In addition, IL-10 also appears to have the ability to protect tumour cells from lysis by cytotoxic T cells (Gatsl et al, 1993). Other tumour-derived mediators may induce local immunosuppression. Increased concentrations of soluble Fas ligand have been measured in tumour cell lines and these elevated concentrations have been associated with increased rates of apoptosis of T cells and DC's in patients with cancer (Liu et al, 1998; Nat et al, 2002). In addition, dendritic cells (DC's) have been shown to be immature and

dysfunctional in gastric cancer and this is thought to be mediated through tumour-derived VEGF production (Saito et al, 1998). DC's are important antigen presenting cells as well as key cells involved in cell-mediated killing and therefore impaired DC function leads to local immune suppression. Several other tumour-derived cytokines and growth factors have been implicated in creating a milieu within the tumour tissue that induces immunosuppression and favours tumour escape, including granulocyte/macrophage-colony stimulating factor (GM-CSF) (Almand et al, 2001).

Figure 2.4.2 Potential mechanisms employed by tumour cells to evade immunosurveilance.



d) Role of immunology in carcinogenesis

Some chronic inflammatory conditions are associated with increased risk of cancer development; for example, ulcerative colitis and colon cancer, and viral hepatitis and hepatocellular carcinoma. In upper gastrointestinal cancer, infection with Helicobacter pylori (H pylori) induces chronic gastritis, which has been linked with increased risk of development of gastric cancer (Forman et al, 1991). Similarly, Barrett's metaplasia is associated with an inflammatory cell infiltrate into the oesophagus and is now recognised as a pre-malignant condition (Jankowski et al, 2000). Such close association between inflammatory conditions and the development of cancer suggests that tissue inflammation may play a role in neoplastic progression. Mice deficient in TNFα (TNFα knockout) are protected from epithelial damage and carcinogenesis from environmental agents, implicating a role for TNFα in cancer development (Moore et al, 1999). In patients with gastro-oesophageal cancer, an inflammatory cell infiltrate into the stomach is associated with increased generation of reactive oxygen species (Naya et al, 1997). These mediators may accumulate in the cell and induce DNA damage, including mutations in oncogenes, such as p53, predisposing to the development of gastric cancer (Farinati et al, 1998). Alternative activation of monocytes/macrophages, principally through stimulation by IL-10 and IL-4, creates a local tissue environment in which cell-mediated immune responses and apoptosis are reduced while angiogenesis is promoted (Dalgleish and O'Byrne, 2002; O'Byrne and Dalgleish, 2001; Sharma et al, 2003). Alternatively activated innate immune cells may therefore actually contribute to carcinogenesis within these tissues (Figure 2.4.2). Furthermore, immune cells may secrete cytokines, growth factors and other active mediators into local tissues promoting malignant transformation. TNFα has been implicated in the progression along the metaplasia-dysplasia-carcinoma sequence in Barrett's oesophagus and IL-1β, IL-6 and IL-10 have been measured at significantly elevated concentrations within Barrett's segments (Tselepis et al, 2002; Fitzgerald et al, 2002; Dvorkova et al, 2004). In addition, possession of an IL-1β polymorphism that is associated with increased tissue IL-1β production is linked with an increased risk of H pylori associated gastric cancer, presumably through acid suppression and stomach atrophy (El-Omar et al, 2000). Cytokines can also regulate matrix-degrading

enzymes, such as metalloproteinases, and reduce adhesion molecule expression, for example, E-cadherin, both of which affect the ability of cells to become invasive (Kim et al, 2000).

Cyclo-oxygenase-2 (COX-2) is an inducible form of cyclo-oxygenase that is involved in the rapid production of prostaglandins during inflammation. COX-2 is over-expressed in a variety of malignant tumours. Increased concentrations of COX-2 have also been measured in gastric and oesophageal cancers and have been associated with adverse outcome (Mobius et al, 2005). Non-steroidal anti-inflammatory agents (NSAIDs), which reduce levels of COX-2 expression, have been associated with lower rates of malignant transformation in Barrett's metaplasia and with lower risk of developing oesophageal and gastric cancers in the general population (Jankowski et al, 1999; Lindblad et al, 2005).

Hypoxia inducible factors (HIF) are key proteins regulating cellular response to hypoxia. Increased HIF expression is observed after exposure of cancer cells to hypoxia and, although increased expression may occur due to oncogene activation, over-expression of HIF in tumours is likely to represent tumour hypoxia (Feldser et al, 1999; Wiesener et al, 1998). Pro-inflammatory cytokines, including IL-1β, IL-6 and TNFα, have also been shown to increase levels of HIF expression (Westra et al, 2007). It is postulated that exposure of cells to cytokine stimulation promotes cellular HIF expression which prepares the cell for the relative tissue hypoxia that is associated with tissue inflammation (Frede et al, 2007). Prolonged activation of HIF under conditions of inflammation, however, may contribute to the survival of damaged tissue and cells, thus promoting the development of tumours. Cytokine induced HIF expression may also provide some resistance of tumour cells to hypoxia and promote tumour survival and growth.

Increasing expression of HIF proteins have been measured in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence and similarly in the gastric carcinogenesis sequence (Griffiths et al, 2007a; Griffiths et al, 2007b). Elevated levels of expression have also been correlated with advanced disease stage and reduced survival duration (Kurokawa T et al, 2003; Griffiths et al, 2007b). Cytokine-induced expression of HIF may be one of the mechanisms linking tumour inflammation with poor outcome.

In contrast to immunosurveillance, immune cells may, therefore, contribute to and facilitate tumour development directly through local immune suppression and secretion of active mediators, such as cytokines and growth factors, which create a local environment favouring carcinogenesis.

e) Role of immunology in tumour progression and spread

Established tumours are commonly infiltrated by immune cells, including macrophages, dendritic cells, NK cells, and T and B lymphocytes. These cells may mount an immune response to the tumour, through mechanisms such as cell-mediated cytotoxic killing in response to TAAg's. Alternatively, these immune cells may be subverted into promoting tumour growth and progression by mediators and cytokines produced locally by the tumour creating a milieu leading to local immune suppression and favouring growth factor production, angiogenesis, and matrix degradation. Two immune cell sub-populations have been primarily investigated for their role in tumour-associated inflammation. These are tumour-associated macrophages (TAM's) and tumour-infiltrating lymphocytes (TIL's).

Up to 60% of the tumour mass may be accounted for by TAM's, which may vary by tumour type but tend to remain constant for a particular individual tumour (van Ravenswaay et al, 1992; Toomey et al, 1999; Evans, 1972). Factors derived from tumour cells themselves appear to play an important role in the regulation of macrophage levels within the tumour (Mantovani et al, 1992). Monocyte chemotactic protein-1 (MCP-1) is a factor produced by tumour cells and concentrations of this mediator have been found to correlate with concentrations of TAM's in oesophageal cancer (Koide et al, 2004). MCP-1 has also been shown to induce IL-β and IL-6 production in monocytes (Jiang et al, 1992). TAM's also demonstrate an increased life-span and proliferative capacity, processes which may contribute to the maintenance of a constant macrophage population within the tumour (Bottazzi et al, 1990). Activated macrophages within the tumour mass have a wide range of biological functions depending on their mode of activation. These functions may promote or inhibit tumour growth depending on the balance of these opposing actions (Figure 2.4.3).

Actions inhibiting tumourigenesis include facilitating T cell-mediated cytotoxic killing of tumour cells by antigen presentation and direct lytic effects through release of toxic mediators, such as oxygen free radicals. Alternatively, macrophage functions favouring the tumour include the production of growth factors (for example, TGF-β and VEGF), release of lytic enzymes facilitating invasion and dissemination (for example, matrix metalloproteinases), and inducing local immunosuppression (for example, secretion of IL-10). The mechanisms controlling of these opposing functions are poorly understood, but local cytokine concentrations are likely to play a key role; for example, alternative monocyte activation by IL-10 and IL-4 favours the tumour through increased production of angiogenic factors, secretion of growth factors and suppression of cell-mediated cytotoxicity (O'Byrne and Dalgleish, 2001).

Figure 2.4.3 Potential actions of tumour-associated macrophages (TAM's). These functions may promote or inhibit tumour growth depending on the balance of these opposing actions.

Actions favouring tumour growth

Enhanced growth
- Growth factors (eg TGF-β)
- Cytokines (IL-1β, IL-6, TNFα)

Enhanced angiogenesis
- Cytokines (IL-6, IL-8)
- Prostanoids

Invasion
- Lytic enzymes (eg MMP)
- Cytokines (IL-1β and TNFα)

Immunosuppression
- Cytokines (IL-10)
- Prostanoids

Actions favouring tumour regression

Direct cellular toxicity
- Cell to cell contact

Antibody-dependent cytotoxicity
- Fc receptor expression

Cytotoxic secretory products
- Cytokines (IL-1β and TNFα)
- Free radicals
- Eicosanoids (prostaglandins)

Macrophage –induced apoptosis
- Cytokines (IL-1β and TNFα)
- Free radicals

Lymphocytes (TIL's) are also commonly found within tumour tissue. These cells appear to be functionally compromised by local (tumour tissue) production of mediators and as a consequence there is a failure of immunosurveillance and the tumour attains a position of immune privilege (Chiou et al, 2005). There is down-regulation of cell-mediated killing due to preferential type 2 T helper cell activation secondary to tumour IL-10 production (Ferguson et al, 2003). Signalling defects have been identified in TIL's resulting in impaired immune functioning (Nakagomi et al, 1993). Regulatory T cells may also play a vital role in the failure of anti-tumour immune activity. Tumour cells may influence regulatory T cells, which in turn produce cytokines and other mediators that inhibit the adaptive immune response towards the tumour (Curiel et al, 2004).

The clinical relevance of TAM's and TIL's to patients with cancer remains unclear, especially among patients with gastro-oesophageal malignancy. An increased macrophage infiltrate is associated with more advanced disease stage in patients with gastric cancer in one study, whereas other studies have suggested a more favourable prognosis associated with a more pronounced macrophage infiltration (Heidl et al, 1987; Ohno et al, 2003; Tsujitani et al, 1987). Similarly, increasing tumour-infiltrating lymphocyte count has been linked with decreased risk of death from gastric cancer in one study, but associated with an adverse prognosis in another (Grogg et al, 2003; Setala et al, 1996). Studies relating to oesophageal cancer are equally contradictory (Koide et al, 2004; Ma et al, 1999). This topic is further explored in Chapter V.

f) Summary

The immune system and tumour biology are intimately associated. Failure of immunosurveillence, either through tumour escape mechanisms or host immunosuppression, allows tumour development. Alternatively, there is evidence that the presence of chronic inflammation may have a direct role in carcinogenesis. An inflammatory cell infiltrate is commonly identified once tumours are established and may include cells of the innate immune system (TAM's) and cells from adaptive immunity (TIL's). The tumour is able to

subvert the normal anti-tumour functions of these cells through the local production of cytokines and other mediators that creates a local environment within the tumour tissue favouring tumour growth and dissemination. The clinical significance of an inflammatory cell infiltrate into the tumour tissue is unclear, especially among patients with gastro-oesophageal cancer. This thesis will investigate the influence of an inflammatory cell infiltrate on prognosis for patients with gastro-oesophageal cancer and compare the magnitude of the inflammatory infiltrate with cytokine concentrations measured in both the tumour tissue and the systemic compartment. Additional studies will attempt to determine whether the aetiology of systemic inflammation in cancer patients is derived from host immune cells or is driven by tumour-derived mediators.

Local tumour-associated inflammation is also associated with systemic inflammation and, as such, cancer patients are exposed to the systemic sequelae of inflammation. This may have an impact on patients' function, quality of life, nutritional status, response to treatment, and prognosis. This is discussed in the following section.

5. TUMOUR-ASSOCIATED SYSTEMIC INFLAMMATION

The presence of cancer within the host may trigger an immune response and lead to the generation of tissue and systemic inflammation. Patients with cancer are, therefore, exposed to the local and systemic sequelae of tumour-associated inflammation. The clinical sequelae of systemic inflammation has already been described in Section 3, however, this Section will describe the sequelae among patients with cancer, with particular emphasis on the effects of systemic inflammation on patients' nutritional status and function (cachexia). Firstly, the incidence of systemic inflammation among patients with cancer will be described and then a discussion on the possible causes of systemic inflammation in cancer patients will follow.

a) The incidence of cancer-associated systemic inflammation

Elevated markers of systemic inflammation have been measured in patients with most types of cancer. Increased pro-inflammatory cytokine concentrations have been measured in sera from patients with cancer and elevated concentrations of IL-1β, IL-6, IL-8 and TNFα have repeatedly been measured in blood collected from patients with gastro-intestinal malignancy, including pancreatic, gastric and oesophageal cancers (Barber et al, 1999; Wang et al, 1999; Kabir and Daar, 1995; Ebrahimi et al, 2004). However, reliable measurement of serum cytokine concentrations is difficult, partly because of short half-lives and the presence of blocking factors, but also due to circadian variations in plasma concentrations (May et al, 1992). The difficulties in reliably measuring serum TNFα concentrations has already been aluded to (Muc-Wierzgon et al, 1996). In addition, the end-organ effects of these systemic cytokines will depend upon binding to transport proteins, receptor expression and receptor affinity. These findings have raised doubt as to the significance of circulating concentrations of pro-inflammatory cytokines as markers of systemic inflammatory activity. It is possible that the local release of pro-inflammatory cytokines into target tissues by peripheral blood mononuclear cells (PBMC) is a more accurate determinant of pro-inflammatory activity in the tissues. Such PBMC's become 'activated' by local cytokine production as the cells pass through the tumour tissue, circulate in the bloodstream, and release cytokines in target tissues thereby increasing local tissue concentrations. PBMC's from patients with pancreatic cancer with an elevated serum acute phase protein response demonstrate greater rates of pro-inflammatory cytokine (IL-6 and TNFα) production when compared with those without a systemic inflammatory response (O'Riordain et al, 1999). These cells are then capable of stimulating increased rates of C-reactive protein (CRP) production by hepatocytes in co-culture systems (O'Riordain et al, 1999).

Serum concentrations of acute phase proteins (APPR) demonstrate more stability within the systemic compartment and may be a more reliable marker for the presence of systemic inflammation in patients (Gabay and Kushner, 1999). Up to 50% of patients with cancer have an APPR at the time of diagnosis, including patients with upper gastro-intestinal malignancy (Chen et al, 1999; Alexandrakis et al, 2003; Barber et al, 1999; Falconer et al,

1994; Falconer et al, 1995). In cancer patients circulating concentrations of IL-6 have been shown to correlate with markers of systemic inflammation such as CRP (Barber et al, 1999). The presence of such an acute phase reaction may then be used as an indirect marker of increased pro-inflammatory cytokine activity and serum concentrations of acute phase proteins may, therefore, be used to confirm the presence of systemic inflammation.

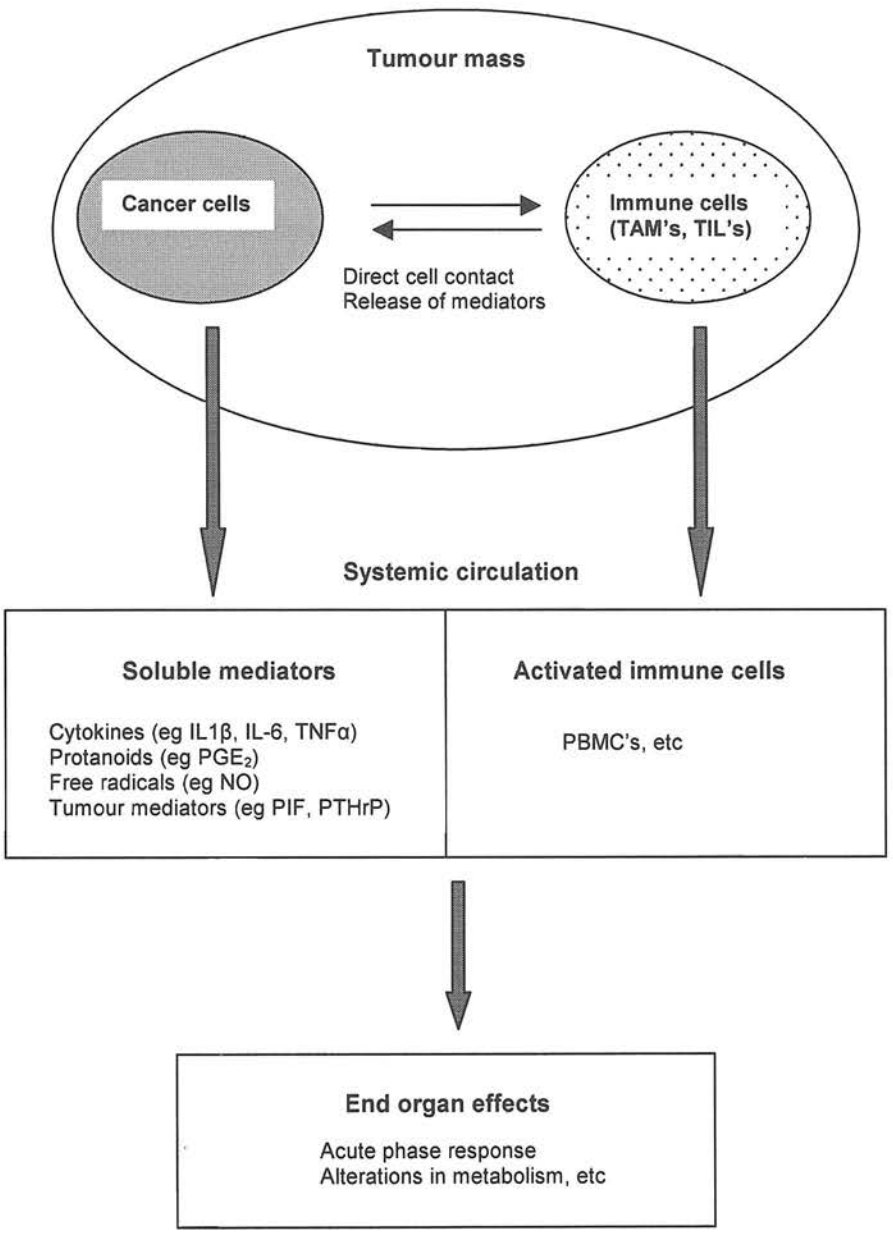
b) The aetiology of systemic inflammation in patients with cancer

The origins of systemic inflammation in patients with cancer remain unclear. The close relationship between tumourigenesis and host immunology has already been described in Section 4(a). The cellular interactions between host immune cells and tumour cells are controlled by locally produced cytokines and other mediators that serve to create a local environment favouring the tumour. Little is known of the systemic relevance of these locally produced mediators. Studies comparing tissue cytokine concentrations with systemic concentrations have mainly involved chronic inflammatory conditions. In Crohn's disease tissue cytokine concentrations correlate with serum CRP concentrations (Raddatz et al, 2005). Similarly, tissue production of inflammatory cytokines (IL-1β, TNFα and IL-6) correlates with the intensity of the systemic inflammatory response in giant-cell arteritis (Hernandez-Rodriguez et al, 2004). Conversely, other groups investigating inflammatory joint disease have failed to demonstrate an association between tissue cytokine concentrations and systemic markers of inflammation (Rioja et al, 2004). In patients with cancer this relationship is equally unclear. Tumour tissue from patients with head and neck cancer demonstrate elevated concentrations of pro-inflammatory cytokines and elevated serum acute phase protein concentrations, but not elevated serum cytokine concentrations (Chen et al, 1999). It is likely that tumour tissue cytokine concentrations have little correlation with serum cytokine concentrations. However, immune cells may become activated as they pass through the inflamed tumour tissue and become exposed to the locally produced cytokines and other inflammatory mediators. These cells are then free to circulate in the systemic circulation and act on target tissues exerting their pro-inflammatory effects. For example, by inducing an

acute phase protein response from the liver (Figure 2.5.1). In support of this, peripheral blood mononuclear cells (PBMC's) from cancer patients with an elevated serum APPR demonstrate greater rates of pro-inflammatory cytokine (IL-6 and TNFα) production when compared with those without a systemic inflammatory response (O'Riordain et al, 1999). These cells are then capable of stimulating increased CRP production by hepatocytes in co-culture systems (O'Riordain et al, 1999). Other groups have also identified the ability of certain tumours to sensitise PBMC's and induce IL-6 expression from these cells (Martignoni et al, 2005).

Whether the main stimulus for the systemic inflammatory response in patients with cancer arises from the tumour cells or the host immune cells remains unknown. Either the tumour cells or the host cells or a combination of the two are responsible for the production of the pro-inflammatory cytokines that induce the APPR. It may simply follow that the host immune system mounts a response secondary to the tumour burden or in response to a necrotic tumour that is behaving like an 'abscess' with associated release of cytokines. Elevated serum CRP concentrations have been linked with increased tumour volume in patients with oesophageal malignancy (Nozoe et al, 2001). However, the complexities of tumour immunology have already been outlined in Section 4(a) and this explanation is likely too simplistic. The interactions between host and tumour cells appear to be crucial to the development of tumour-associated inflammation and, therefore, also crucial to the development of systemic inflammation. Furthermore, the extent of leucocyte infiltration into tumour tissue (TAM's and TIL's) does not reliably correlate with either tumour tissue cytokine concentrations or with markers of systemic inflammation (Chen et al, 1999). Local immune cell immunosuppression and alternative activation of monocytes (type 2 activation) within the tumour tissue has already been aluded to and it is possible that these host immune cells do not contribute to the generation of systemic inflammation. Alternatively, tumour cells are capable of constitutive production of pro-inflammatory cytokines. Oesophageal cancer cells have been shown capable of producing increased pro-inflammatory cytokine mRNA and protein concentrations (Yuan et al, 2000; Martignoni et al, 2005; Nozoe et al, 2003). Gastric cancer cells are also capable of producing a range of pro-inflammatory cytokines (Yasumoto et al, 1992). The main stimulus for systemic inflammation in patients with cancer may therefore be driven by the tumour cells. This topic will be further explored in Chapter V.

Figure 2.5.1 An overview of the possible mechanisms underlying the generation of a systemic inflammatory response in patients with cancer. The cellular interactions between host immune cells and cancer cells within the tumour mass and the inflammatory mediators produced by these cells appear to be crucial in the development of systemic inflammation.



c) The clinical sequelae of systemic inflammation in patients with cancer

The clinical sequelae of systemic inflammation have already been described in detail in Section 3. The chronic sequelae are of particular relevance to patients with cancer and these effects are characterised by changes in protein and fat metabolism, elevated energy expenditure and other metabolic changes, mediated by pro-inflammatory cytokines and changes in hormonal expression. These metabolic alterations are commonly identified in patients with cancer and lead to a cluster of common symptoms, such as anorexia, fatigue and weakness, collectively known as systemic immune-metabolic syndrome (SIMS). These symptoms and the metabolic changes underlying are important due to their negative impact on quality of life, performance status, response to treatment and overall prognosis among these patients (Deans and Wigmore, 2005). This section will describe the effects of systemic inflammation on nutritional status (cachexia). The role of systemic inflammation in determining prognosis in patients with cancer has already been discussed in Section 1(e).

Cachexia

Cachexia is a syndrome characterised by a group of symptoms including anorexia, early satiety, weight loss, weakness and anaemia (Fearon, 1992). The syndrome is associated with alterations in metabolism and also changes in patient functioning and performance status. More recently a definition of cachexia has evolved to encompass these two aspects of cachexia (Fearon et al, 2006). A 3-factor profile incorporating weight loss (greater or equal to 10%), low food intake (less than or equal to 1500 kcal/d), and systemic inflammation (CRP greater or equal to 10 mg/L) identifies patients with both adverse function and prognosis. Cachexia is not exclusive to cancer, but is also seen in chronic illness, such as rheumatoid arthritis and AIDS. The syndrome of cachexia is, however, common among patients with cancer, especially among patients with malignancy affecting the gastrointestinal tract (DeWys et al, 1980). Up to 85% of patients with pancreatic cancer have symptoms relating to cachexia at the time of diagnosis and almost all of these patients develop symptoms close to the time of death (Wigmore et al, 1997).

The clinical sequelae of cachexia may have significant detrimental effects on the host and cachexia remains an important cause of morbidity and mortality among cancer patients. Cachexia is associated with reduced quality of life scores, reduced performance status, lower response rates to chemotherapy, and overall poorer outcomes in patients with cancer (DeWys et al, 1980; Barber et al, 1999). Weight loss has been associated with adverse outcome in cancer patients and it has been suggested that around 20% of deaths from cancer are directly related to cachexia (Studley, 1936; Inagaki et al, 1974).

The metabolic changes underlying cachexia are similar to those seen in SIMS (section 3(b)). There is weight loss secondary to reduction in lean body mass and fat mass. There is preferential loss of skeletal muscle protein with relative sparing of visceral protein, which contrasts with the pattern of protein loss seen through starvation (Fearon and Preston, 1990). Skeletal muscle is depleted through reduced rates of protein synthesis and increased rates of proteolysis (Baracos et al, 1983; Acharyya et al, 2004). Some authors have also suggested muscle cells may undergo apoptosis during cachexia also contributing to loss of muscle mass (Carbo et al, 2002). Fat is lost through reduced dietary intake, increased rates of lipolysis, and reduced rates of lipogenesis (Jeevanandam et al, 1986). Cachexia is also associated with hypermetabolism. Rates of resting energy expenditure are significantly elevated in patients with pancreatic cancer, and higher still in those patients with evidence of an acute phase response (Falconer et al, 1994). There is a reduction in food intake due to anorexia, early satiety and alterations in taste that are associated with the syndrome (Fearon, 1992). There are also alterations in endocrine function that may contribute to overall tissue catabolism (Fearon et al, 1998).

The metabolic similarities underlying SIMS and cancer cachexia suggest that pro-inflammatory cytokines also play a role in the mechanisms of cachexia. Animal studies initially suggested a role for pro-inflammatory cytokines in the generation of cancer-associated cachexia. IL-1β, IL-6 and TNFα among others have been associated with anorexia and weight loss in rodent tumour models (Ghelin et al, 1991; Strassman et al, 1993). These pro-inflammatory cytokines have also been implicated in cachexia among human subjects, where elevated serum cytokine concentrations have been linked with weight loss in cancer patients (Martignoni et al, 2005). Martignoni and co-workers identified IL-6 over-expression in the

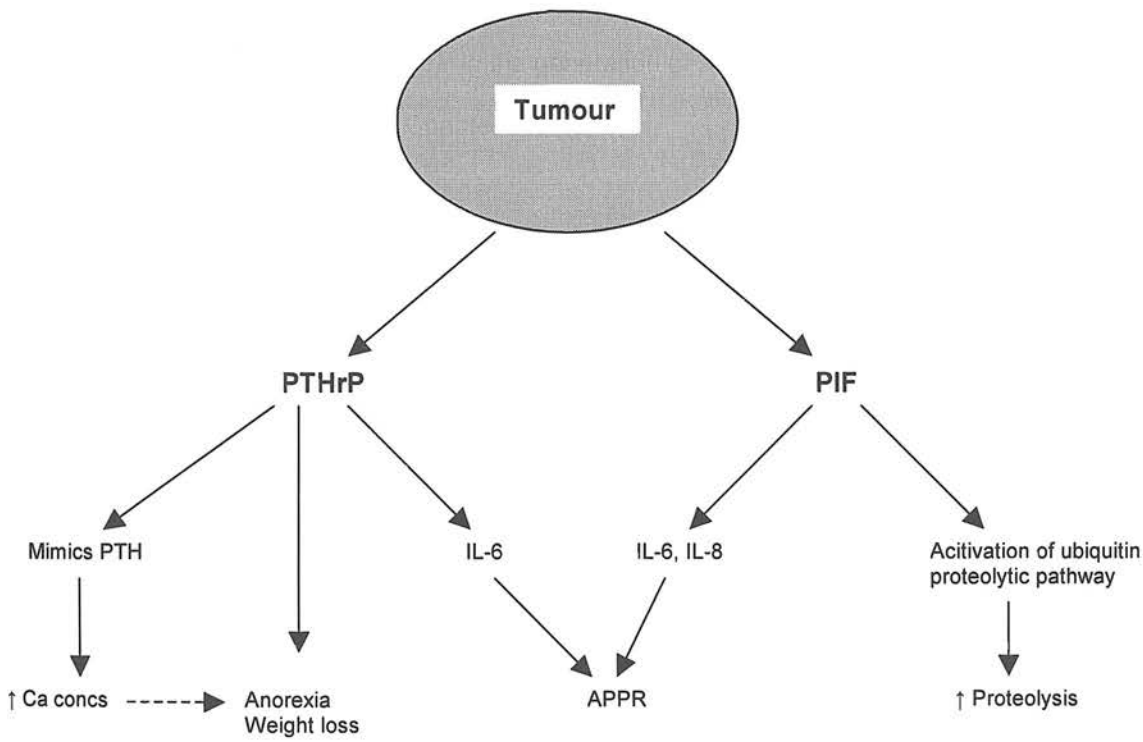
serum and also in the tumour specimens of cachectic patients with pancreatic cancer. They identified IL-6 mRNA expression to be significantly increased in tumour samples of cachectic patients compared with non-cachectic patients or pancreas samples from normal controls. These authors have suggested that IL-6 over-expression in cachectic pancreatic cancer patients is related to the ability of certain IL-6 producing tumours to sensitise PBMC and induce IL-6 expression in PBMCs. In patients with inoperable oesophageal cancer treatment with thalidomide, an inhibitor of TNFα synthesis, was associated with weight gain and increased muscle mass after only two weeks (Khan et al, 2003).

The acute phase response has also been linked with cachexia. Serum CRP concentrations have been correlated positively with weight loss in cancer subjects (Falconer et al, 1995; Wigmore et al, 1997; O'Gorman et al, 1999). Resting energy expenditure (REE) in patients with pancreatic cancer and evidence of an APPR have greater rates of REE compared with cancer patients without an APPR (Falconer et al, 1994). Cancer patients with an APPR have elevated fibrinogen synthesis rates in both the fed and fasting states (Preston et al, 1998; Barber et al, 2000). The protein substrate to fuel this APPR is thought to be mainly derived from muscle stores and it is this reprioritisation of protein metabolism that accompanies the APPR that may also contribute to the wasting observed in cancer cachexia (Fearon et al, 1999).

The aetiology of cancer cachexia is almost certainly mutifactorial, although elevated pro-inflammatory cytokines and the presence of systemic inflammation are likely to play a role. Additional potential mediators of cachexia are tumour-derived products, such as parathyroid hormone-related peptide (PTHrP) and proteolysis-inducing factor (PIF) (Figure 2.5.2). Parathyroid hormone-related peptide (PTHrP) is a tumour-derived circulating factor that has been associated with hypercalcaemia of malignancy, affecting up to 20% of patients during the advanced stages of cancer (Twycross, 1997; Suva et al, 1987). PTHrP expression has been found in association with both gastric and oesophageal malignancy (Jais et al, 1997; Tachimori et al, 1991; Abdeen et al, 1995; Engelich et al, 2000; Alipov et al, 1997). PTHrP may contribute to the pro-inflammatory cytokine cascade and several studies have identified elevated serum PTHrP concentrations in association with elevated serum cytokine concentrations in cancer patients (Ogata, 2000; Takahashi et al, 2003). PTHrP has been

shown to stimulate IL-6 production from osteoblasts *in vivo* and administration of PTHrP to mice increased serum acute phase protein levels (Pollock et al, 1996; Funk et al, 1998). PTHrP has also been linked with cachexia. PTHrP is produced by a human lung cancer model (HARA-B) and, when implanted into mice, results in a significant reduction in body weight and tissue mass, an effect that is reversed by addition of an antibody to PTHrP (Iguchi et al, 2001). The relevance on PTHrP in the generation of systemic inflammation, weight loss and prognosis will be explored in Chapter VII.

Figure 2.5.2 Tumour-derived mediators, such as proteolysis-inducing factor (PIF) and parathyroid hormone-related peptide (PTHrP), may also be involved in the aetiology of cachexia and systemic inflammation in patients with cancer.



PIF is a tumour-derived mediator that may be linked with systemic inflammation, weight loss and prognosis in cancer patients. Initially isolated from a murine cachexia tumour model (MAC16), a human homologue of PIF was subsequently identified in human urine from weight losing cancer patients, but not from weight-stable cancer patients or patients with weight loss secondary to benign disease (Todorov et al, 1996; Wigmore et al, 2000; Cariuk et al, 1997). PIF has been named as such because it has been shown to induce skeletal muscle proteolysis through activation of the ubiquitin proteolytic pathway (Lorite et al, 1997; Lorite et al, 2001; Todorov et al, 1997). PIF has been identified within gastrointestinal cancer cells and its presence was associated with increased weight loss among these patients (Cabal-Manzano et al, 2001). PIF has also been shown to stimulate production of IL-6, IL-8 and CRP in hepatocytes via induction of transcription factors NF-kB and STAT3 (Watchorn et al, 2001). PIF may, therefore, contribute to the inflammatory state observed in conjunction with cancer cachexia in addition to its proteolytic function. The role of PIF in the generation of systemic inflammation, weight loss and prognosis in patients with gastro-oesophageal cancer will be investigated in Chapter VIII.

In summary, cancer cachexia is commonly associated with malignancy of the gastrointestinal tract and is an important cause of morbidity and mortality among patients with cancer. Metabolic and clinical similarities with SIMS suggest that pro-inflammatory cytokines are linked with cachexia, although other mediators, such as PIF and PTHrP may be involved. Markers of systemic inflammation may therefore be linked with markers of cachexia, which in turn may be linked with reduced prognosis. Examining the link between systemic inflammation and cachexia may lead to improved estimations of prognosis among patients with gastro-oesophageal cancer, allow earlier identification of patients at risk of cachexia, and lead to the identification of novel therapeutic targets. This topic will be explored in Chapter X.

6. SUMMARY

This chapter has highlighted the increasing incidence of gastric and oesophageal cancer in the UK and outlined the current staging modalities and therapeutic strategies available. Current practices are failing to stage accurately these patients and treatment modalties are ultimately of a palliative nature. Improved staging accuracy would enable better treatment selections for individual patients, moreover, novel therapeutic strategies are much needed. Markers of systemic inflammation may have a prognostic role in gastro-oesophageal cancer and improved understanding of the mechanisms involved in the generation of systemic inflammation in cancer patients may provide insights into novel therapeutic targets and enable individualised patient care. The important cells and mediators involved in the generation of inflammation have been described, including key cytokines and the acute phase response. The role of immunology in carcinogenesis, tumour progression and spread has been described and possible mechanisms underlying the aetiology of systemic inflammation in patients with cancer have been discussed. The adverse clinical sequelae of systemic inflammation on the host have been outlined with particular relevance to alterations in metabolism, nutritional status (cachexia), and prognosis.

Therefore, the overall aim of this thesis is to describe the genesis, mediators and sequelae of systemic inflammation in patients with gastro-oesophageal cancer. The usefulness of systemic inflammation as a prognostic indicator and the role of cachexia as a factor in the adverse prognosis associated with systemic inflammation are expanded in detail. The key hypothesis being that tumour cells produce mediators (such as cytokines, PIF and PTHrP), which can either directly or indirectly (via systemic inflammation) induce a catabolic state in the peripheral tissues of the host. Such wasting may be one of the mechanisms linking systemic inflammation with adverse prognosis in patients with cancer. Potential novel therapeutic targets may also be identified.

CHAPTER III

MATERIALS AND METHODS

This thesis aims to describe the genesis, mediators and sequelae of systemic inflammation in patients with gastro-oesophageal cancer. The usefulness of systemic inflammation as a prognostic indicator, and the associated role of cachexia, are explored in more detail. This Chapter outlines the main methods undertaken to fulfil these tasks.

The Chapter describes a prospective cohort study to characterise the natural history of patients with gastro-oesophageal malignancy, documents the prevalence of elevated serum markers of systemic inflammation, examines the role of tumour tissue in the generation of systemic inflammation, explores various hypotheses related to novel mediators/factors giving rise to systemic inflammation, and provides data for the development of a prognostic model involving markers of systemic inflammation and cachexia.

Firstly, an overview of the study protocol is outlined, including details on patient recruitment to the study and subsequent episodes of patient contact, timings of sample collection, and follow-up data collection. A detailed description of assays performed on blood samples will be described, including measurements of serum cytokine concentrations, serum acute phase protein concentrations, and routine clinical chemistry. The contribution of tumour tissue to the generation of systemic inflammation was investigated by measuring concentrations of cytokine mRNA and protein within tissue homogenates, using real-time polymerase chain reaction (PCR) and cytometric bead array analysis. Cytokine genotyping was undertaken to examine the potential influence of host genotype on the generation of a systemic inflammatory response. Finally, the potential roles of tumour-derived mediators (PIF and PTHrP) on systemic inflammation and cachexia were investigated. Western blotting and mass spectroscopy were used to detect urinary PIF. Tumour tissue PIF mRNA was measured by real-time PCR and systemic PTHrP was determined by radio-immunoassay.

1. STUDY PROTOCOL

a) Ethical approval

All aspects of this study received full ethical permission from the Lothian Research Ethics Committee (reference LREC/2002/5/9). In every instance patients provided written informed consent and patients were issued with appropriate information sheets (see Appendix A).

b) Statistics and Power Estimation

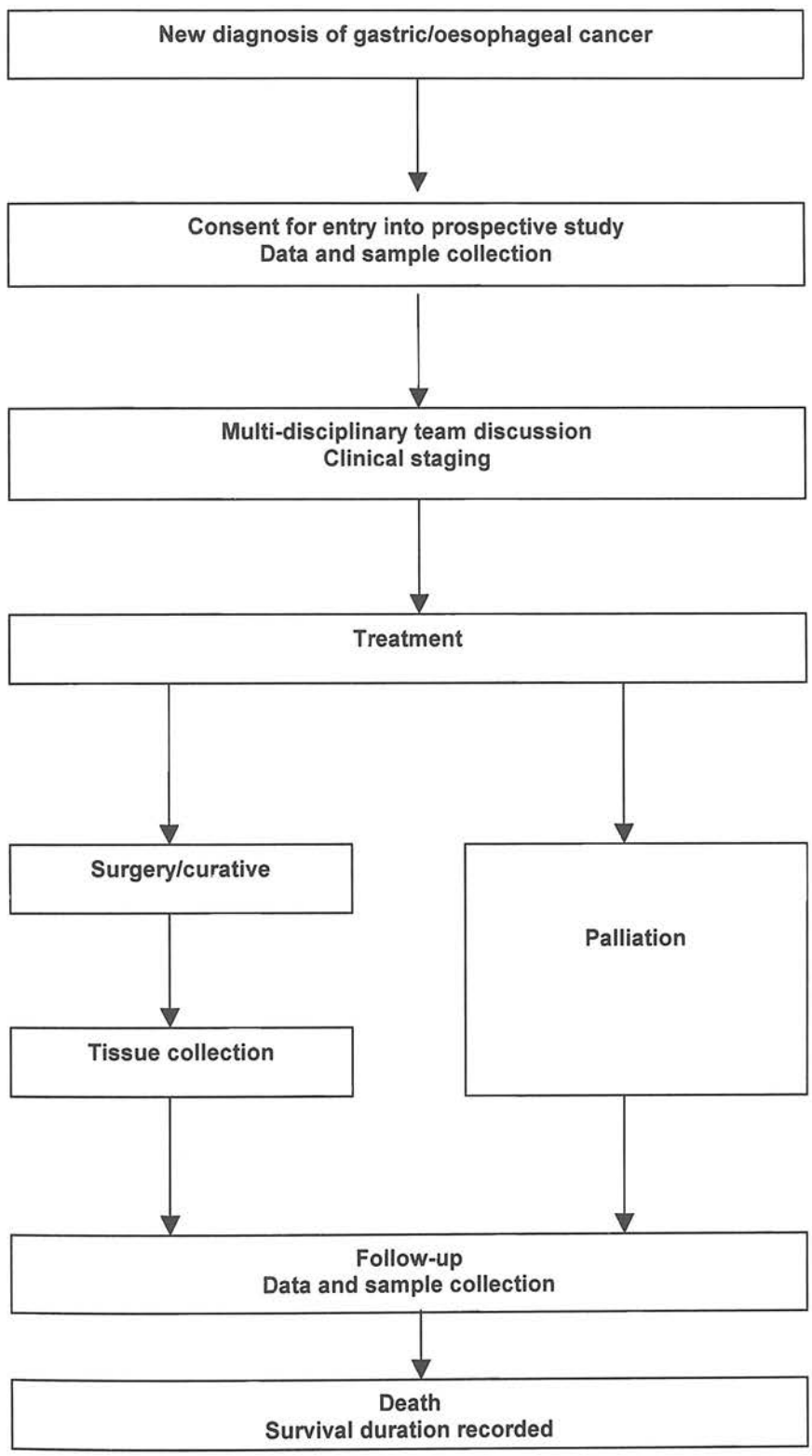
Prior to commencing the prospective collection of patient data/samples a power calculation was performed. In addition to investigating the origin of systemic inflammation in gastro-oesophageal cancer, the latter part of this thesis is to examine the prognostic role of systemic inflammation and to develop a novel prognostic model. Therefore, a nomogram was used to estimate the sample size for this study, which is specifically designed for calculating patient numbers for studies using survival as an end point (Schoenfeld and Richter, 1982). Previous studies in pancreatic cancer patients have suggested a 50% survival difference between patients with systemic inflammation and those without (Falconer et al, 1995). Assuming this to be true for patients with gastro-oesophageal cancer, and based on a two-sided test with a significance level equal to 0.05 and a power of 0.9, a 2-year study with a mean follow-up period of 6 months would require two hundred patients to demonstrate a 50% survival difference. Therefore, over two hundred patients were recruited to the study.

Statistical analyses are described in detail within context in each Chapter. In general, data were considered not to be normally distributed and non-parametric tests were applied. Log-rank analysis was used to determine prognostic value for univariate analysis and Cox's proportional hazard's model was used to test for multivariate analysis. A p value ≤ 0.05 was considered statistically significant in each case. Analysis was performed using SPSS® software (SPSS, Chicago, Illinois, USA).

c) Patient recruitment and follow-up

An overview of the study protocol is shown in Figure 3.1.1.

Figure 3.1.1 An overview of the study protocol and the patient journey following diagnosis of gastro-oesophageal cancer.



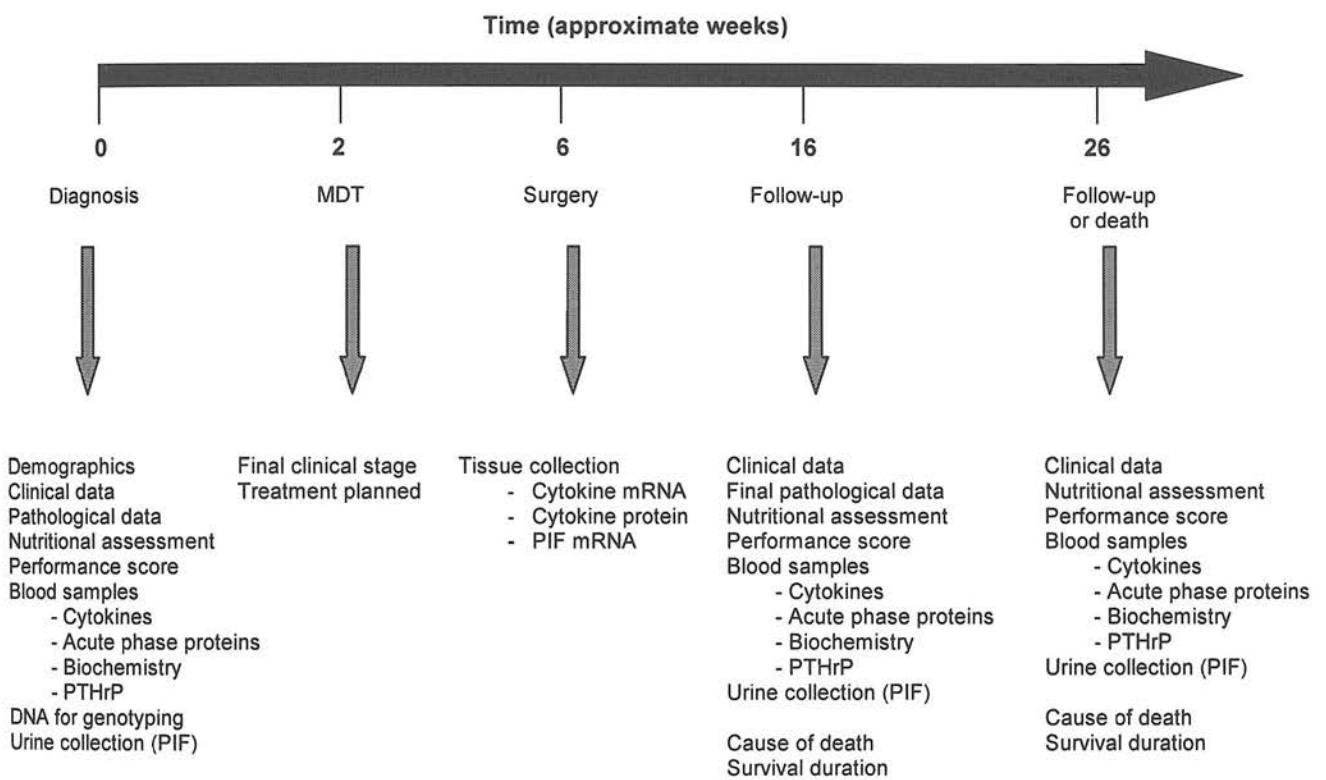
Patients were recruited to the study between 25 March 2002 and 25 June 2004. All patients were enrolled before any treatment had been initiated and within 1-2 weeks from the time of diagnosis. Newly diagnosed patients were identified principally from the multi-disciplinary team meetings. The Lothian and Borders Oesophago-Gastric Cancer Group is a multi-disciplinary team (MDT) comprising specialist oesophago-gastric surgeons, gastroenterologists, medical and radiation oncologists, radiologists, pathologists, dieticians, clinical nurse specialists, data collectors, researchers and trainee medical/surgical staff. This team provides a regional referral service for patients with oesophageal and gastric cancer from the Lothian and Borders regions (population approximately 1 million). Patients newly diagnosed with gastric or oesophageal cancer within these regions are referred to the MDT, which meets on a weekly basis to discuss all new referrals and to decide on individual patient management strategies. Alternatively, suitable patients were identified in outpatients, on the wards (when admitted as emergencies), and from the clinical nurse specialists. It is estimated that through these methods most patients newly diagnosed with gastro-oesophageal malignancy in these regions would have been identified (approximately 150 patients per year). There were a few known cases where patients were diagnosed in hospitals out-with Edinburgh Royal Infirmary and were not referred to the MDT due to extreme frailty of the patient and these patients were not recruited to the study. No patients refused entry into the study.

Once identified, suitable patients were interviewed and, following written informed consent, numerous demographic, clinical and pathological data were recorded using a standard data collection sheet (see Section 1(d) and Appendix B). The timings of data recording and sample collection are shown in Figure 3.1.2.

After the initial interview at the time of recruitment to the study patients were followed-up when possible and repeat information was collected on the data sheet. Patients who underwent surgical treatment were reviewed approximately three months following surgery, and those patients who received pre-operative chemotherapy were additionally reviewed at the completion of neoadjuvant therapy and prior to surgery (Figure 3.1.2). Where possible patients who received palliative treatments were similarly reassessed a various time points during their disease progression. Dates and cause of death were identified from the upper GI

cancer data manager, the patients' General Practitioners, and the National Registry of Births, Deaths and Marriages.

Figure 3.1.2 Approximate timing of data recording and sample collection during the study. Further information of the type of data collected and samples collected is described in the text and in Appendix B.



d) Clinical and Pathological data collection

The various timings of data collection and the type of information gathered are shown in Figure 3.1.2. Demographic data was recorded and included age and sex for each patient. Co-morbidity was documented for each patient as well as current health and medications taken

(including over the counter preparations), which may modify the systemic inflammatory response. Duration of symptoms and the date of diagnosis, defined as the date of histological confirmation of disease, were also recorded in all instances.

All patients underwent endoscopic investigation and histological analysis of tissue prior to entry into the study. Following histopathological confirmation of disease patients followed a staging protocol devised by the MDT, which included investigation by computerised tomography (CT) and, provided there was no metastatic disease, endoscopic ultrasound (EUS). In addition, patients with tumours involving the distal oesophagus and stomach also underwent laparoscopic assessment including laparoscopic ultrasound (LUS) to identify potential peritoneal disease. Patients considered too frail for interventional treatment may not have progressed further than a tissue diagnosis. Each patient was staged according to the International Union Against Cancer (UICC) (Sobin and Wittekind, 2003). Final histopathological stage (pTNM) was used where available, but was only available for those patients who underwent surgical resection. In all other cases the final clinical stage (cTNM), as agreed at the unit MDT meeting, was recorded. Tumours around the oesophago-gastric junction were classified according to Siewert and Stein and those classified as type I and II were staged as oesophageal tumours and type III as gastric cancers (Siewert and Stein, 1998).

In addition to staging information, the anatomical position of the tumour was recorded as well as the histological type, the degree of differentiation, and any other relevant features on histological analysis (including the presence of signet ring cells, tumour necrosis, Helicobacter pylori, and peri-neural or vascular tumour invasion). Treatment modality undertaken by each patient was recorded as well as intent (palliative versus curative). Survival duration, defined as time from histological diagnosis until death, was documented and where possible the cause of death was confirmed as either disease related or due to another cause.

2. NUTRITIONAL ASSESSMENT

Patients underwent a nutritional assessment to record changes in body composition and dietary intake at the time of diagnosis and later in the course of the disease. This was to document changes in nutritional status in a cohort of patients with gastro-oesophageal cancer from the time of diagnosis, through the advancement of disease and, in some cases, up to the time of death. These findings could then be used to explore any association between adverse nutritional status (cachexia) and the presence of systemic inflammation and, in turn, examine the role of cachexia in explaining the adverse prognosis associated with systemic inflammation in patients with gastro-oesophageal cancer. This topic is further explored in Chapter X.

The assessment of nutritional status included calculation of body mass index (BMI) and anthropometry in all patients. In a subgroup of patient's body composition analysis was also undertaken using electrical impedance. Dietary intake was recorded in dietary diaries and also estimated by patients. The severity of dysphagia was also assessed and documented.

Calculation of body mass index (BMI) and rate of weight-loss

Height was measured using a wall-mounted stadiometer without shoes. Patients were weighed on spring balance scales without shoes and wearing light clothing. Body mass index (BMI) was calculated following height and weight measurements. Pre-morbid patient weight was recalled by the patient and verified where possible from the medical notes. Individual weight loss was calculated and expressed as percentage of pre-illness body weight lost per month of symptoms.

Anthropometry

Anthropometry was undertaken on each patient and included measurement of mid-arm circumference (MAC) and triceps skinfold thickness. MAC was measured at the mid-point between the acromion and the olecranon processes. Triceps skinfold thickness was measured with Harpenden skin callipers (Holtain, Byberian, UK) and mid-arm muscle

circumference (AMC) was calculated by means of Jelliffe's equation (Jelliffe, 1966). Values were normalised using standardised reference tables (Bishop et al, 1981).

Body composition analysis

Body composition analysis was performed on a randomly selected patient sub-group attending the outpatient clinic. Bioelectrical impedance analysis was performed using a four-terminal multi-frequency bioelectrical impedance analyser (Xitron 4000 MFBIA, Xitron Technologies, San Diego, USA), operated at a current of 200μA root mean square. All values were recorded with the subject supine with limbs apart. Values for total body water were obtained using equations validated in a similar patient group (Hannan et al, 1995). Lean body mass was calculated from total body water assuming a hydration coefficient of 0.732.

Estimation of dietary intake

Dietary intake was estimated by all patients and scored as 1 = normal, 2 = reduced and 3 = poor/minimal. The smaller group of randomly selected patients provided detailed 3-day food diaries for comparison. Patients were instructed to record all food and drink consumed over a 3-day period, which included a weekend day and the data were analysed by a senior dietician who provided information on the level of intake of macronutrients using computer software (CompEat®, Nutrition Systems, Grantham, UK). Comparisons were made to dietary reference values issued by the Department of Health (Department of Health, 1991).

Dysphagia score

The severity of dysphagia was assessed at each interview and was scored according to Knyrim *et al* as follows (Knyrim et al, 1993):

0	=	able to eat normal diet / no dysphagia.
1	=	able to swallow some solid foods
2	=	able to swallow only semi solid foods
3	=	able to swallow liquids only
4	=	unable to swallow anything / total dysphagia

3. Assessment of Performance Status

Patient performance status was scored at each assessment using the scoring system devised by Karnofsky (Karnofsky and Burchenal, 1949):

Normal, no complaints	100
Able to carry out normal activities, minor signs of disease	90
Normal activity but with effort	80
Self-caring but unable to carry out normal activity or work	70
Requires occasional assistance but able to care for most needs	60
Requires considerable assistance and frequent medical care	50
Disabled, requires special care and assistance	40
Severely disabled, hospitalisation indicated although death not imminent	30
Very sick, requires hospitalisation. Active supportive treatment necessary	20
Moribund	10
Dead	0

4. Collection of Biological Samples

Biological samples were collected at different time points during the disease process (see Figure 3.1.2). At the time of recruitment (diagnosis) whole blood was collected for determination of serum concentrations of acute phase proteins, cytokines, albumin, alkaline phosphatase, urate, parathyroid hormone-related peptide (cPTHrP), and calcium. The cellular component was separated and used for extraction of DNA for cytokine genotyping. Patients also provided a sample of urine for proteolysis-inducing factor (PIF) determination.

At each follow-up interview an additional blood sample was collected for determination of serum concentrations of C-reactive protein (CRP), albumin, alkaline phosphatase, urate, cPTHrP, and calcium. Additional urine samples were also collected.

Paired samples of tumour tissue and adjacent normal mucosa were collected from patients at the time of surgery for measurement of tissue cytokine mRNA and protein concentrations and for PIF mRNA analysis. In order to preserve as much pathological staging information as possible the resected specimens were transferred fresh from the operating theatre to the pathology department where representative tissue samples were cut by a Consultant Pathologist before the samples were snap frozen in liquid nitrogen prior to storage at -80°C.

5. Blood Assays

As part of the investigation into the genesis and mediators of systemic inflammation in patients with cancer, concentrations of serum cytokine and acute phase proteins were measured.

a) Serum acute phase protein measurement

C-reactive protein (CRP), haptoglobin, α_1-antichymotrysin (ACT), albumin, and transferrin were selected as the acute phase proteins for investigation. CRP, haptoglobin, and ACT represent positive acute phase proteins and albumin and transferrin represent negative acute phase reactants (see Chapter II, Section 2 (c) for further details).

C-reactive protein (CRP)

Serum CRP concentration was measured using an automated immuno-turbidimetric assay within the Clinical Biochemistry Department, Edinburgh Royal Infirmary, UK. Using this assay

a concentration of 10mg/l represents the upper limit of the normal range with most healthy individuals having a serum concentration <2mg/l (Gabay and Kushner, 1999). The coefficient of variation was less than 3%.

Serum concentrations of CRP begin to rise within 24 hours of the onset of the inflammatory stimulus and continue to rise for 3-4 days. Elevated serum concentrations continue until the systemic inflammatory stimulus resolves (Gabay and Kushner, 1999).

Haptoglobin

Serum haptoglobin concentrations were determined by sandwich enzyme-linked immunosorbent assay (ELISA). Whole blood was collected into lithium-heparinised tubes and samples were centrifuged at 1500g for 5 minutes at 4°C. The serum was stored at –80°C until batch analysis. Samples were diluted to 1 in 15,000 in dilution buffer (see Appendix C for reagent recipes) prior to analysis. 96-well plates (Costar) were coated with 100µl primary antibody (polyclonal rabbit anti-human, Dako, Ely, UK) at a concentration of 10 mg/l, covered in aluminium foil, and incubated overnight at 4°C. The plates were washed with dilution buffer 3 times using an automated washing device. 100µl of diluted sera was added to the coated wells and incubated at room temperature for 2 hours. Standards were prepared in a similar manner. All reactions were performed in duplicate. Plates were washed as before and 100µl of peroxidase-conjugated secondary antibody diluted 1 in 5,000 (polyclonal goat anti-rabbit, Dako) was added to each well and incubated for 1 hour at room temperature. Plates were washed again before 100µl of chromogenic substrate was added. The substrate used was OPD (*o*-Phenylenediamine dihydrochloride, Dako) in substrate buffer and the reaction was stopped after 15 minutes with 100µl of 0.5M sulphuric acid. Plates were read at 490 nm using a Dynatech MR5000 automated plate reader (Billinghurst, UK). Standard curves were generated using standards of known concentration supplied by the manufacturer (Dako, UK). Taking into account the sample dilutions the lower limit of sensitivity for the assay was 100 pg/ml. The coefficient of variation was less than 10%.

Serum haptoglobin concentrations start to rise with 24-48 hours of stimulus and continue to increase steadiliy for 7-10 days (Gabay and Kushner, 1999). Elevated concentrations may continue for as long as the inflammatory stimulus is maintained.

α_1-antichymotrysin (ACT)

Serum concentrations of ACT were determined by sandwich ELISA using the same methodology described for haptoglobin measurement. Serum samples were diluted 1 in 4,000. The primary antibody was a polyclonal rabbit anti-human (Dako, Ely, UK) used at a concentration of 10 mg/l. The secondary antibody was diluted 1 in 2,000 and was the same as that used for haptoglobin. The lower limit of sensitivity for this assay was 50 pg/ml. The coefficient of variation was less than 10%.

Albumin

Serum albumin concentration was measured by the automated bromocresol green dye-binding technique in the Clinical Biochemistry Department, Edinburgh Royal Infirmary, UK. Normal serum concentrations are 35-50 g/l. The coefficient of variation was less than 3%.

Albumin is a negative acute phase reactant and serum concentrations decline after 24 hours of an inflammatory stimulus and continue to decline for several days or weeks depending on the duration of inflammation (Gabay and Kushner, 1999).

Transferrin

Serum transferrin measurement was performed by ELISA in the same manner to that described for haptoglobin and ACT. Serum samples were diluted 1 in 17,000. The primary antibody was a polyclonal rabbit anti-human (Dako, Ely, UK) used at a concentration of 10 mg/l. The secondary antibody was diluted 1 in 5,000 and was the same as that used for haptoglobin. The lower limit of sensitivity for this assay was 30 pg/ml. The coefficient of variation was less than 10%. Transferrin is also a negative acute phase protein and serum concentrations follow the same pattern as albumin (Gabay and Kushner, 1999).

b) Serum cytokine measurement

Serum interleukin-1β (IL-1β), IL-6, IL-8, IL-10 and soluble tumour necrosis factor receptor 55 kDa (sTNF-R) were chosen for analysis. IL-1β, IL-6 and IL-8 are key pro-inflammatory

cytokines and IL-10 is an important anti-inflammatory cytokine (see Chapter II). Serum TNFα concentrations are not reliably detected within the circulation, however, TNF receptors are shed into the circulation in response to TNFα release, and concentrations of TNF receptors are more reliably detected in the circulation (Muc-Wierzgon et al, 2003). Therefore, serum TNF receptors provide a more robust indirect measure of TNFα production and consequently this study measured TNF receptor (sTNF-R) concentrations instead of TNFα concentrations (Spinas et al, 1992). Serum cytokine concentrations were determined by ELISA using module kits and were performed according to the manufacturers instructions (Caltag, Bender MedSystems, Towcester, UK).

96-well plates (Costar) were coated with 100µl of the primary antibody. The primary antibody concentrations were; IL-1β 1 µg/ml, IL-6 2.5 µg/ml, IL-8 5 µg/ml, IL-10 0.5 µg/ml, and sTNF-R 3 µg/ml. Plates were covered and stored at 4°C overnight. The wells were then washed 3 times with buffer (0.5ml Tween 20 in 1 litre of phosphate buffered saline) using an automated washer. The wells were then blocked with the addition of 250 µl of assay buffer (see Appendix C) and kept at room temperature for 2 hours. The plates were washed as described previously before the addition of 100 µl of the test sample into each well. Patients' serum was diluted 1 in 2 in all cases except for sTNF-R where the samples were diluted 1 in 10. Standards were prepared by serial dilutions of a lyophilised standard protein provided by the manufacturer. All samples were prepared in duplicate. 50 µl of biotin-conjugate (diluted 1 in 1000 with the assay buffer) was added to each well before incubation at room temperature for a further 2 hours on a plate shaker set at 200 rpm. Following another wash, 100 µl of streptavidin-HRP (diluted 1 in 10,000 with the assay buffer) was added to each well for 1 hour on a microplate shaker (200 rpm) at room temperature. The plates were washed again and 100 µl of substrate solution (1 in 2 mixture of H_2O_2 and tetramethylbenzidine) was added to each well. The plates were incubated in the dark at room temperature for a further 15 minutes before the reaction was stopped by the addition of 100 µl of 4N sulphuric acid. Plates were then read at 450 nm using an automated plate reader (Dynatech MR5000, Billinghurst, UK). The lower limit of sensitivity for each assay was; IL-1β <1 pg/ml, IL-6 1.4 pg/ml, IL-8 11 pg/ml, IL-10 0.8 pg/ml, and sTNF-R 5.8 pg/ml. The coefficient of variation for each assay was: L-1β 5.1%, IL-6 5.2%, IL-8 8.7%, IL-10 5.6%, and sTNF-R 8.6%.

c) Clinical chemistry

Routine serum clinical chemistry (CRP, albumin, alkaline phosphatase, and calcium) was kindly performed by the Clinical Biochemistry Department, Edinburgh Royal Infirmary. Serum total calcium was measured by an automated analyser. Calcium levels were adjusted for serum albumin concentration using the following formula (Truong et al, 2003):

$$\left\{\frac{40 - \text{albumin concentration}}{4}\right\} \times 0.1 + \text{measured serum calcium concentration}$$

The normal range for corrected calcium is 2.12 – 2.62 mmol/l. The normal range for alkaline phosphatase is 40-125 units/l and normal ranges for serum urate concentrations are 0.12-0.42 mmol/l for males and 0.12-0.36 mmol/l for females. The coefficient of variation of all automated assays was 3% or less.

d) Serum Parathyroid hormone-related peptide (PTHrP) measurement

Parathyroid hormone-related peptide (PTHrP) is a tumour-derived product that is postulated to play a role in the generation of systemic inflammation and cachexia in patients with cancer (Ogata, 2000; Takahashi et al, 2003). To investigate this potential role in patients with gastro-oesophageal cancer serum concentrations of PTHrP were measured in the study population. This topic is explored in Chapter VIII.

Whole blood was collected into lithium-heparinised tubes and samples were centrifuged at 1500g for 5 minutes at 4°C. The serum was stored at –80°C until batch analysis when all samples were analysed at the same time. PTHrP has a very short half-life and is unstable unless stored in plasma with esterase inhibition. However, its breakdown products include a c-terminal region, which is stable when stored in serum and demonstrates

direct correlation to circulating intact serum PTHrP concentrations (Suehiro et al, 1994). This is a direct breakdown product of PTHrP and, therefore, its presence indicates PTHrP in plasma. In this thesis the c-terminal fragment (cPTHrP) was measured by radio-immunoassay (Daiichi, Tokyo, Japan).

The assay was performed according to the manufacturers instructions and followed national safety guidelines on the use and handling of radioactive substances. 200µl of each standard (0 pmol/l, 10 pmol/l, 30 pmol/l, 100 pmol/l, 300 pmol/l, and 1000 pmol/l) was pipetted into a clean test tube. Similarly, 200µl of a positive control provided in the kit was also prepared in addition to 200µl of dH_2O as an additional negative control. Patient serum samples were thawed and diluted by a factor of ten by adding 180µl of dilution reagent to 20µl of serum. 100µl of iodinated (I^{125}) cPTHrP was then added to 200µl of each of the test samples and to each standard. In addition, 100µl of I^{125} cPTHrP was added to an empty test tube, which was used to provide the total count measurement. All standards and test samples were prepared in duplicate. 100µl of cPTHrP antibody (sheep antiserum) was added to each tube, shaken well, and then stored at room temperature for 24 hours. The following day 500µl of B/F reagent (sheep IgG donkey antiserum) was added to each tube, mixed well and incubated at room temperature for a further 1 hour. Tubes were then centrifuged at 2000g at 4 °C for 30 minutes and the supernatants were removed by careful aspiration. Counting rates were measured by a gamma-ray counter and a standard curve was generated using the counts obtained from the standards. cPTHrP concentrations for each patient sample were then read from the standard curve and multiplied by the dilutional factor of 10. Samples were re-analysed if there were any count discrepancies and results were obtained for all patient samples.

This assay has no demonstrable cross-reactivity with parathyroid hormone (PTH). The sensitivity of this test is 10 pmol/l and the intra-assay variability is 4.1% and the inter-assay variability is 3.3% (Daiichi, Tokyo, Japan). Using this assay, serum cPTHrP levels in healthy volunteers were (±2 SD from the mean) 13.8 - 55.3 pmol/l for males and 13.9 - 54.0 pmol/l for females (Fukunaga, 1992). No female patients had a cPTHrP serum concentration between 54.0 - 55.3 pmol/l, and therefore for simplicity serum levels above 55.3 pmol/l were considered above the normal range for both sexes.

6. CYTOKINE GENE POLYMORPHISM GENOTYPING

As part of the investigation into the aetiology of systemic inflammation in cancer, the influence of host cytokine genotype was examined. Comparisons between host genotype, tissue cytokine concentrations and systemic markers of inflammation are investigated in Chapter VI.

a) Isolation of genomic DNA from whole blood

Blood was collected from every patient for genotyping. Genomic DNA was extracted from samples of lithium-heparinised blood using the Wizard® Genomic DNA purification kit (Promega, Southampton, UK). Whole blood was centrifuged at 1,500g for 5 minutes at 4°C. 300 µl of the cellular layer was added to 900 µl of cell lysis solution in a 1.5 ml microcentifuge tube and mixed thoroughly by inverting the tubes several times. The mixture was incubated for 10 minutes at room temperature followed by centrifugation at 15,000g for 20 seconds also at room temperature. The supernatant was discarded and the process of adding cell lysis solution, incubation and centrifugation was repeated once more. 300 µl of nuclei lysis solution was added to the pellet and the tube was vortexed to resuspend the white cells. The solution was pipetted gently several times to lyse the white cells. 1.5 µl of RNase solution (Promega) was added to each tube, mixed and incubated at 37°C for 15 minutes. After allowing the tubes to cool to room temperature, 100 µl of protein precipitation solution was added and mixed by vortex for 15 seconds. Samples were then centrifuged at 15,000g for 3 minutes at room temperature. The supernatants were then transferred to a clean 1.5 ml microcentrifuge tube and mixed with 300 µl of pure isopropanol. Gentle mixing reveals the appearance of the DNA as white thread-like strands. Further centrifugation at 15,000g for 1 minute at room temperature allows the DNA to be collected as a pellet at the bottom of the tube. The supernatant is then decanted and 300 µl of ethanol 70% is added to wash the pellet. The samples were then centrifuged again for a further minute at 15,000g and the ethanol was carefully aspirated away. 100 µl of DNA rehydration solution (10mM Tris-HCl and 1mM

EDTA) was added to each tube and samples were incubated at 65°C for 1 hour. DNA samples were then stored at 4°C.

The quality and quantity of the isolated DNA was assessed by optical density (OD) readings of the samples taken at 260 nm and 280 nm using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Bucks, UK) employing Beer-Lambert's law. Typical yields were between 20-80 μg (in 100 μl total volume) with OD_{260}/OD_{280} ratios between 1.5-2.0. In addition, the quality of the DNA was confirmed by conventional polymerise chain reaction (PCR) prior to shipment to Southampton for genotyping. 2 μl of DNA was mixed with 1 μl $MgCl_2$ (25mM), 2.5 μl 10x Taq DNA polymerase buffer with added $MgCl_2$, 2.5 μl dNTP (10mM), 5 μl forward and reverse primers (10 μM), 11 μl DEPC treated water and 1 μl Taq DNA polymerase (5u/μl) (all reagents Promega, Southampton, UK). Primers for Cytochrome b were used to confirm adequate quality DNA. The forward primer sequence was GGTTCTGGAATAAGAATATAGG and the reverse primer sequence GACAACACAGTAAGAACCAGG, giving a product of 367 bp if adequate DNA was present.

b) Cytokine genotyping

Single nucleotide polymorphism (SNP) genotyping was performed by TaqMan® allelic discrimination genotyping in collaboration with Professor Robert Grimble, Dr Martin Howell, and Dr Matthew Rose-Zerilli (Histocompatibility and Immunogenetics Laboratory and Institute of Human Nutrition, School of Medicine, University of Southampton, UK). The following single nucleotide polymorphisms were selected for genotyping due to their documented but variable association with cytokine production: IL-1β -511, IL-6 –174, IL-10 –1082, TNFα -308, and LTα +252. Genotyping was carried out on the 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK). Primers and TaqMan probes were designed using Primer Express version 2.0 software (sequences and cycler conditions shown in Table 3.6.1) and synthesised and supplied by Applied Biosystems, UK. 10 μl PCR reactions containing 20 ng of DNA, 0.9 μM and 0.2 μM probes (final concentrations) were performed in 384 well plates. Each genotyping plate contained no DNA Template (water) controls; SDS version 2.1

software was used to analyse real-time and end-point fluorescence. Around 50 samples (~25% of the sample group) were randomly selected and included as replicates for each genotype tested. All replicates agreed. Only between 3-8 samples failed to genotype for each SNP (96.1-98.5% completion rate).

Table 3.6.1 Cytokine TaqMan genotyping primer and probe sequences. (A) TaqMan MGB Probe Assays (Ta = 60 °C), (B) TaqMan TAMRA probe Assays (Ta = 62°C). For each probe, the position of SNP is marked in lower case.

(A)

SNP	Sequence
TNFα-308	
Forward Primer:	5'-CCAAAAGAAATGGAGGCAATAGGTT-3'
Reverse Primer:	5'-GGACCCTGGAGGCTGAAC-3'
G allele Specific Probe:	5'-CCCGTCCcCATGCC-3 (VIC-labelled)
A allele Specific Probe:	5'-CCCGTCCtCATGCC-3' (FAM-labelled)
LTα+252	
Forward Primer:	5'-CAGTCTCATTGTCTCTGTCACACATT-3'
Reverse Primer:	5'-ACAGAGAGAGACAGGAAGGGAACA-3'
G allele Specific Probe:	5'-CCATGgTTCCTCTC-3' (FAM)
A allele Specific Probe:	5'-CTGCCATGaTTCC-3' (VIC)
IL-6-174	
Forward Primer:	5'-GCTGCACTTTTCCCCCTAGTT-3'
Reverse Primer:	5'-GCTGATTGGAAACCTTATTAAGATTGT-3'
G allele Specific Probe:	5'-CTTTAGCATcGCAAGAC-3' (VIC)
C allele Specific Probe:	5-CTTTAGCATgGCAAGAC-3' (FAM)
IL-1β-511	
Forward Primer:	5'-GGTCTCTACCTTGGGTGGTGTT-3'
Reverse Primer:	5'-TCCTCAGAGGCTCCTGCAAT-3'
C allele Specific Probe:	5'-TGCCTCgGGAGCT-3' (VIC)
T allele Specific Probe:	5'-TCTGCCTCaGGAGC-3' (FAM)

(B)

SNP	Sequence
IL-10-1082	
Forward Primer:	5'-ACA CAC AAA TCC AAG ACA ACA CTA CTA A-3'
Reverse Primer:	5'-GGA GGT CCC TTA CTT TCC TCT TAC C-3'
G Allele Specific Probe:	5'-ATC CCT ACT TCC CCc TCC CAA AGA A-3' (VIC)
A Allele Specific Probe:	5'-CCC TAC TTC CCC tTC CCA AAG AAG C-3' (FAM)

7. DETECTION OF URINARY PROTEOLYSIS-INDUCING FACTOR (PIF)

Proteolysis-inducing protein (PIF) is a tumour-derived product that has been proposed as a candidate cachectic factor (Todorov et al, 1996). *In vitro* studies have also suggested a role for PIF in the stimulation of pro-inflammatory cytokine production and the generation of an acute phase response (Watchorn et al, 2001). This thesis will therefore investigate the expression of PIF in patients with gastro-oesophageal cancer and relate levels of expression to markers of systemic inflammation, nutritional status and outcome. This topic is investigated in Chapter VIII.

a) Isolation of urinary protein

At the time of study recruitment and at each subsequent follow-up visit patients were requested to provide a specimen of urine. Initially, patients were asked to provide 24-hour urinary samples to avoid possible problems that may relate to any unknown diurnal variation that may occur in PIF production. However, patients were reluctant to provide 24-hour samples and owing to the often-large distances to travel to the hospital from the large

geographical catchment area, retrieval of samples was also difficult. Therefore, 4-5 months into the study patients were asked to provide a single urinary sample at the time of clinic visit. Samples were collected into clean containers without preservative and specimens were stored at -20°C until processing.

As a consequence of the difficulties we had in reliably detecting urinary PIF (see following section), several alternative methods to isolate urinary protein were tried.

(i) Ammonium sulphate precipitation

This was the first method that was tried and followed exactly the method described in the original publications on the identification of PIF (Todorov et al, 1996; Wigmore et al, 2000). In addition, we followed a detailed protocol kindly provided by Professor M Tisdale (Aston University, Birmingham).

After thawing, 20 ml of urine was centrifuged at 16,000g for 30 minutes at 4°C to remove cellular debris. The supernatant was then transferred into a 100ml glass beaker containing a magnetic follower and placed on a magnetic stirrer in a cold room at 4°C. Over a period of about 6 hours aliquots of ammonium sulphate (16g; 80% w/v) was added and the beaker was covered with aluminium foil and left with continuous stirring overnight. The next morning, the sample was centrifuged at 3,000g for 30 minutes at 4°C before transferring the precipitate into a 100ml Amicon® filtration cell (Amicon, Stonehouse, UK) for washing. The Amicon® unit was attached to nitrogen for pressure filtration at 60 psi and each sample was washed four times with distilled water. A membrane filter with a molecular weight cut-off of 10,000 was used in each case. The protein was collected from the membrane using a pipette and the samples were then concentrated to around 200 µl using another Amicon® unit.

(ii) Ammonium sulphate method modification

Samples were prepared as described above for the addition of ammonium sulphate (80% w/v). After continuous stirring overnight at 4°C, samples were centrifuged using a centrifugal filtration device (Centriplus® spin columns, Millipore, Watford, UK) instead of Amicon® filtration units to wash and concentrate the samples. The columns were chosen with10 kDa MW cut-off filtration membranes. This method of protein isolation has been the preferred technique in

recent PIF studies owing to the increased ease of use compared with the Amicon® filtration units (Williams et al, 2004).

The 20ml urine sample was divided between two filtration devices, which were centrifuged at 3,000g for 60 minutes at 4°C. The filtrate was removed and this cycle was repeated once more. This process usually yielded approximately 500 µl of retentate. Care was taken not to spin the tubes to dryness.

(iii) Acetone precipitation

This method was tried as an alternative to the ammonium sulphate precipitation method and has been used previously with success for the isolation of urinary proteins (Thongkoonkerd et al, 2002).

Urine was spun as before to remove cellular debris. 10ml of urine was mixed with 20 ml of cold acetone containing 20mM dithioreitol (DTT). Samples were stored at -20°C overnight then spun at 13,000g for 15 minutes. The pellet was re-suspended in sodium dodecylsulphate (SDS) buffer. This technique was also used without the initial spin at 16,000g to try and preserve as much protein as possible.

Determination of protein concentration

Urinary protein concentrations were determined by the Lowry method (DC Protein assay, Bio-Rad, Hemel Hempstead, UK) (Lowry et al, 1951). Samples were diluted 1 in 10 and 5 µl of each sample was added into the wells of a 96-well microtiter plate (Costar). Standards were prepared similarly following 1 in 2 serial dilutions of a known concentration of bovine serum albumin (Bio-Rad, Hemel Hempstead, UK). All test samples were performed in duplicate. 20 µl of Reagent S (SDS) was mixed with 1 ml of Reagent A (sodium hydroxide) and 25 µl of this mixture was added to the samples in each well. 200 µl of Reagent B (Folin reagent) was then added to each well. The microtiter plate was placed on a gentle shaking device for 15 minutes before absorbance was read at 750 nm (Dynatech MR5000, Billinghurst, UK).

b) Western blotting

Identification of urinary PIF was initially attempted by Western blot. Once again, owing to the difficulty in achieving reliable and consistent results several modifications of the method were tried. An outline of the general methodology that was used is described below:

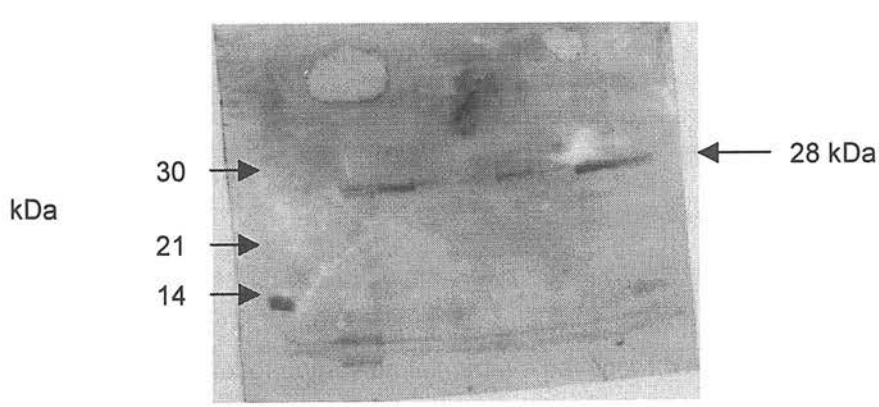
Identification of urinary PIF was attempted by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was performed using materials and equipment obtained from Bio-Rad Laboratories (Hemel Hempstead, UK). 4% stacking gels and 12.5% resolving gels were used in each run (see Appendix D for gel recipes). Urinary proteins were isolated by ammonium sulphate precipitation, or by one of the other methods described above. The proteins were denatured prior to blotting by heating to 95°C for 5 minutes. 5 µg of protein was loaded onto each blot with loading dye and molecular weight markers. Electrophoresis was performed at 200v for approximately 40 minutes before the proteins were transferred onto nitrocellulose membranes (Hoefer Scientific Instruments, San Francisco, USA). Effective transfer was confirmed by staining the membranes with Ponceau S solution (0.1 % (w/v) in 5% acetic acid (v/v); Sigma-Aldrich, Gillingham, UK) and the lanes and molecular weight markers were marked. Membranes were rinsed with Tris-buffered saline (TBS; 0.15M NaCl, 10mM TrisCl, pH 8.0) then blocked for non-specific binding with 5% non-fat milk powder (Marvel®, Premier Beverages, Adbaston, UK; 1g dissolved in 20ml TBS). After 1 hour the Marvel® was rinsed off and the membranes were incubated at room temperature for a further hour with the primary antibody dissolved in TBS with 0.5% Tween 20 (Sigma, UK) at a concentration of 10µg/ml. The main antibody used was a mouse monoclonal antibody from the same stock of antibody used in the original studies leading to the identification of PIF and was a kind gift from Professor M Tisdale (Todorov et al, 1996; Wigmore et al, 2000). This antibody had been purified with a protein A sepharose column (Sigma, UK) and biotinylated using the ECL protein biotinylation module (Amersham, Little Chalfont, UK) prior to use. The primary antibody was rinsed off with 3 washes of TBS-Tween and the membranes were then incubated for 1 hour with the secondary antibody (rabbit anti-mouse streptavidin-horseradish peroxidase conjugate; Amersham, UK) at a 1 in 1,500

dilution. The membranes were washed 3 more times with TBS-Tween before they were transferred to the dark room. Bands were detected with an emission chemiluminescence system (Amersham, UK).

Some of the urine samples were also analysed for PIF in Professor M Tisdale's laboratory at Aston University, Birmingham. Samples from 51 patients with a range of weight loss were investigated for PIF expression by the method described above. Blots were interpreted by an investigator who was blinded to the weight loss data and to our own results from the same group of patients.

The Western blot protocol was optimised with the following modifications: isolating urinary protein by the ammonium sulphate precipitation method, loading 7-10 µg of protein onto the gels, blocking with 5% Marvel® for 1 hour, using the primary monoclonal antibody at a concentration of 10 µg/ml with 1% Marvel® and incubating with the membranes for 1 hour, exposing the membrane to the photographic film for 60 seconds. These modifications resulted in bands appearing around 28 kDa, which differs to the previously published size of PIF (24 kDa or 66 kDa – see Chapter IX) (Figure 3.7.1). Therefore, we attempted to identify the 28 kDa product by mass spectroscopy.

Figure 3.7.1 Western blot of urinary protein from a patient with oesophageal cancer using the monoclonal antibody. Bands appeared at 28 kDa and were identified as CD59 by mass spectrometry.



Identification of the protein band by mass spectrometry

Protein samples were loaded onto gels (as described above) and, following electrophoresis, were washed three times with ultrapure water for 15 minutes each before staining with 20 ml colloidal based Coumassie blue (GelCode; Pierce) for 1 hour. Gels were washed again three times with ultrapure water as before. The band at 28 kDa was cut out the gel and stored in an Eppendorf tube at -20°C until analysis.

Subsequent analysis was performed by Dr A Cronshaw (Institute of Structural and Molecular Biology, University of Edinburgh). The proteins were subjected to trypsin digestion prior to sequencing the protein product by matrix-assisted laser desorption/ionisation, time-of-flight mass spectroscopy (MALDI-TOF).

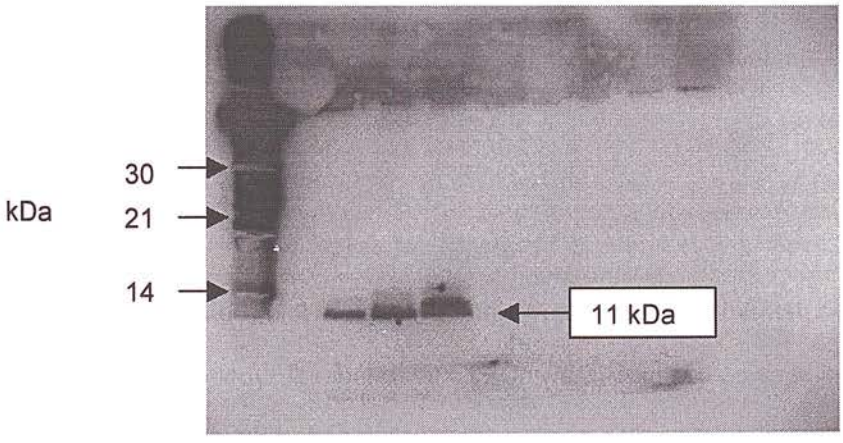
The results of the sequencing failed to confirm the presence of PIF and identified the 28 kDa band as CD59. CD59 (also known as Protectin) is widely expressed molecule found on cells on all tissues and is essential in protecting cells through inhibition of membrane attack complex (MAC) formation and complement mediated cell lysis (Davies and Lachmann, 1993). In addition, our results for the PIF Westerns (appearance of the 28 kDa band) differed from those obtained for the same group of patients when performed at Aston University (see Chapter VIII). This suggested that our Western blot was not identifying PIF. Blots were repeated with fresh monoclonal antibody received from Aston and Professor V Baracos' Laboratory in Alberta, Canada, to exclude any problems with our own antibody, but the results were similar. Other antibodies were therefore tried, including the development of a new PIF antibody.

A commercially available polyclonal antibody was initially tried (Alpha Diagnostic, San Antonio, USA). This was a rabbit polyclonal antibody raised against the core peptide sequence for PIF and called the 'cachectic factor antibody'. Western blotting was performed according to the manufacturers instructions. Urinary protein was isolated and gels were prepared as previously described. The primary antibody was used at a range of concentrations (1:100 to 1:2000). Goat anti-rabbit 2° antibody labelled with horseradish-peroxidase (HRP) was used with ECL detection. No bands were detected with this antibody.

Two other polyclonal antibodies were tested (CAC1 and CAC2). These antibodies were developed by Astra-Zeneca as part of their studies into PIF and were gifted to our Department by Dr I Waddell. The antibodies were raised against predicted antigenic sites within the peptide core. Blots were performed as described for the 'cachectic factor antibody' using a secondary antibody labelled with HRP. Once again no bands were detected.

Finally, our own anti-PIF polyclonal antibody was designed against the peptide sequence HEASAAQKENAGEDPC, which lies within the PIF core peptide. The antigenic peptide and the antibody, named CAC3, were developed from immunised rabbits by Sigma-Aldrich, Gillingham, UK. Western blots were performed according to the methods already described for the other polyclonal antibodies. This antibody generated a band at 11 kDa (Figure 3.7.2). This band was also subjected to identification by MALDI-TOF as previously described and was identified as casein, presumably from the Marvel milk protein used in the blocking process.

Figure 3.7.2 Western blot of urinary protein from a patient with gastric cancer using the CAC3 polyclonal antibody. The bands at 11 kDa were identified as casein by mass spectrometry.



In addition to analysing urinary protein, blots were also run using recombinant PIF and *in vitro* synthesised PIF. These samples were hoped to be useful as positive controls and to confirm the effectiveness of the PIF antibody.

Recombinant PIF (rPIF) is a 6-His-tagged recombinant peptide from bacteria that was developed by Astra-Zeneca. This peptide is not glycosylated and runs as two bands of 17 kDa and 12 kDa on SDS gels.

In vitro translated PIF was kindly gifted by Prof SJ Wigmore. The system involved cloning the cDNA sequence into pcDNA 3.1 (Invitrogen) and transcription and translation were performed using a T7 promoter system (Promega). Peptides underwent post-translation processing following the addition of canine pancreatic microsomal membranes (Promega). This process should produce the fully glycosylated PIF molecule.

In this study none of the PIF antibodies generated bands when blotted with either 6-his-rPIF or *in vitro* translated PIF.

c) Mass spectroscopy (MALDI-TOF)

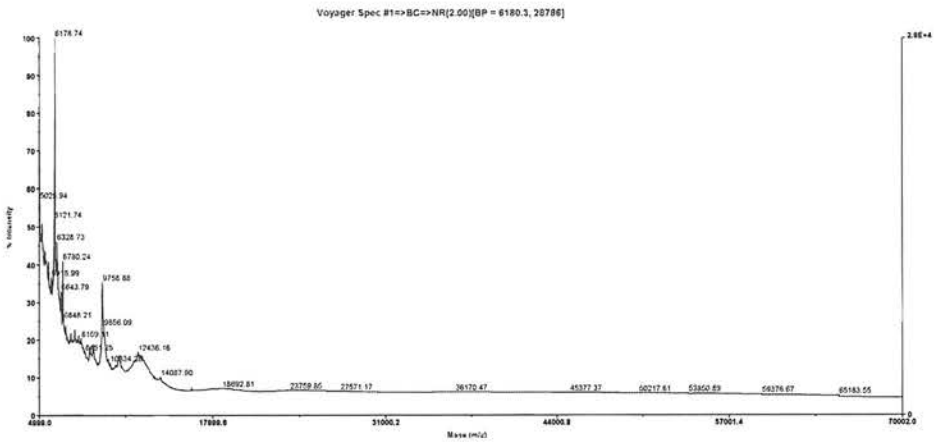
Mass spectroscopy was used as an alternative method to Western blotting for the identification of urinary PIF. The principle of matrix-assisted laser desorption/ionisation, time-of-flight mass spectroscopy (MALDI-TOF) involves converting a sample of unknown proteins into gaseous ions, which are accelerated by high voltage under a vacuum through a magnetic field (Guilhaus, 1995). A detector separates the ions according to mass, charge, signal intensity, and time of flight (time to travel through the machine). This generates a spectrum of characteristic peaks that represent the 'fingerprint' of the molecule. Specialised software matches the appearances of the peaks to known molecules and allows identification of the unknown molecule.

Isolation of urinary protein was performed by either ammonium sulphate precipitation or acetone precipitation as already described. Mass spectroscopy was performed by Dr A Cronshaw (Proteomics Facility Manager, Institute of Structural and Molecular Biology, Edinburgh University). Initially, concentrated urine samples were run directly on MALDI-TOF

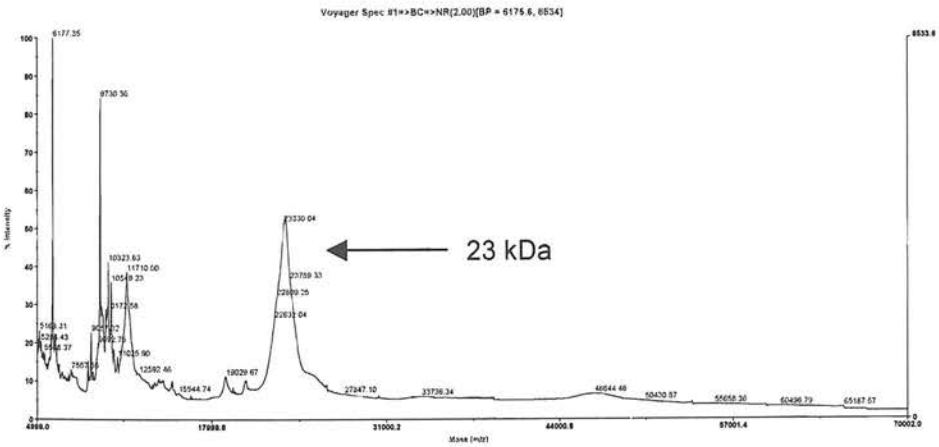
(Voyager DE-STR, Applied Biosystems). We were able to repeat the work of Choudhary *et al* but found that the 24 kDa glycoprotein factor they reported was in fact Ig kappa light chain (Figure 3.7.3) (Choudhary et al, 1999). A novel list of the proteins identified in urine from patients with gastro-oesophageal cancer and weight loss are listed in Appendix E.

Figure 3.7.3 MALDI-TOF mass spectra generated from two different patients with oesophageal cancer. Protein was isolated from urine by ammonium sulphate precipitation and the samples were loaded into a mass spectrometer (Voyager DE-STR, Applied Biosystems) without tryptic digestion or deglycosylation. Patient (A) represents a normal urinary protein trace. Patient (B) has a large peak at 23.3 kDa, which has been previously attributed to PIF. Subsequent analysis has identified this peak to represent Ig kappa light chain.

(A)



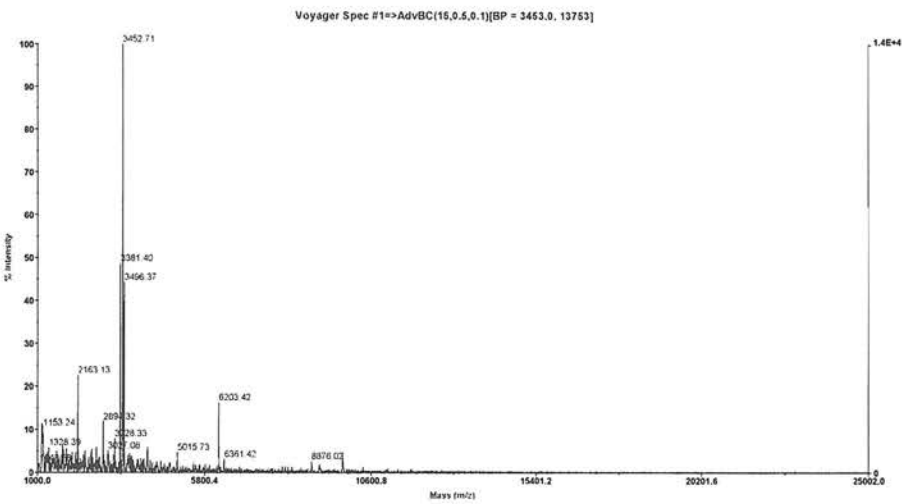
(B)



Therefore, owing to the complex mix of biological compounds within the urine samples it was necessary to run the protein samples on 1-dimensional and 2-dimensional SDS-PAGE gels (15%) before performing the mass spectrometry. The 2D gels allowed greater resolution of the 24 kDa peak in particular. The gel lanes were then pixelated and digested with trypsin (Promega). Peptides from the digested bands were then run on a liquid chromatograph mass spectrometer (LC-MS) and MALDI-MS instruments (Figure 3.7.4). The LC-MS consisted of a Famos autosampler, Switchos column switching unit and an Ultimate nanoLC (Dionex). The digests were loaded onto a PepMAP C18 column (3 µm particle size, 75um x 15cm) at a flow rate of 200 µl/min. The output was monitored at 214nm. The column was equilibrated with solvent A (0.1% formic acid) and eluted with a linear gradient from 0 to 70% solvent B (0.1% formic acid in 100% acetonitrile) over 45min. The flow was controlled by Chromelion software and interfaced to the MS with a PicoTip (New Objective). The LCQdeca mass spectrometer (ThermoFinnigan) was fitted with a nanoLC ESI source. Data dependent acquisition was controlled by Xcalibur software and database searching was achieved using TurboSequest.

Aliquots of 0.5 µl digests were mixed with 0.5 µl CHCA matrix on a MALDI sample plate. The samples were then analysed on a Voyager DE-STR MALDI-MS (Applied Biosystems) and the processed spectra searched against the NCBI non-redundant database using Protein Prospector.

Figure 3.7.4 Mass spectra of urinary protein from a patient with weight loss and oesophageal cancer. Protein was isolated from urine by ammonium sulphate precipitation. The proteins were run on 2D gels and the spots at 24 kDa were selected for tryptic digestion. These proteins were then run on the MALDI-TOF analyser. PIF was not identified.

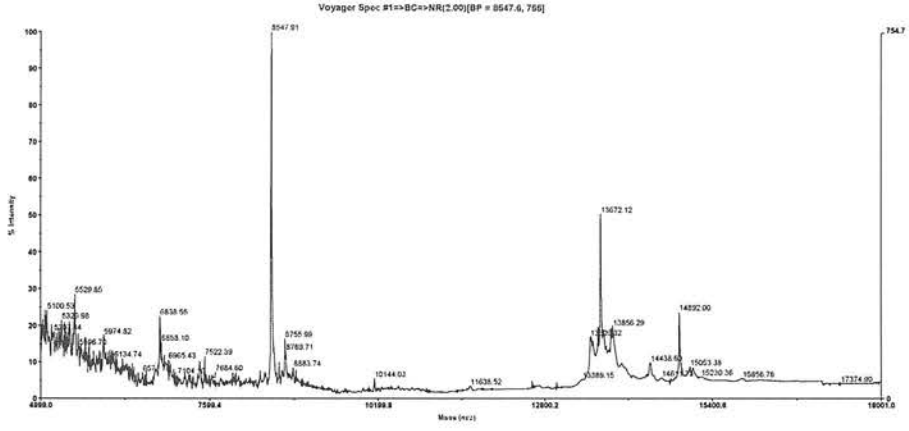


Given the high degree of glycosylation of the PIF molecule, protein samples were also de-glycosylated prior to analysis by mass spectrometry. N-linked de-glycosylation was performed using peptide N-glycosidase-F (PNGaseF) provided in kit form (GlycoProfile, Sigma). A 1D gel was run and the products were stained with Coomassie blue and the 24 kDa band was cut from the gel. 200 µl of destaining solution was added to the band in an Eppendorf tube and incubated for 30 minutes at 37°C. This was repeated twice and the gel was dried. 5 units of PNGaseF was added to the gel and incubated for 30 minutes at 37°C. 20 µl of ultrapure water was added to the tube and incubation was continued overnight. The tube was centrifuged briefly and the enzyme was aspirated out. 200 µl of water was added and the mix was incubated at room temperature for 30 minutes before briefly centrifuging and removing the supernatant. This was repeated three more times. The sample was then trypsinised as before.

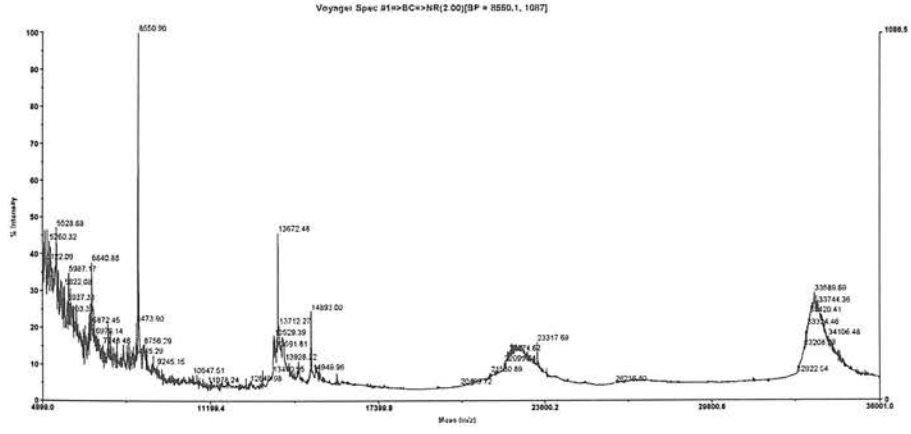
In addition to performing mass spectrometry on patients' urine samples, we also analysed the recombinant PIF (rPIF) and *in vitro* synthesised PIF samples. In particular the rPIF sample was analysed with and without peptidase digestion (Figure 3.7.5). This was necessary to demonstrate that the methodology was indeed able to identify the PIF core peptide when present. MALDI-TOF was able to detect normal urine samples 'spiked' with rPIF. However, *in vitro* synthesised PIF was not identified.

Figure 3.7.5 Mass spectra generated from running the recombinant PIF (rPIF) in the MALDI-TOF analyzer: (A) without tryptic digestion and (B) following tryptic digestion. These spectra identified PIF core peptide within urine samples from healthy subjects 'spiked' with PIF.

(A)



(B)



d) Summary

Detection of urinary PIF was initially attempted by Western blot by following the published methodology and using the same monoclonal antibody. Using these methods bands at 28 kDa were detected, which when sequenced were identified as CD59. Several polyclonal antibodies were also tried and one of these resulted in the generation of bands at 11 kDa, which were subsequently identified as casein. In addition, Western blot results from Edinburgh, Birmingham and Canada differed for the same group of patients. Mass spectroscopy was then tried as a more robust method of detecting urinary PIF. Spectral peaks at 24 kDa were found to be Ig kappa light chain. Separation of urinary proteins by 2D electrophoresis followed by tryptic digestion with and without deglycosylation has also so far failed to identify PIF in patients' urine. However, the PIF synthetic peptide core has been successfully detected in normal urine that has been spiked with the peptide.

8. Tissue Studies

To investigate the contribution of the tumour tissue towards the generation of systemic inflammation, cytokine mRNA and protein concentrations were measured within tissue homogenates. PIF mRNA concentrations were also measured in the tissues. Samples of tumour tissue and benign tissue were collected from patients with gastro-oesophageal cancer for analysis. In addition, the degree of inflammatory cell infiltrate into the tissues was determined by histological studies. This topic is explored in detail in Chapter V. The methods relating to the measurement of these mediators is described in the following Section.

a) Isolation of RNA

Tissue collection

Tissue was obtained from patients at the time of surgical resection or endoscopic biopsy. A representative sample of tumour tissue and adjacent benign mucosa was collected from each patient and tissues were snap frozen in liquid nitrogen prior to storage at -80°C until further analysis. The average time from tissue collection to freezing for patients who underwent surgical resection was 15 minutes (range 10-25 minutes). Tissue collected at the time of endoscopy was immediately snap frozen. An additional 12 patients were recruited as healthy controls. These patients underwent endoscopy as an elective investigation and in all instances the result of the procedure was normal, including both macroscopic and microscopic assessment.

Total RNA isolation protocol

Laser capture microdissection was not successful at isolating RNA of sufficient quality to perform reliable quantitative PCR. Therefore, whole tissue samples were homogenized and total RNA was isolated from samples using the RNeasy kit (Qiagen, Crawley, UK).

Approximately 30-50 mg of frozen tissue was placed into 600 µl of buffer RLT (containing guanidine thiocyanate and β-mercaptoethanol) in a glass test tube. Tissues were disrupted using a hand-held electric homogeniser using pulses of 10-15 seconds. Care was taken to avoid tissue thawing prior to homogenisation. Once the tissue was completely disrupted, tubes were centrifuged at 15,000 rpm for 3 minutes at room temperature. The supernatant was transferred to a fresh microcentrifuge tube and 600 µl of 70% ethanol was mixed by pipetting. The sample was then loaded into an RNeasy mini-column and centrifuged at 8,000g for 15 seconds and the flow-through was discarded. The mini-columns were washed with 350 µl of buffer RW1 (ethanol) and tubes were spun once again at 8,000g for 15 seconds. 80 µl of DNase I (Qiagen) was loaded into each mini-column and the tubes were incubated at 25°C for 20 minutes. The columns were then washed with buffer RW1 as already described before transferring the columns into fresh collection tubes. 500 µl of buffer RPE was then added to each column and the tubes were centrifuged at 8,000g for 2 minutes.

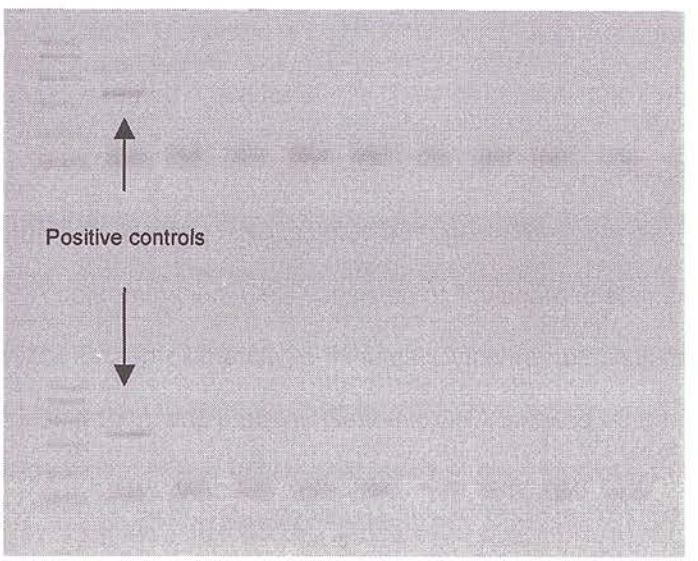
The columns were then placed into fresh microcentrifuge tubes once again and the RNA was eluted with the addition of 30 µl of RNase-free water into the columns and spinning at 8,000g for 1 minute. The elution process was repeated once more.

RNA concentration was measured by optical density (OD) readings taken at 260 nm and 280 nm using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Bucks, UK). Typical yields were around 10-15 µg of RNA from 50 mg of frozen tissue.

Reverse Transcription

Reverse transcription was performed using 1 µg of total RNA. All RNA samples were checked for genomic DNA contamination prior to reverse transcription using conventional polymerase chain reaction. 2 µl of total RNA was mixed with 1 µl $MgCl_2$ (25mM), 2.5 µl 10x Taq DNA polymerase buffer with added $MgCl_2$, 2.5 µl dNTP (10mM), 5 µl forward and reverse primers (10 µM), 11 µl DEPC treated water and 1 µl Taq DNA polymerase (5u/µl) (all reagents Promega, Southampton, UK). Primers for Cytochrome b were used to detect DNA contamination. The forward primer sequence was GGTTCTGGAATAAGAATATAGG and the reverse primer sequence GACAACACAGTAAGAACCAGG, giving a product of 367 bp if contamination was present (Figure 3.8.1). Cycling conditions were: 94°C for 5 minutes; 40 cycles of 94°C for 20 seconds, 55°C for 20 seconds, 72°C for 30 seconds; then 72°C for 10 minutes. Contaminated samples were re-treated with DNase I (as described above).

Figure 3.8.1 Non-RT-PCR using RNA isolated from gastro-oesophageal tumour tissues to exclude genomic DNA contamination. Gels consisted of 1% agarose stained with ethidium bromide and run at 70V for approximately 35 minutes. Cytochrome b was used for the reaction giving a 367 bp if DNA was present. Genomic DNA isolated from blood was used as the positive control.



Reverse transcription was performed once DNA contamination had been excluded. The reaction mixture included the RNA (1 μg in 10 μl DEPC treated water), 4 μl $MgCl_2$ (25 mM), 2 μl 10 x reverse transcriptase buffer, 2 μl dNTP's (10 mM), 1 μl random hexamers (500 μg/ml), 1.5 μl AMV reverse transcriptase (10 u/μl), and 0.5 μl recombinant RNase inhibitor (40 u/μl) (all reagents Promega, Southampton, UK). Reverse transcription was performed at 42°C for 60 minutes followed by 95°C for 5 minutes.

b) Quantitative (real-time) polymerise chain reaction

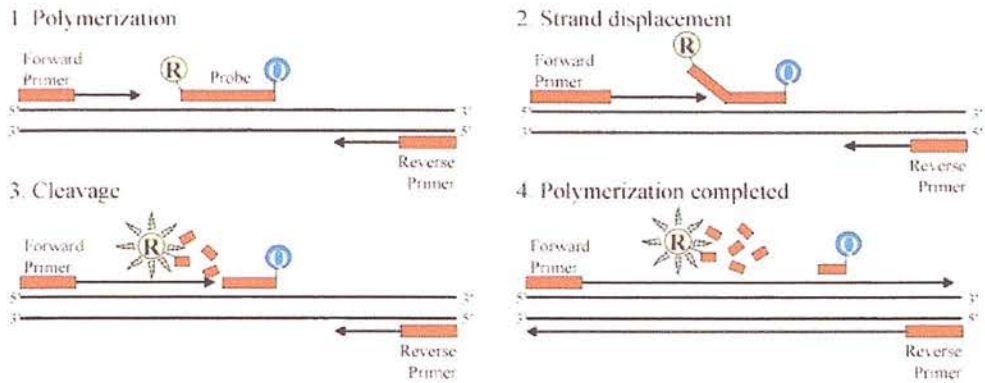
Quantitative polymerise chain reaction (PCR) was performed using the ABI PRISM 7700 real-time Sequence Detection System (Applied Biosystems, Warrington, UK). This system contains a 96-well thermal cycler and is able to detect fluorescence emitted during the PCR reaction in 'real-time'. Each reaction mixture contains a forward and reverse primer and a fluorogenically labelled probe. The probe is an oligonucleotide with a reporter fluorescent dye (FAM) attached to the 5' end and a quencher dye (TAMRA) attached to the 3' end (Figure 3.8.2). The probe is cleaved by the DNA polymerase during the reaction separating the quencher from the reporter dye allowing increased fluorescence. This fluorescence is measured in 'real-time' during the PCR reaction and an amplification plot is generated (Figure 3.8.3). Quantification of gene expression is then determined from this graph.

This system was used to quantify gene expression in tissues collected from patients with gastro-oesophageal cancer and to compare levels of gene expression with tissue collected from healthy controls. The following genes were chosen for study; cytokines (IL-1β, IL-6, IL-8, TNFα), acute phase protein (CRP), and proteolysis-inducing factor (PIF).

Primer design

Primers and probes were designed using Primer Express® software (version 2, Applied Biosystems). A forward primer, reverse primer and a fluorogenic-labelled probe were required for each gene to be studied. For optimum performance of the real-time PCR machine certain guidelines for the design of the primers and probes had to be followed. The primers were designed to be around 20 nucleotides in length with the aim of generating an amplicon product of 50-150 nucleotides. The melting temperature (Tm) of each was between 58-60°C and there were fewer than two G or C residues within last 5 nucleotides at the 3' end (to minimise non-specific extension). The primers were chosen to lie as close to the probe as possible, but without overlapping.

Figure 3.8.2 Generation of fluorescence during real-time PCR. 1) The primers and probe anneal to the template cDNA. 2) The primer strand disrupts the probe allowing 3) cleavage by DNA polymerase. 4) The quencher is separated from the reporter dye allowing increased fluorescence, which is detected by the PCR machine during the reaction. An amplification plot is generated allowing the level of gene expression to be quantified. (Reproduced from the Applied Biosystems website.)



The probe was also designed to be 20 nucleotides in length with a Tm 10°C higher than the primers (68-70°C). It was also a condition that the 5' end was not a G residue as this nucleotide may continue to quench the fluorescence despite cleavage.

Primer and probe sequences for PIF and IL-6 were designed as described above and manufactured by Applied Biosystems (Table 3.8.1). BLAST searching the human genome with the primer/probe sequences confirmed good specificity for the gene of interest. The primers/probes for CRP and ZAG were designed and manufactured by Applied Biosystems and the sequences were not disclosed by the Company. The other cytokines were available as a commercially produced reaction plate (TaqMan cytokine gene expression plate 1, Applied Biosystems). The cytokines included on the plate were IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, gamma interferon (γIFN), and TNFα.

Ribosomal 18S RNA was used as the internal control. Therefore, within each well there were two reactions simultaneously undergoing PCR (multiplex PCR); the target gene of interest (labelled with FAM), and the 18S internal control (labelled with VIC). The two reporters fluoresce at different wavelengths and, therefore, can be discriminated from each other by the real-time machine. Ribosomal RNA was chosen as the internal control as their levels are least likely to vary under conditions that affect the expression of mRNA (Barbu and Dautry, 1989). The internal control allows the efficiency of the PCR reaction to be assessed and also enables comparison of levels of gene expression between different samples by normalising for different starting concentrations of cDNA. The primers and probe for ribosomal 18S were provided by Applied Biosystems.

Table 3.8.1 Real-time primer and probe sequences for interleukin-6 and proteolysis-inducing factor.

Gene	Sequence	Tm (°C)
Interleukin-6		
Forward primer	5'-AGC CCA CCG GGA ACG A-3'	60
Reverse primer	5'-AGG CGC TTG TGG AGA AGG A-3'	60
Probe	(FAM) 5'-CTC GCC TCC AGG AGC CCA GCT ATG-3' (TAMRA)	70
Proteolysis-inducing factor		
Forward primer	5'-CAA AAG GAA AAT GCA GGT GAA GA-3'	59
Reverse primer	5'-CTC CGT CTA GGC CTT TTT CCA-3'	59
Probe	(FAM) 5'-ACA GGC ACC AAA GCC AAG GAA GCA-3' (TAMRA)	69

PCR reaction conditions

Reactions were performed in 96-well optical reaction plates (Applied Biosystems). Primers were used at an initial concentration of 25 μM and probes were used at an initial concentration of 5 μM. 60 μl of forward primer, 60 μl of reverse primer, and 200 μl of probe

were added to 1014 μl of nuclease-free water. Following gentle mixing, 14 μl of this primer/probe mix was added to each well. Reactions were performed in 50 μl total volume, consisting of; 25 μl Taqman universal PCR master-mix (UNG x2), 14 μl primer/probe mix, 2.5 μl ribosomal 18S primer/probe mix (all reagents Applied Biosystems), 3.5 μl DEPC treated water, and 5 μl cDNA. Once all the reagents were added, the plate was covered and spun for 5 minutes in a plate spinner at 1000 rpm before being loaded into the PCR machine.

The TaqMan cytokine gene expression plate consisted of a 96-well optical reaction plate with each well pre-loaded with primers and probes for the detection of one cytokine cDNA target and an 18S endogenous control. Therefore, 25 μl Taqman universal PCR master-mix (UNG x2), 20 μl of nuclease-free water, and 5 μl of cDNA was added to each well. The plates were spun as above before loading into the TaqMan machine.

The cycler conditions were 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles with 15 seconds at 95°C and 1 minute at 60°C. Samples were analysed in duplicate and reactions were repeated if there were any discrepancies between the two results.

Positive controls, negative controls and quality control

Each plate contained negative controls (no cDNA) and positive controls. If the negative controls suggested contamination, all the samples on the 96-well plate were repeated. In addition, approximately 5-10 randomly selected cDNA samples from previously analysed reactions were run again on each plate to ensure that results were consistent between runs. Inter-assay variability between plates was less than 15%.

RNA derived from endotoxin-stimulated white blood cells was used as the positive control for the cytokine reactions. 10 ml of whole blood was collected from each of three healthy donors and mixed with equal volumes of phosphate-buffered saline (PBS). 15 ml of histopaque (Sigma, Dorset, UK) was pipetted into each tube to underlay the blood. The tubes were centrifuged at 1,000g for 20 minutes. The creamy interface was carefully pipetted from the 3 tubes and transferred to a single tube, which was filled with PBS and centrifuged at 3,000g for 7 minutes. The supernatant was discarded and the pelleted cells were resuspended in 10 ml culture medium (RPMI supplemented with L-glutamine (2 mmol/l), penicillin (5 units/ml), streptomycin (50 μg/ml), β-mercaptoethanol, and 10% fetal calf serum).

The cells were counted by trypan blue exclusion and additional culture medium was added to give a final concentration of approximately 1 x 10^6 cells per ml. The cells were cultured in lipo-polysaccharide (LPS) and Con A (Sigma, Dorset, UK) each at a concentration of 1 µg/ml for 48 hours before isolation of the RNA.

Total RNA was isolated using Trizol (Gibco BRL, Paisley, UK). The culture medium was poured off and 1 ml of trizol was added for each 10 cm^2 of well surface area and incubated for 5 minutes. The cells were scraped off the well surface using a sterile pipette tip and the mixture was incubated for a further 5 minutes. The mixture was then transferred into microcentrifuge tubes and 200 µl of chloroform was added and the tubes were shaken vigorously for 15 seconds. The tubes were then centrifuged for 15 minutes at 12,000g. After spinning, the aqueous phase was carefully transferred to a clean tube and 500 µl of isopropanol was added and the tubes were left to stand at room temperature for 10 minutes, before centrifuging again at 12,000g for 10 minutes. The supernatant was carefully removed and the pellet was washed with 1 ml of 70% ethanol. After a final spin at 7,500g for 5 minutes the RNA pellet was resuspended in nuclease-free water. The final RNA concentration was 210 µg/ml. The total RNA was reverse transcribed as described above and the total cDNA was mixed then aliquoted into separate tubes each containing 20 µl of the stock cDNA. Tubes were stored at –20°C until use. Each real-time reaction used an aliquot from the stock solution of cDNA as a positive control.

The MIA PaCa-2 pancreatic cancer cell line was used as a positive control for PIF analysis. Previous work in our Laboratory had identified that this cell line synthesised PIF mRNA (personal communication). The cells were obtained from the Public Health Laboratory Service Centre for Applied Research (Porton Down, UK). Cells were cultured in Dulbecco's modification of Eagle's medium (Life Technologies, Inchinan, UK) supplemented with L-glutamine, penicillin, streptomycin and fetal calf serum (as above) at 37°C in 95% humidified air-5% CO_2. RNA isolation and reverse transcription were performed as already described. The total RNA concentration was 1.7 µg/ µl. Once again 20 µl aliquots of cDNA were stored at –20°C until use.

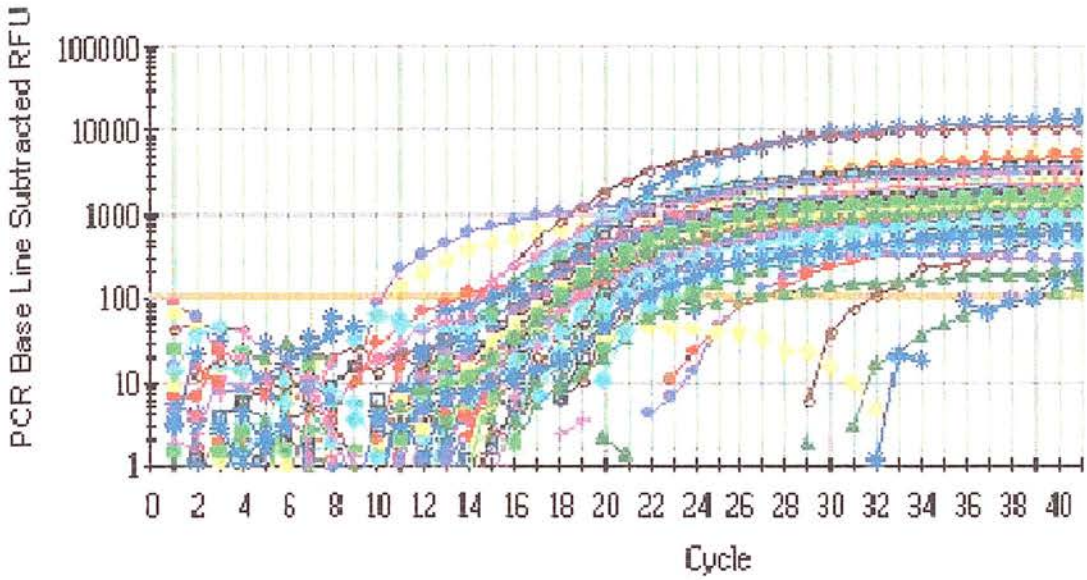
Quantification of gene expression

Quantification of gene expression was calculated using the comparative ($\Delta\Delta C_T$) method, where samples were compared with the positive control (Bustin, 2000). The threshold cycle (C_T) is defined as the number of cycles required for the level of fluorescence to rise significantly above the background level (maximum = 40) (Figure 3.8.3). Lower C_T values represent higher initial copy numbers of the gene. The C_T value is generated automatically by the real-time machine and two values per reaction well are given; one for FAM (target gene of interest) and he other for VIC (the internal control, 18S). Samples that generated cycle numbers above 23 for the endogenous control were discarded and the samples were repeated. Similarly, cycle numbers greater than 36 for the gene of interest were considered to have no expression of the gene.

The level of gene expression within each sample was expressed as percentages of the level of gene expression within the control sample. Analysis was performed as follows; the average C_T FAM and C_T VIC values for each sample were calculated and the mean VIC C_T values were subtracted from the mean FAM C_T values (FAM C_T - VIC C_T = ΔC_T). $\Delta\Delta C_T$ was calculated by subtracting the ΔC_T value of the positive control sample from the ΔC_T value for each sample (ΔC_T - ΔC_T control = $\Delta\Delta C_T$). The relative level of expression, normalised to the endogenous control and relative to the positive control, is then calculated by the following formula: $2^{-\Delta\Delta CT}$. The level of gene expression of the positive control was assigned an expression value of 1 and levels of gene expression in the samples were expressed as percentages of the control level.

To ensure inter-plate reproducibility, samples were repeated on different plates and serial dilutions of the positive control cDNA were performed. Reproducibility was between 3.7-15.5%.

Figure 3.8.3 An amplification plot generated following real-time PCR of cDNA samples obtained from patients with gastro-oesophageal cancer. In this example TNFα is the gene of interest. Each colour on the graph represents a single well on the 96-well plate. The threshold cycle (C_T) number can be read from the graph and the level of TNFα expression may be determined.



c) Tissue cytokine protein measurement

Extraction of tissue protein

Tissue samples were collected and stored as described previously. Sections of frozen tissue were cut and tissues were disrupted using a hand-held electric homogeniser. Care was taken to avoid tissue thawing prior to homogenisation. Tissue lysates were prepared by homogenising 50 mg of tissue in 400 μl tissue homogenising buffer (0.4ml 500mM Tris, 0.2 ml 100 mM ATP, 1 ml 50 mM $MgCl_2$, 10 μl dithiothreitol (DTT), 1x protease inhibitor, 8.4 ml water

– Sigma, Dorset, UK). Samples were heated to 95°C for 5 minutes before centrifuging at 13,000 rpm for 30 minutes at 4°C. The supernatants were transferred to fresh Eppendorf tubes and the protein concentrations were determined by the Bradford method (Bio-Rad, Hemel Hempstead, UK) (Bradford, 1976). Mean protein concentrations were 17.0 mg/ml (S.D.=11.3 mg/ml). Samples were stored at -80°C until further analysis.

Determination of tissue cytokine protein concentrations

Cytokine protein concentrations were determined using the Cytometric Bead Array System according to the manufacturers instructions (Human Inflammation Kit, BD Biosciences, Oxford, UK). This kit allows the simultaneous measurement of concentrations of the following cytokines; IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNFα. The system combines flow cytometry and sandwich ELISA for the determination of cytokine concentrations. Advantages include increased sensitivity, smaller reaction volumes and simultaneous measurement of six cytokines during the same reaction (multiplexing). Six beads coated with cytokine antibodies are mixed with the protein supernatant. Each bead-coated antibody fluoresces at an individual wavelength, which is read by a flow cytometer (BD FACScan; BD Biosciences, UK) and cytokine concentrations are determined from a standard curve.

Standards were prepared by serial dilutions according to the manufacturers instructions. 50 μl of neat tissue extract was added to the reaction mix containing 50 μl of the antibody-coated microbeads and 50 μl of the PE detection reagent. Samples were incubated at room temperature for 3 hours in the dark. Following incubation, 1 ml of wash buffer was added and tubes were centrifuged at 200g for 5 minutes. The supernatant was carefully aspirated and discarded and the pellet was resuspended in 100 μl of wash buffer. Cytokine concentrations were determined by flow cytometry (BD FACScan, Oxford, UK). Care was taken to gently vortex the tubes immediately prior to analysis. Results were calculated to take into account the total protein concentration of the tissue lysate and are expressed as pg/mg of total protein. Assay sensitivities, intra-assay variability, and inter-assay variability are shown in Table 3.8.2.

Table 3.8.2 Assay sensitivities, intra-assay variability, and inter-assay variability of rindividual cytokines using the Cytometric Bead Array System (BD Biosciences, Oxford, UK).

Cytokine	Sensitivity (pg/ml)	Intra-assay variability (%)	Inter-assay variability (%)
IL-1β	7.2	5.7	10
IL-6	2.5	6.3	9
IL-8	3.6	3.7	5
IL-10	3.3	5.7	9.3
IL-12p70	1.9	3.7	7.3
TNFα	3.7	8.3	12

Chapter Summary

This Chapter has described the main materials and methods undertaken during the completion of this thesis. It describes the processes of patient recruitment and the timings of data recording and sample collection. It lists the clinical, pathological and investigative variables collected on each patient, including the assessment of nutritional status and performance status. It provides detailed methodology regarding the various laboratory procedures performed, such as measurement of serum cytokines and acute phase protein concentrations. Techniques relating to the isolation of genomic DNA and cytokine genotyping are described. The processes of tissue collection and measurement of tissue cytokine mRNA and protein concentrations are outlined. Finally, the methods used in the identification of tumour-derived products (PIF and PTHrP) are described as well as the difficulties encountered by these techniques.

The following Chapters in his thesis describe the results of these methods and a discussion relating to each of these results is provided at the end of every Chapter. Firstly, the prevalence of systemic inflammation in the cohort of patients with gastro-oesophageal cancer

will be explored (Chapter IV). Investigation into the aetiology of such systemic inflammation in these patients will examine the contribution of the tumour tissue. Tissue cytokine concentrations will be measured and an assessment of host immune cell infiltrates into the tissues will be performed. These values will then be correlated with systemic inflammatory markers (Chapter V). The influence of host cytokine genotype on cytokine concentrations in the tumour tissue and systemic circulation will be investigated, and any additional value that these genotypes may have on prognosis will be explored (Chapter VI). The role of tumour-derived products, PTHrP and PIF, towards the generation of systemic inflammation, adverse nutritional status and prognosis among these patients will be investigated (Chapters VII, and VIII). The association between these elevated markers of systemic inflammation and adverse prognosis will then be investigated (Chapter IX). Changes in nutritional status in patients with gastro-oesophageal cancer will be documented and any association between systemic inflammation and cachexia will be determined. In addition, the role of cachexia as an aetiological factor in the link between systemic inflammation and adverse prognosis in patients with gastro-oesophageal cancer will be investigated (Chapter X). Finally, the usefulness of systemic inflammation as a prognostic indicator, and the role of cachexia as a contributory factor, will be used to develop a novel prognostic model to aid clinical management decisions for patients with gastro-oesophageal cancer (Chapter XI).

CHAPTER IV

PREVALENCE OF SYSTEMIC INFLAMMATION AT DIAGNOSIS AND FOLLOWING TREATMENT IN PATIENTS WITH GASTRO-OESOPHAGEAL CANCER

ABSTRACT

This Chapter investigated the prevalence of systemic inflammation in patients with gastro-oesophageal cancer. Serum cytokine and acute phase protein concentrations were measured as markers of systemic inflammation and their concentrations were related to numerous demographic, clinical, and pathological data. The effects of treatment and disease progression on levels of expression of these markers were also explored.

Patients newly diagnosed with gastric or oesophageal cancer were recruited at the time of diagnosis. Blood was collected from patients and healthy controls for determination of serum cytokine (IL-1β, IL-6, IL-8, IL-10, and soluble TNF receptor – sTNF-R) and serum acute phase protein (CRP, haptoglobin, α1-antichymotrypsin, transferrin, and albumin) concentrations by ELISA. Serum concentrations were compared with controls and with various demographic, clinical and pathological data. Further samples were collected at different time points following initiation of treatments and later in the course of the disease.

Two hundred and twenty patients were studied. Serum positive acute phase protein concentrations were significantly elevated compared with controls and at the time of diagnosis 43% of patients with gastro-oesophageal cancer had evidence of systemic inflammation (CRP concentration greater than 10 mg/l). The presence of systemic inflammation was associated with advanced disease stage, poor performance status, increasing age, and female sex. Surgical resection was associated with an increase in serum CRP concentrations and pre-operative chemotherapy was associated with a reduction in CRP concentrations. In contrast, serum cytokines demonstrated little association with clinical and pathological variables or with serum acute phase protein concentrations, except for sTNF-R, which appears to behave like an acute phase reactant. IL-1β and IL-10 concentrations were generally not detectable in the serum of patients or healthy controls.

This study confirms systemic inflammation is present in patients with gastro-oesophageal cancer. Investigation into the aetiology of systemic inflammation and the association between systemic inflammation and adverse prognosis in patients with gastro-oesophageal cancer is explored in later Chapters.

INTRODUCTION

This Chapter investigated the prevalence of systemic inflammation in a cohort of patients with gastro-oesophageal cancer. Serum cytokine and acute phase protein concentrations were measured as markers of systemic inflammation in the study population at the time of diagnosis and later in the course of the disease. The effects of treatment and disease progression on the levels of expression of these markers were explored. Comparisons were also made between elevated markers of systemic inflammation and various clinical, pathological and demographic data. Such information may then be used to investigate the aetiology of systemic inflammation in these patients and to explore the association between systemic inflammation and adverse prognosis.

Elevated serum pro-inflammatory cytokine concentrations have been identified in association with a number of animal tumour models (Oliff et al, 1987; Strassmann et al, 1992; Ghelin et al, 1991; McIntosh et al, 1989) and various cancer cell lines have also demonstrated an ability to produce pro-inflammatory cytokines (Wigmore et al, 2002; Wigmore et al, 2001; Wigmore et al, 1997). Elevated markers of systemic inflammation have also been measured in human cancer patients. Increased pro-inflammatory cytokine concentrations have been measured in sera from patients with cancer and elevated concentrations of IL-1β, IL-6, IL-8 and TNFα have repeatedly been measured in blood collected from patients with gastro-intestinal malignancy, including gastric and oesophageal cancers (Barber et al, 1999; Wang et al, 1999; Kabir and Daar, 1995). However, results and conclusions of these studies are often inconsistent and contradictory making meaningful conclusions difficult. Such inconsistent findings are likely to be related to the difficulties in reliably measuring serum cytokine concentrations, as previously described (Muc-Wierzgon et al, 2003). In addition, the end-organ effects of these systemic cytokines will depend upon binding to transport proteins, receptor expression and receptor affinity. These findings have raised doubt as to the significance of circulating concentrations of pro-inflammatory cytokines as markers of systemic inflammatory activity. In contrast, serum concentrations of acute phase proteins (APPR) demonstrate more stability within the systemic compartment and may be a more reliable marker for the presence of systemic inflammation in patients (Gabay and Kushner,

1999). Up to 50% of patients with cancer have an APPR at the time of diagnosis, including patients with upper gastro-intestinal malignancy (Chen et al, 1999; Alexandrakis et al, 2003; Barber et al, 1999; Falconer et al, 1994; Falconer et al, 1995). In patients with cancer circulating concentrations of IL-6 have been shown to correlate with markers of systemic inflammation such as CRP (Barber et al, 1999). The presence of such an acute phase reaction may then be used as an indirect marker of increased pro-inflammatory cytokine activity and serum concentrations of acute phase proteins may, therefore, be used to confirm the presence of systemic inflammation.

MATERIALS AND METHODS

Study Population

All patients diagnosed with gastric or oesophageal cancer within the Lothian and Borders regions between March 2002 and June 2004 were eligible for inclusion into the study. No patients were excluded and subjects were recruited to the study within 2 weeks of diagnosis. All patients had histological confirmation of their disease following endoscopic biopsy. Data were prospectively collected. Patients were staged as per Department policy with a combination of computerised tomography (CT), endoscopic ultrasound (EUS) and laparoscopy/laparoscopic ultrasound (LUS) according to the International Union Against Cancer (UICC) (Sobin and Wittekind, 2003), and final histopathological stage (pTNM) was used when available. Final stage (pTNM) was only available for those patients who underwent surgical resection. In all other cases the final clinical stage (cTNM), as agreed at the unit multidisciplinary team meeting (MDT), was recorded.

Clinical information such as duration of symptoms, smoking habit and patient age and sex were recorded in every case. Performance status was assessed using the Karnofsky index (Karnofsky and Burchenal, 1949). Pathological information was also recorded for each patient, including treatment modality, tumour site, histological type, and tumour grade. In every case documentation of the use of non-steroidal anti-inflammatory drugs (NSAIDs) and any other therapeutic agents that may influence the inflammatory response was recorded.

Twenty-two healthy controls were also recruited to the study and provided blood samples for determination of serum cytokine and acute phase protein concentrations. This group comprised 15 (68%) males with a mean age 31 years (range 22-56 years).

Measurement of serum acute phase protein and cytokine concentrations

Whole blood was collected from each patient for determination of serum acute phase protein concentrations and serum cytokine concentrations. C-reactive protein (CRP), haptoglobin, and α1-antichymotrypsin (ACT) were chosen as representatives of the positive acute phase response, and albumin and transferrin were selected as negative acute phase proteins. Pro-inflammatory cytokines IL-1β, IL-6 and IL-8 were also measured. Soluble tumour necrosis factor receptor (60 kDa) (sTNF-R) was measured as an indirect marker of circulating TNFα levels (Spinas et al, 1992). IL-10 was chosen as an anti-inflammatory cytokine. Serum acute phase protein concentrations and serum cytokine concentrations were determined by ELISA, as previously described in the materials and methods Chapter (Chapter III).

Sample collection

Blood samples were collected within two weeks of diagnosis of cancer and before any treatment was commenced. All patients were free from systemic infection at the time of blood collection. A second serum CRP measurement was undertaken a few days later in a subgroup of patients at the commencement of the study to investigate the stability of CRP concentrations. In addition, a further blood sample was collected from patients at a later stage in the disease process to investigate any variation in serum acute phase protein concentrations that may occur due to the disease progression or treatment intervention. The second follow-up samples were collected in 77 (35%) patients after a mean time interval of 95 days (range 41-151 days) from the time of the original measurement. Between the initial and follow-up measurements 48 patients had undergone surgical resection, 20 patients had received pre-operative chemotherapy but had not yet undergone surgery, and 9 patients had been palliated by non-surgical methods (for example, stenting). These measurements were performed a minimum of 92 days following surgery and a minimum 41 days following completion of pre-operative chemotherapy.

Statistics

Non-parametric continuous variables were analysed by the Spearman rank test and independent variables were analysed by the Mann-Whitney U test or the Kruskal-Wallis test. Categorical data was analysed by the Chi-square test. The paired sample T-test was used to compare serial measurements for each patient.

RESULTS

1. The Study Population – Patient demographics

Two hundred and twenty patients were recruited to the study between March 2002 and June 2004. Patient demographics are shown in Table 4.1. The median age was 71 years (range 26-95 years, inter-quartile range 62-78 years) and two-thirds of patients were male. Seventeen (8%) patients were asymptomatic at the time of diagnosis and were diagnosed following endoscopic investigation of iron deficiency anaemia. Most patients (40%) were diagnosed within 2 months of the onset of symptoms, such as dysphagia or epigastric pain. 23 (10%) patients had experienced symptoms for at least 6 months before a diagnosis was made. Reasons for diagnostic delay were mostly due to reluctance of the patient to seek medical help. Approximately one-third of patients had a normal performance status at the time of diagnosis (Karnofsky score 100) and another third of patients were able to undertake normal activities but with some degree of effort (Karnofsky 80 or 90). Ten (5%) patients required frequent medical care or hospitalisation (Karnofsky 30-50).

Table 4.1 Study patient demographics (n=220)

		Number [%]
Age (years) [+]		71 (62-78)
Sex	Male	145 [66]
	Female	75 [34]
Smoker	Current	63 [29]
	Ex-smoker	68 [30]
	Never	89 [41]
Symptom duration	Asymptomatic	17 [8]
	<2 weeks	15 [7]
	2-4 weeks	25 [11]
	1-2 months	48 [22]
	2-4 months	51 [23]
	4-6 months	41 [19]
	>6 months	23 [10]
Karnofsky score	30	4 [2]*
	40	1 [1]
	50	5 [2]
	60	17 [8]
	70	25 [12]
	80	36 [17]
	90	50 [24]
	100	70 [34]
	Unknown	12
Tumour Site	Oesophageal	101 [46]
	Proximal third	2
	Middle third	13
	Distal third	86
	Oesophago-gastric junction	40 [18]
	Gastric	79 [36]
	Proximal	25
	Body	26
	Distal	28
Histology	Adenocarcinoma	185 [84]
	Squamous cell carcinoma	30 [14]
	Small cell	2 [1]
	Indeterminate	3 [1]
Grade	Well differentiated	3 [2]*
	Moderately differentiated	63 [34]
	Poorly differentiated	118 [64]
	Not commented	36
Helicobacter pylori	Present	13 [12]*
	Absent	95 [88]
	Not recorded	112
Treatment undertaken	Surgery alone	70 [32]
	Pre-operative chemotherapy/Surgery	25 [11]
	Chemoradiotherapy with curative intent	7 [3]
	Palliative Chemotherapy	28 [13]
	Palliative Radiotherapy	6 [3]
	Stent/dilatation/laser/symptomatic	84 [38]
UICC Stage	1	25 [11]
	2	34 [16]
	3	86 [39]
	4	75 [34]
Status	Alive	73 [33]
	Dead	147 [67]

[+]Values are median (interquartile range). * Values are expressed as percentages of known results.

The primary tumour sites were oesophageal (n=101, 46%), gastric (n=79, 36%) and those arising from the gastro-oesophageal junction [OGJ] (n=40, 18%). Most oesophageal tumours were located in the distal third, whereas the gastric tumours were equally distributed throughout the stomach. Approximately two-thirds (69%) of all the cancers occurred in or around the gastro-oesophageal junction (distal oesophagus, OG junction, or proximal stomach) reflecting the increasing incidence of tumours located in this area.

Adenocarcinoma was the commonest histological type overall (n=185; 84%), including oesophageal cancers alone (70%). Most tumours were poorly differentiated (n=118; 64%). The presence of Helicobacter pylori was recorded only for those patients who underwent surgical resection or where status was documented if the bacterium was noted on the biopsy specimen. Data were available for 108 patients and only 13 (12%) patients were noted to be positive. Prior treatment with eradication therapy was not known.

95 (43%) patients underwent surgical resection (Ivor-Lewis two-stage oesophago-gastrectomy, left thoraco-abdominal resection, transhiatal oesophageactomy, partial gastrectomy, or total gastrectomy) and 25 of these received pre-operative chemotherapy. All patients had a potentially curative resection except 5 patients, 3 patients had unexpected metastatic disease identified at laparotomy and therefore did not proceed to resection and 2 patients had residual macroscopic disease (R2 resection). Seven (3%) patients, all of whom had squamous cell carcinoma of the oesophagus, received chemo-irradiation with curative intent. The remaining patients (n=118; 54%) were not suitable for curative therapy and received palliative chemo/radiotherapy or underwent alternative palliative treatments, such as insertion of a stent, endoscopic dilatation, or endoscopic laser therapy.

One-third (34%) of patients had metastatic disease (stage IV) at the time of presentation and most other patients (n=86; 39%) had locally advanced (stage III) disease at diagnosis.

Patients were followed-up for an average 32 months (range 18-45 months). At the time of censoring the data 147 (67%) patients had died. Three patients died in the post-operative period and one patient died from injuries sustained following a fall. Information obtained from death certificates indicated that all other deaths were disease related. Overall median survival was 12.6 months.

2. Serum markers of systemic inflammation

a) Serum acute phase protein concentrations

Comparison with healthy controls

The serum acute phase protein concentrations of patients and healthy controls are shown in Table 4.2. When compared with healthy controls, at the time of diagnosis cancer patients had significantly elevated serum concentrations of positive acute phase proteins (CRP, $p<0.001$; α1-antichymotrypsin, $p<0.001$; and haptoglobin, $p<0.001$; Mann-Whitney U test), but similar concentrations of negative acute phase reactants (albumin, $p=0.290$ and transferrin, $p=0.477$). The median CRP concentration was 7 mg/l (interquartile range 2-25 mg/l) and at the time of diagnosis 95 (43%) patients had a CRP concentration greater than 10 mg/l. This value is used as the definition of systemic inflammation in this thesis.

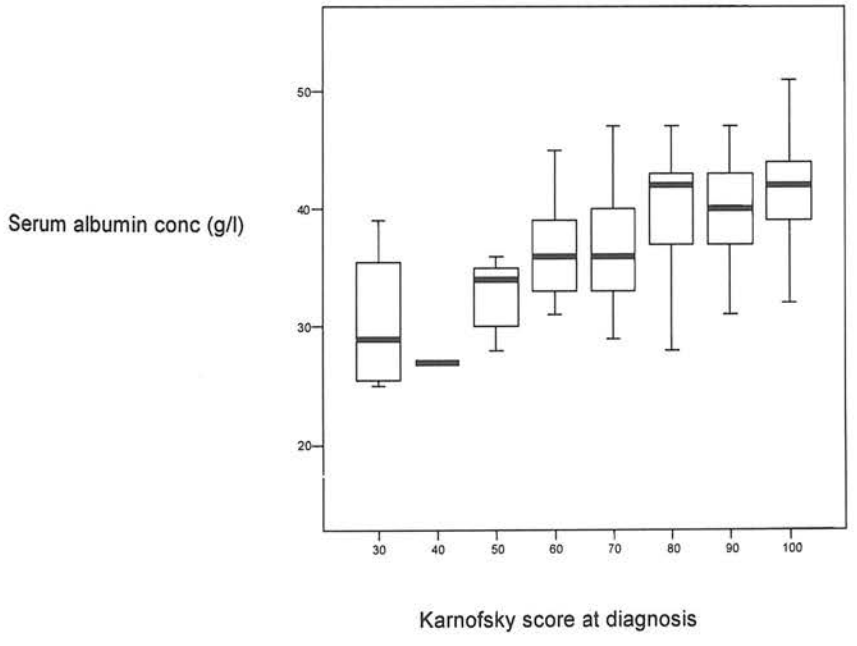
Table 4.2 Comparison of serum acute phase protein concentrations between healthy controls and cancer patients at the time of diagnosis. Cancer patients had significantly elevated serum concentrations of positive acute phase proteins, but similar concentrations of negative acute phase reactants. *Mann-Whitney U test. Median (inter-quartile range).

	Controls (n=22)	Patients (n=220)	P value*
CRP (mg/l)	1 (1-2)	7 (2-25)	<0.001
α1-Antichymotrypsin (mg/l)	245 (213-261)	446 (359-585)	<0.001
Haptoglobin (mg/l)	821 (627-1157)	2264 (1518-2950)	<0.001
Albumin (g/l)	40 (39-42)	40 (36-43)	0.290
Transferrin (mg/l)	2197 (1861-2451)	2056 (1550-3138)	0.477

Comparison with patient demographics and clinical and pathological characteristics

Increasing patient age at the time of diagnosis was associated with increasing serum CRP concentration ($p=0.009$, $r= 0.18$; Spearman rank) and α1-antichymotrypsin concentration ($p=0.016$, $r= 0.17$) and with reduced serum albumin concentrations ($p<0.001$, $r= -0.32$). Similarly, female sex was also associated with elevated serum CRP ($p=0.062$; Mann-Whitney U test) and α1-antichymotrypsin ($p=0.005$) concentrations and reduced albumin concentration ($p=0.022$). Smoking status was not associated with serum acute phase protein concentrations. Karnofsky performance score was associated with serum acute phase protein concentrations (Figure 4.1). A reduced performance status was associated with increased serum CRP ($p<0.001$; Kruskal Wallis test) and α1-antichymotrypsin ($p=0.003$) concentrations, and reduced albumin ($p<0.001$) and transferrin ($p=0.005$) concentrations. Acute phase protein concentrations were similar between those patients taking NSAID's or other therapeutic agents (for example, steroids) that may modify the inflammatory response and patients who were not (data not shown).

Figure 4.1 Karnofsky performance score was associated with serum acute phase protein concentrations. A reduced performance status was associated with an elevated acute phase response ($p<0.001$; Kruskal Wallis test).

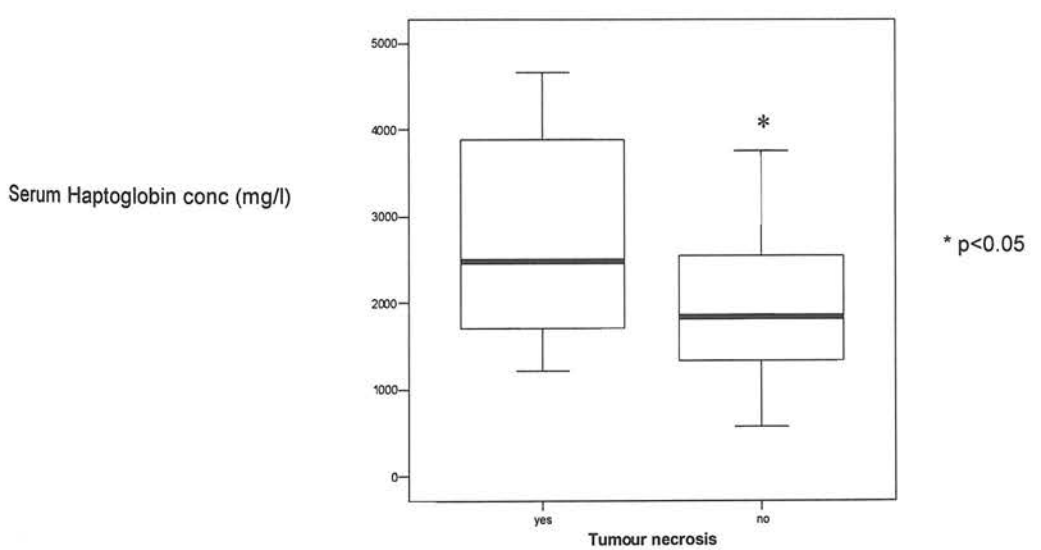


Advanced disease stage was associated with increased concentrations of the positive acute phase reactants CRP ($p<0.001$; Kruskal Wallis test), α1-antichymotrypsin ($p=0.050$) and haptoglobin ($p=0.023$), and reduced concentrations of albumin ($p=0.027$), but not transferrin ($p=0.403$). In addition, poorly differentiated tumours were associated with significantly elevated serum concentrations of positive acute phase proteins (CRP, $p<0.001$; α1-antichymotrypsin, $p<0.001$; haptoglobin, $p=0.025$; Mann-Whitney U test), but not with the negative acute phase reactants (albumin, $p=0.151$; transferrin, $p=0.141$).

The histological presence of tumour necrosis and abscess formation was documented in 54 resection specimens. Tumour necrosis was evident in 13 (24%) of these samples and was associated with elevated serum haptoglobin concentrations ($p=0.045$; Mann-Whitney U test) and CRP concentrations, but this did not quite reach significance ($p=0.07$) (Figure 4.2).

Neither the site of the primary tumour (oesophageal, gastric or junctional) nor histological subtype (adenocarcinoma or squamous cell carcinoma) were associated with serum acute phase protein concentrations. Similarly, serum acute phase concentrations were not associated with Helicobacter pylori infection.

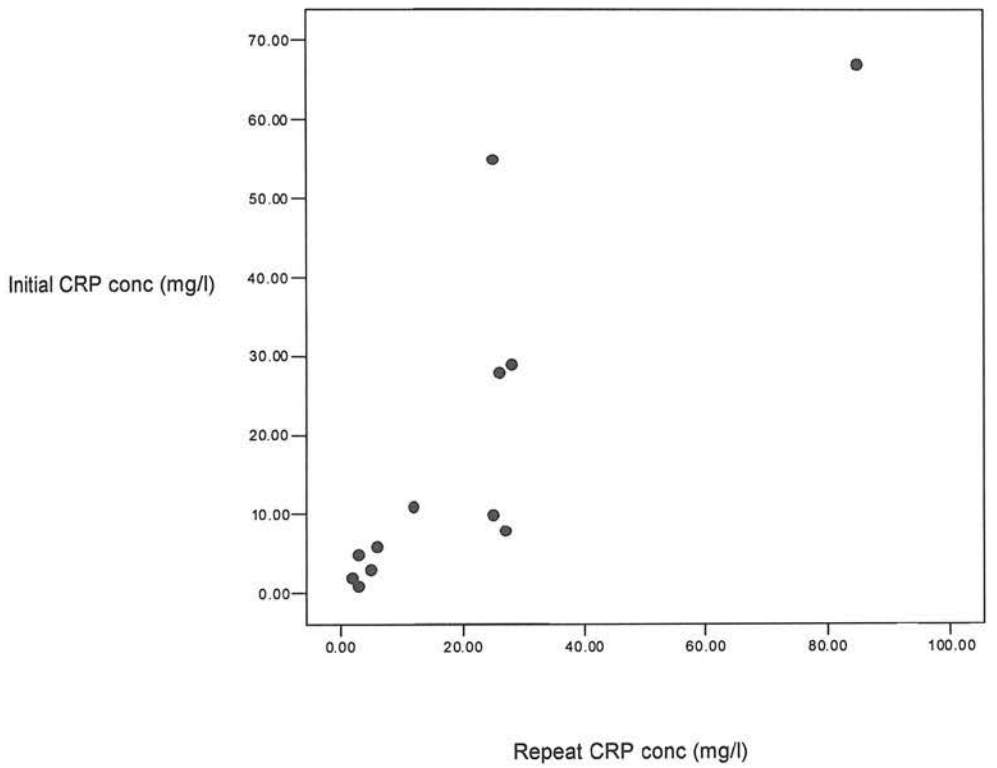
Figure 4.2 Tumour necrosis was evident in 13 out of 54 (24%) resected specimens. Serum haptoglobin concentrations were significantly higher in patients with histological evidence of necrosis present in their resected specimen ($p=0.045$; Mann-Whitney U test).



Stability of serum acute phase protein measurements

A repeat serum CRP measurement was undertaken for the initial patients recruited to the study (n=12) in order to investigate the stability of CRP concentrations. This second sample was collected at a median time interval of 7 days (inter-quartile range 3-16 days) following the collection of the first sample. All samples were collected prior to the initiation of treatment. There was good correlation between the two measurements ($p<0.0001$, $r= 0.87$; Spearman rank test) (Figure 4.3).

Figure 4.3 A scatter plot representing the correlation between CRP concentrations measured a median 7 days apart (n=12) [$p<0.0001$, $r= 0.87$; Spearman rank test].



Serial measurements of acute phase proteins

A second CRP and albumin measurement was performed in 77 (35%) patients after an average 95 days (range 41-151 days) from the time of the original measurement at diagnosis. Between the initial and follow-up measurements 48 patients had undergone surgical resection, 20 patients had received pre-operative chemotherapy but had not yet undergone surgery, and 9 patients had been palliated by non-surgical methods (for example, stenting). These measurements were performed a minimum of 92 days following surgery and a minimum 41 days following completion of pre-operative chemotherapy.

Over the average 95-day time interval there was a significant increase in paired serum CRP concentrations among those patients who had undergone surgical resection [median CRP at diagnosis (pre-operative) = 3 mg/l versus median CRP 95 days later (post-operative) = 9 mg/l; p=0.03, 95% confidence interval –23.0 to –1.1; Paired T test] (Table 4.3). This was associated with a significant decrease in serum albumin concentrations [median albumin at diagnosis (pre-operative) = 42 g/l versus median albumin 95 days later (post-operative) = 38 g/l; p<0.001, 95% confidence interval 2.7 to 5.9] (Table 4.3).

In contrast, patients who had received pre-operative chemotherapy, but who had not yet undergone surgery, experienced a significant reduction in paired serum CRP concentrations [median CRP at diagnosis (pre-chemotherapy) = 5 mg/l versus median CRP 95 days later (post-chemotherapy) = 2 mg/l; p=0.05, 95% confidence interval 0 to 12.2]. However, there was no significant change in serum albumin concentrations among these patients [median albumin at diagnosis (pre-chemotherapy) = 44 g/l versus median albumin 95 days later (post-chemotherapy) = 42 g/l; p=0.40, 95% confidence interval –1.3 to 3.2].

There were no significant changes in either CRP or albumin concentrations among those patients who were treated by palliative means alone (see Table 4.3).

Table 4.3 Serial measurements of serum CRP and albumin concentrations stratified according to intervening therapeutic intervention. CRP/albumin 1 = serum CRP/albumin concentration at diagnosis. CRP/albumin 2 = serum CRP/albumin concentration measured an average 95 days later and following initiation of treatment. CRP concentrations increased and albumin concentrations decreased in those patients who had undergone surgical resection. In contrast, CRP concentrations decreased in those patients who received pre-operative chemotherapy between CRP 1 and CRP 2, but without any significant changes in serum albumin concentrations. There were no significant changes in either CRP or albumin concentrations in those patients who were treated by palliative methods.

	CRP 1 (mg/l)	CRP 2 (mg/l)	95% CI of the difference	P value*	Albumin 1 (g/l)	Albumin 2 (g/l)	95% CI of the difference	P value*
Surgery (n=48)	3 (2-8)	9 (2-34)	-23.0 to -1.1	0.03	42 (40-44)	38 (33-40)	2.7 to 5.9	<0.001
Pre-operative chemotherapy (n=20)	5 (1-16)	2 (1-6)	0 to 12.2	0.05	44 (40-46)	42 (38-46)	-1.3 to 3.2	0.40
Palliation (n=9)	16 (5-22)	7 (6-21)	-17.1 to 36.8	0.42	39 (34-42)	41 (37-42)	-5.9 to 3.9	0.65

Median (inter-quartile range). * Paired T-test.

b) Serum cytokine concentrations

Comparison with healthy controls

The serum cytokine concentrations of patients and healthy controls are shown in Table 4.4. IL-1β and IL-10 were not detectable in the serum of any of the controls and were measured in only 4 (2%) and 10 (5%) patient's serum respectively. Serum concentrations of IL-6 and IL-8 were similar between patients and controls (p=0.80 and p=0.91, respectively; Mann-Whitney U test). Serum soluble tumour necrosis factor receptor (sTNF-R) concentrations were

elevated in cancer patients compared with healthy controls, but this did not quite reach statistical significance (p=0.056; Mann-Whitney U test). Given the low level of expression, IL-1β and IL-10 were excluded from subsequent analysis.

Table 4.4 Comparison of serum cytokine concentrations between cancer patients and healthy controls.

	Controls (n=22)	Patients (n=220)	P value*
Interleukin-1β (pg/ml)	0 (0-0)	0 (0-0)	-
Interleukin-6 (pg/ml)	11.2 (0-214)	8.7 (0-88)	0.911
Interleukin-8 (pg/ml)	0 (0-118)	0 (0-115)	0.799
Interleukin-10 (pg/ml)	0 (0-0)	0 (0-0)	-
Soluble TNF receptor (ng/ml)	2.8 (1.3-3.6)	3.3 (1.9-5.8)	0.056

*Mann-Whitney U test. Values are median (inter-quartile range).

Comparison with patient demographics and clinical and pathological characteristics

Similar to patterns of acute phase protein expression, elevated sTNF-R concentrations correlated with increasing patient age (p<0.001; Spearman rank), female sex (p=0.05; Mann-Whitney U test), and reduced Karnofsky performance scores (p<0.001; Kruskal-Wallis test). In contrast to acute phase protein concentrations, sTNF-R concentrations were similar between patients irrespective of duration of symptoms prior to diagnosis. IL-6 and IL-8 were not associated with or anti-inflammatory drug consumption or any other demographic data (data not shown for simplicity).

In contrast with serum acute phase protein concentrations, serum cytokine concentrations were not associated with stage of disease or the presence of tumour necrosis. However, sTNF-R concentrations were significantly elevated in patients with poorly differentiated tumours (p=0.002; Mann-Whitney U test). Elevated serum sTNF-R concentrations were also associated with gastric tumours (p=0.046; Kruskal-Wallis test) when compared with serum concentrations in patients with oesophageal or OG junctional tumours (Table 4.5) and elevated serum IL-6 concentrations were associated with oesophageal

tumours (p=0.050). In addition, serum IL-6 concentrations were increased in patients with squamous cell histology [SCC median concentration = 51.6 pg/ml (inter-quartile range 1.2-194.4 pg/ml) versus ACC median concentration = 4.8 pg/ml (inter-quartile range 0-69.5 pg/ml); p=0.005; Kruskal-Wallis test]. Serum cytokines concentrations were not associated with Helicobacter pylori status (data not shown).

Table 4.5 Comparison of serum sTNF-R and IL-6 concentrations according to tumour site.

	Tumour site			P value*
	Oesophagus (n=101)	OG junction (n=40)	Gastric (n=79)	
IL-6 (pg/ml)	23.7 (0-114)	0 (0-28)	8.9 (0-83)	0.050
sTNF-R (ng/ml)	2.9 (1.8-5.3)	2.8 (1.4-6.2)	4.1 (2.4-7.8)	0.046

* Kruskal-Wallis test. Values are median (inter-quartile range).

c) Relationship between serum cytokine concentrations and serum acute phase protein concentrations

The correlations between serum cytokine concentrations and serum acute phase protein concentrations are shown in Table 4.6. Concentrations of serum sTNF-R correlated with all the acute phase protein concentrations and IL-6 and IL-8 concentrations. Serum IL-6 concentrations were positively correlated with serum haptoglobin (p=0.020, r=0.16; Spearman rank) and were negatively correlated with serum albumin concentrations (p=0.055, r=-013). IL-6 concentrations also correlated with and serum IL-8 concentrations (p<0.001, r=0.52). Serum IL-8 concentrations did not correlate with any of the serum acute phase proteins. IL-1β and IL-10 were excluded from analysis due to the low levels of expression.

Table 4.6 Correlations between serum cytokine concentrations and serum acute phase protein concentrations (r = correlation co-efficient; Spearman rank analysis).

	CRP		Haptoglobin		ACT		Albumin		Transferrin		IL-8		sTNF-R	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
IL-6	0.09	0.185	0.16	0.020	0.12	0.085	-0.13	0.055	-0.04	0.586	0.52	<0.001	0.23	0.001
IL-8	0.05	0.513	0.08	0.217	0.04	0.566	-0.01	0.907	-0.9	0.881	-	-	0.16	0.019
sTNF-R	0.41	<0.001	0.20	0.003	0.39	<0.001	-0.38	<0.001	-0.18	0.007	-	-	-	-

CRP = C-reactive protein, ACT = α1-antichymotrypsin

Discussion

At the time of diagnosis patients with gastro-oesophageal cancer had significantly elevated serum concentrations of positive acute phase proteins and soluble TNF receptor concentrations when compared with healthy controls, but similar concentrations of negative acute phase proteins and serum cytokines. Forty-three percent of patients had evidence of systemic inflammation (CRP >10 mg/l) at diagnosis. Elevated acute phase protein and sTNF-R concentrations, but not cytokine concentrations, were associated with reduced Karnofsky performance scores, poorly differentiated tumours, and advanced disease stage. Soluble TNF-R concentrations correlated with serum acute phase protein concentrations, but there was little correlation between the other serum cytokines and acute phase proteins. Surgical resection was associated with an increase in CRP concentrations and pre-operative chemotherapy was associated with a reduction in serial serum CRP concentrations.

Patient demographics

The patient cohort described in this thesis is comparable to equivalent patient groups elsewhere in the United Kingdom and patient demographics are similar to those published in national statistics (Cancer Research UK, 2004). The results presented here confirm that

gastro-oesophageal malignancy is more common in males and with increasing patient age. Almost 70% of tumours were located in or around the oesophago-gastric junction and the majority of tumours were adenocarcinomas (84%) [70% of oesophageal tumours were adenocarcinomas]. This supports the claim of the rising incidence of these tumours observed throughout Europe and North America and has been attributed to an increase in the incidence of Barrett's metaplasia (Jankowski et al, 2000; Coleman et al, 1999). As is typical in the UK, most patients presented with locally advanced disease (stage III) or metastatic disease (stage IV) at the time of diagnosis, with fewer than half of all patients suitable for surgical resection (Cancer Research UK, 2004).

Serum acute phase protein concentrations

Cancer patients had significantly elevated serum concentrations of positive acute phase proteins at the time of diagnosis. In addition, 43% of patients had systemic inflammation, defined as a serum CRP concentration greater than 10 mg/l. An APPR has been identified in association with the most malignancies and the prevalence of systemic inflammation is comparable with those quoted in previous studies relating to gastric and oesophageal cancers (range 23-32%) (Shimada et al, 2003; Nozoe et al, 2001; Crumley et al, 2006).

An APPR was associated with increasing patient age and female sex. Many studies have recognised that healthy patients greater than 65 years of age have significantly elevated serum CRP concentrations when compared with younger patients (Ballou et al, 1996; Wener et al, 2000). It is not clear why serum acute phase protein concentrations should increase with aging. Some authors have suggested an association with atherosclerosis (Ballou et al, 1996), while others have proposed dysfunction of the cytokine regulation network (Wener et al, 2000). This study has not controlled for cardiovascular disease. The association between female sex and elevation of some of the serum acute phase reactant concentrations has also been identified previously and is thought to be related to increased circulating oestrogen concentrations (Wener et al, 2000). Serum acute phase protein concentrations may also be influenced by race and ethnicity, however, only 2 (1%) patients were non-Caucasian in this study and therefore these differences were not explored.

Karnofsky performance score was inversely associated with serum acute phase protein concentrations. This association has been well documented among patients with malignancy, including gastrointestinal malignancy, and may represent a clinical measure of the disease burden (O'Gorman et al, 2000). However, the Karnofsky score and markers of systemic inflammation have also been shown to be independently associated with outcome in malignant disease (O'Gorman et al, 2000).

Among the pathological variables, the presence of elevated serum acute phase protein concentrations were associated with advanced disease stage and poor tumour grade, but not with histological subtype or tumour location. The presence of an elevated APPR and advancing disease stage has been widely documented and has been linked with the presence of both overt and occult metastases (Barber et al, 1999). This association may simply represent the generation of inflammation in response to the tumour burden in the host. Previously, elevated serum CRP concentrations have been associated with increased tumour volume in patients with oesophageal cancer (Nozoe et al, 2001). However, prognostic studies have shown that markers of systemic inflammation are prognostic independent of disease stage suggesting that the inflammatory response is more than simply a marker of disease bulk (Rashid et al, 1982; Shimada et al, 2003; Nozoe et al, 2001; Falconer et al, 1994). The absence of any association between tumour location or histological subtype is perhaps not surprising given that the presence of systemic inflammation has been identified in a variety of malignant diseases of diverse location and cellular origin. This suggests that cancer-associated inflammation is not cancer type specific.

Although tumour necrosis was evident in 24% of the specimens that were studied, serum acute phase protein concentrations were only weakly associated with histological necrosis/abscess formation and many patients without tumour necrosis had significantly elevated serum APP concentrations. This would also suggest that cancer-associated systemic inflammation is more than simply a passive response to the presence of tumour abscess formation.

Helicobacter pylori infection incites a chronic inflammatory response within the stomach that may lead to atrophic gastritis, achlorhydria and malignant transformation (El-Omar et al, 2000). We identified a low incidence of Helicobacter pylori infection in this study

(12%), however, H pylori status was reported in fewer than half the study patients and prior eradication treatment was not recorded. We therefore found no association between serum acute phase protein concentrations and Helicobacter pylori infection.

Serum cytokine concentrations

IL-1β and IL-10 were not detectable in the serum of any of the controls and were infrequently measured in patient's serum (2% and 5%, respectively). Only very low levels of IL-1β have been reported in normal serum and IL-10 is rarely detectable in healthy individuals (Paul and Seder, 1994). Previous work in our Department has similarly found low serum IL-1β concentrations in pancreatic cancer patients, where only 2 out of 64 patients had measurable serum IL-1β levels (Barber et al, 2000). Another group, however, have identified elevated serum IL-1β concentrations in patients with advanced gastric cancer (Kabir and Daar, 1995). To our knowledge serum IL-1β levels have not been directly investigated among oesophageal cancer patients. Elevated serum IL-10 concentrations have been identified in a number of gastrointestinal (and other) malignancies and elevated concentrations have been associated with advanced disease and adverse outcome (De Vita et al, 1999; Fortis et al, 1996). We found no association between elevated serum IL-10 concentrations and advanced disease stage. It is possible that the serum concentrations of these cytokines were below the limit of sensitivity for our assay.

Concentrations of IL-6 and IL-8 were similar among cancer patients and healthy controls. There is extensive documentation relating serum pro-inflammatory cytokine concentrations to numerous clinical and pathological endpoints with conflicting and inconsistent conclusions. Some of these conflicting results may relate to difficulties in reliably measuring serum cytokine concentrations and this has been highlighted in previous Chapters. In addition, there is doubt over the relevance of the systemic concentrations of cytokines when compared with the tissue compartment concentrations where such mediators may exert most of their biological effects. The significance of circulating cytokine concentrations in cancer patients remains unclear. However, serum IL-6 concentrations were significantly elevated among patients with oesophageal tumours when compared with tumours arising at other anatomical positions. Serum IL-6 concentrations were also positively associated with

squamous cell histology, which accounted for approximately one-third of the cases of oesophageal cancer. Squamous cell cancers have been associated with significantly higher serum IL-6 concentrations when compared with adenocarcinomas in lung cancer patients and squamous tumour cells have been shown to produce IL-6 mRNA, which has been correlated with elevated serum IL-6 levels in oesophageal cancer patients (Yamaguchi et al, 1998; Wang et al, 1999). The increased serum IL-6 concentrations identified in the oesophageal cancer group is, therefore, likely to be secondary to the squamous cell histology within this group.

In contrast with the other serum cytokines measured, sTNF-R levels were significantly higher in the cancer patient group compared with healthy controls. In this respect sTNF-R behaves more like an acute phase reactant than a cytokine. As such, we found that sTNF-R concentrations were also associated with increasing patient age, female sex, reduced Karnofsky performance scores, and poor tumour grade, but in contrast to the acute phase proteins, sTNF-R concentrations were not associated with stage of disease or histological evidence of tumour necrosis in this study.

Correlation between serum cytokine concentrations and acute phase protein concentrations.

Soluble TNF-R concentrations correlated with all the serum acute phase protein concentrations and our group has previously demonstrated this association among patients with advanced pancreatic cancer (Barber et al, 1999). These findings are most likely due to the increased stability of the molecule compared with TNFα and the consistent serum levels. Interleukin-6 levels correlated with two of the five acute phase reactants that were measured in this study. Many previous studies, including work from our own group, have identified correlations between serum IL-6 concentrations and serum acute phase reactants in cancer patients (Barber et al, 1999), however these findings are often inconsistent and relate to the problems already mentioned regarding the validity of measuring circulating cytokine concentrations. Interleukin-8 concentrations did not correlate with any of the acute phase proteins.

Effects of treatment and disease progression on markers of systemic inflammation.

CRP concentrations at the time of diagnosis demonstrated good stability and this reproducibility is essential in order to develop a robust measure that may be used to define systemic inflammation in this patient group and later for the development of a prognostic model (see Chapter XI).

A second CRP and albumin measurement was performed in a subgroup of patients approximately 3 months following diagnosis. Analysis of the paired values demonstrated that CRP concentrations increased and albumin concentrations decreased in patients who had undergone surgery. These measurements were performed a minimum of 92 days following surgery and no patient had signs of active infection or post-operative complications at the time of sample collection. All these patients had apparently undergone curative resection with removal of all the macroscopic disease. The on-going systemic inflammatory response in these patients may not, therefore, be directly related to the tumour, but represent subclinical post-operative complications driving the pro-inflammatory response. Previously, reductions in serum acute phase protein concentrations have been identified in gastric cancer patients who had undergone surgical resection and increasing concentrations were measured in those patients with evidence of tumour recurrence (Fujita et al, 1999; Bernacka et al, 1993). Similarly, in colorectal cancer patients there was a reduction in CRP values following curative resection (28% elevated pre-operatively versus 17% elevated post-operatively) and an elevated CRP post-operatively was associated with adverse outcome (McMillan et al, 2003). In contrast, serum CRP concentrations were significantly reduced in those patients who received pre-operative chemotherapy without any changes in serum albumin concentrations. We did not evaluate whether a reduction in CRP concentration was associated with a histological evidence of response to the chemotherapy and it is possible that this may be the case. It is also possible that the chemotherapy had an anti-inflammatory/immunosuppressant action resulting in reduced concentrations of serum acute phase reactants. The effect of neo-adjuvant chemotherapy on serum acute phase protein concentrations has not previously been investigated in gastro-oesophageal cancer patients. However, studies in head and neck cancers and lung cancer found a significant reduction in serum concentrations of acute phase reactants following pre-operative chemotherapy (Kolebacz et al, 1999; Endo et al, 2004).

There was no significant change in serum acute phase concentrations in patients who received palliative treatments alone. This may reflect the fewer patient numbers involved. Previously, an increase in CRP concentrations of 15 mg/l per month has been noted among patients with pancreatic cancer patients with almost all patients demonstrating a systemic inflammatory response towards the time of death (Barber et al, 1999). That study examined patients with advanced disease who were near to death and it is possible that our study would show a similar trend were the patients followed up for longer and closer death.

Conclusions

The data from this Chapter has established that 43% of patients with gastro-oesophageal cancer have evidence of systemic inflammation at the time of diagnosis. The presence of systemic inflammation is associated with advanced disease stage, poor performance status, increasing age, and female sex. Surgical resection is associated with an increase in serum CRP concentrations and pre-operative chemotherapy is associated with a reduction in CRP concentrations. In contrast, serum cytokines demonstrate little association with clinical and pathological variables or with serum acute phase protein concentrations, except for sTNF-R, which appears to behave like an acute phase reactant. The next Chapter investigated levels of cytokine expression within tumour tissue and related these concentrations to markers of systemic inflammation and outcome in patients with gastro-oesophageal cancer.

CHAPTER V

PATTERNS OF TISSUE CYTOKINE EXPRESSION AND THEIR ASSOCIATION WITH AN IMMUNE CELL INFILTRATE AND MARKERS OF SYSTEMIC INFLAMMATION IN PATIENTS WITH GASTRO-OESOPHAGEAL CANCER

Deans DAC, Wigmore SJ, Gilmour H, Paterson-Brown S, Ross JA, Fearon KCH
Tumour tissue Interleukin-1β is associated with systemic inflammation – a marker of reduced survival in gastro-oesophageal cancer.
British Journal of Cancer 2006; 95(11): 1568-1575

ABSTRACT

Markers of systemic inflammation were elevated in 43% of patients with gastro-oesophageal malignancy at the time of diagnosis. Serum cytokine concentrations demonstrated little correlation with markers of systemic inflammation, suggesting that circulating concentrations of cytokines do not represent the true levels of cytokine activity driving the systemic inflammatory response. This Chapter investigated the role of tumour tissue in the genesis of systemic inflammation. Tissue cytokine concentrations were compared with serum cytokine and acute phase protein concentrations. In addition, tumour tissue cytokine concentrations were compared with concentrations measured in tissue from healthy controls and in non-neoplastic tissue also collected from cancer patients. The presence of an inflammatory cellular infiltrate into the tumour tissue was also documented and the extent of cellular infiltrate was compared with levels of tissue and systemic cytokine expression.

Tumour tissue was collected from 56 patients with gastro-oesophageal cancer at the time of surgery. At the same time non-neoplastic mucosal tissue adjacent to the tumour was also sampled from each patient. Tissue was also collected from 12 healthy controls during upper GI endoscopy. Tissue cytokine mRNA concentrations were measured by real-time PCR and tissue protein concentrations were determined by a cytometric bead array system. The degree of chronic inflammatory cell infiltrate into the tissues was recorded. Serum cytokine and acute phase protein concentrations were determined by ELISA.

Pro-inflammatory cytokine concentrations (mRNA and protein) were significantly elevated in tumour tissue compared with concentrations measured in non-malignant tissue sampled from healthy controls. Tumour tissue IL-1β concentrations were significantly elevated and were positively associated with markers of systemic inflammation ($p=0.05$, $r=0.31$). In addition, a chronic inflammatory cellular infiltrate was identified in 75% of tumours and was also associated with elevated markers of systemic inflammation (CRP; $p=0.01$), but was not associated with elevated tissue cytokine mRNA or protein concentrations.

A chronic inflammatory cell infiltrate and tumour tissue IL-1β over-expression are potential key initiators of systemic inflammation in patients with oesophagogastric cancer. Over-expression of IL-1β appears not to be related to the presence of a host immune cell infiltrate and may be host/tumour dependent.

INTRODUCTION

The previous Chapter has demonstrated that patients with gastro-oesophageal cancer have significantly elevated concentrations of serum (positive) acute phase proteins and 43% of these patients have evidence of systemic inflammation at the time of diagnosis. In contrast, serum cytokine concentrations demonstrate little association with clinical or pathological variables or with serum acute phase protein concentrations (except for sTNF-R). These data suggest that circulating concentrations of cytokines do not represent the true levels of cytokine activity that are driving the systemic inflammatory response in these patients. In order to investigate the aetiology of systemic inflammation in these patients, this Chapter investigated patterns of cytokine expression within tumour tissue. Tissue cytokine concentrations (mRNA and protein concentrations) were compared with serum cytokine and acute phase protein concentrations. In addition, concentrations of cytokines measured in the tumour tissue were compared with concentrations measured in tissue from healthy controls and in non-neoplastic tissue also collected from the same cancer patients. The presence of an inflammatory cellular infiltrate into the tumour tissue was also documented and the extent of cellular infiltrate was compared with levels of tissue and systemic cytokine expression. This Chapter, therefore, aimed to determine the role of the tumour tissue in the generation of systemic inflammation in patients with gastro-oesophageal cancer.

MATERIALS AND METHODS

Study Patients and Controls

Patients diagnosed with gastric or oesophageal cancer within the Lothian and Borders regions between March 2002 and June 2004 were invited into the study (see Chapter IV for further details on the patient cohort). For this study, only those patients who underwent surgical resection were eligible for inclusion due to the requirement for tissue sampling.

Therefore, patients not suitable for surgery, for example, due to advanced disease or co-morbidity, were excluded from this study. No patients eligible for entry refused consent and no patients who underwent surgery were excluded. An additional 12 patients were recruited as healthy controls. These patients underwent endoscopy as an elective procedure for investigation of dyspeptic-type symptoms. In all instances the result of the procedure was normal, including both macroscopic and microscopic assessment. All control subjects were considered healthy without established co-morbidity or taking any regular medications.

Measurement of serum acute phase protein and cytokine concentrations

Blood was collected from patients at the time of diagnosis and prior to any therapeutic intervention. Samples were simultaneously collected from 22 healthy controls for comparison. Analysis was performed by ELISA as previously described in the Materials and Methods section (Chapter III).

Measurement of tissue cytokine mRNA and protein concentrations

Tissue was obtained from patients at the time of surgical resection. Representative samples of tumour tissue and adjacent non-neoplastic mucosal tissue were cut from the specimens within 20 minutes of resection by a Consultant pathologist. Samples were immediately snap frozen in liquid nitrogen prior to storage at -80°C until further analysis. An additional 12 patients were recruited as healthy controls and tissue was collected at the time of endoscopy. Mucosal tissue (7 oesophageal and 5 gastric) samples were collected from these patients with biopsy forceps at the time of endoscopy and samples were snap frozen for storage. Tissue samples were homogenised prior to analysis. Concentrations of IL-1β, IL-6, IL-8 and TNFα were measured in the tissue samples. Quantification of cytokine mRNA was performed by real-time PCR and tissue cytokine protein concentrations were measured by a cytometric bead array system. Both these techniques are described in detail in the Materials and Methods Chapter (Chapter III).

Histological Analysis

Representative sections of tumour tissue from each patient were fixed with formalin and stained with haematoxylin and eosin. A single Consultant pathologist reviewed all the tissue sections and samples were classified into one of three groups according to the degree of chronic inflammatory cellular infiltrate present. Group 1 was defined as having a scanty diffuse (occasional) chronic inflammatory cell infiltrate, Group 2 samples had a diffuse chronic inflammatory cellular infiltrate present throughout the whole tissue, and Group 3 had focal lymphoid aggregates only (see Figure 5.3). In addition, the presence of Helicobacter pylori and tissue necrosis was also noted. The pathologist was blinded to the clinical data, serum acute phase protein/cytokine concentrations, tissue cytokine concentrations, and the clinical outcome for each patient.

Statistical Analysis

Comparisons between groups of continuous variables were made by the Mann-Whitney U test. Categorical variables were compared by Fisher's exact test. Correlations between continuous variables were assessed by linear regression following natural logarithmic transformation of the data. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Study Patients

Patient demographics are shown in Table 5.1. Fifty-six patients were recruited to the study. The primary tumour sites were oesophageal (n=26, 46%), gastric (n=17, 30%) and those arising from the gastro-oesophageal junction [OGJ] (n=13, 23%). The predominant histological subtype was adenocarcinoma (93%). All patients underwent surgical resection and 13 (23%) of these received pre-operative chemotherapy. At the end of the study 19 (34%) patients had died.

Sub-group analysis confirmed no significant differences in either tissue mRNA or protein levels between patients who received pre-operative chemotherapy and those who did

not. Similarly, there were no differences in tissue mRNA or protein levels or serum cytokine or acute phase protein levels among those patients taking NSAID's or any other therapeutic agents that may modify the inflammatory response. Therefore, all patients were included as a single group for analysis.

Table 5.1 Study patient demographics (n=56)

		Number [%]
Age (years)*		66 (58-75)
Sex	Male	40 [71]
	Female	16 [29]
Tumour Site	Oesophageal	26 [46]
	Oesophago-gastric junction	13 [23]
	Gastric	17 [30]
Histology	Adenocarcinoma	52 [93]
	Squamous cell carcinoma	4 [7]
Grade	Well differentiated	4 [7]
	Moderately differentiated	24 [43]
	Poorly differentiated	28 [50]
UICC Stage	1	17 [30]
	2	13 [23]
	3	21 [38]
	4	5 [9]
Treatment undertaken	Oesophagectomy	25 [45]
	Gastrectomy	18 [32]
	Pre-operative Chemotherapy followed by surgery	13 [23]
Status	Alive	37 [66]
	Dead	19 [34]

*Values given are median (inter-quartile range)

Concentrations of serum cytokine and acute phase proteins

Similar to the findings described in Chapter IV relating to the whole patient cohort, this study also identified significantly elevated serum concentrations of positive acute phase proteins, but not negative acute phase proteins, in the patient population when compared with controls. Ten (18%) patients had evidence of systemic inflammation (serum CRP concentration >10

mg/l). Serum cytokine concentrations were not significantly different between patients and controls and serum cytokine concentrations did not correlate with serum acute phase protein concentrations (data not shown for brevity).

Tissue cytokine mRNA and protein concentrations

Healthy Controls

IL-1β, IL-6 and IL-8 mRNA were not measurable in any of the gastro-oesophageal mucosa samples collected from healthy controls and TNFα was only detectable at low concentrations (Table 5.2) (Figure 5.1A). Similarly, IL-6 protein was not detected in tissue samples from healthy controls and IL-1β, IL-8, and TNFα were also only measured at low concentrations (Table 5.2) (Figure 5.1B).

Table 5.2 Cytokine mRNA and cytokine protein concentrations measured in tissue from healthy controls compared with concentrations measured in tissue (tumour tissue and benign tissue) from cancer patients.

	Tissue cytokine mRNA conc (% of PCR control)			**Tissue cytokine protein conc (pg/mg total protein)**		
	Healthy control	Cancer patient		Healthy control	Cancer patient	
	Benign tissue	Benign tissue	Tumour tissue	Benign tissue	Benign tissue	Tumour tissue
IL-1β	0	0.8 (0.2-12.1)	3.2 (0.2-12.1)	2.6 (2.2-4.9)	44.5 (23.0-89.2)	135.8 (40.5-424.7)
IL-6	0	1 (0-5.4)	1.9 (0.3-13.6)	0	0 (0-6.7)	3 (0-45.8)
IL-8	0	0 (0-0.1)	0.2 (0.1-1.0)	0 (0-7.7)	8.4 (2.8-23.7)	56.2 (22.6-158.6)
TNFα	0 (0-2.1)	1.3 (0-7.8)	2.7 (0.7-15.6)	0 (0-1.0)	3 (0-5.8)	6.6 (1.1-25.8)

Values are median (inter-quartile range)

Cancer Patients - cytokine mRNA concentrations

Tumour tissue cytokine mRNA concentrations were detected at significantly higher concentrations compared with those measured in tissue from healthy subjects: IL-1β 3.2% of control (inter-quartile range (IQR) 0-12.1), $p<0.001$; IL-6 1.9% of control (IQR 0-13.6), $p<0.001$; IL-8 0.2% of control (IQR 0-1), $p<0.001$; TNFα 2.7% of control (IQR 0-15.6), $p=0.006$ (Table 5.2) (Figure 5.1A). Similarly, IL-1β, IL-6, and TNFα mRNA concentrations were also measured at significantly elevated concentrations in non-neoplastic tissue sampled from cancer patients when compared with tissue from healthy controls. IL-8 concentrations were also elevated but did not reach statistical significance ($p=0.30$). Although cytokine mRNA concentrations were higher in tumour tissue when compared with benign tissue from cancer patients, only IL-8 was statistically significant ($p<0.001$).

Cancer Patients - cytokine protein concentrations

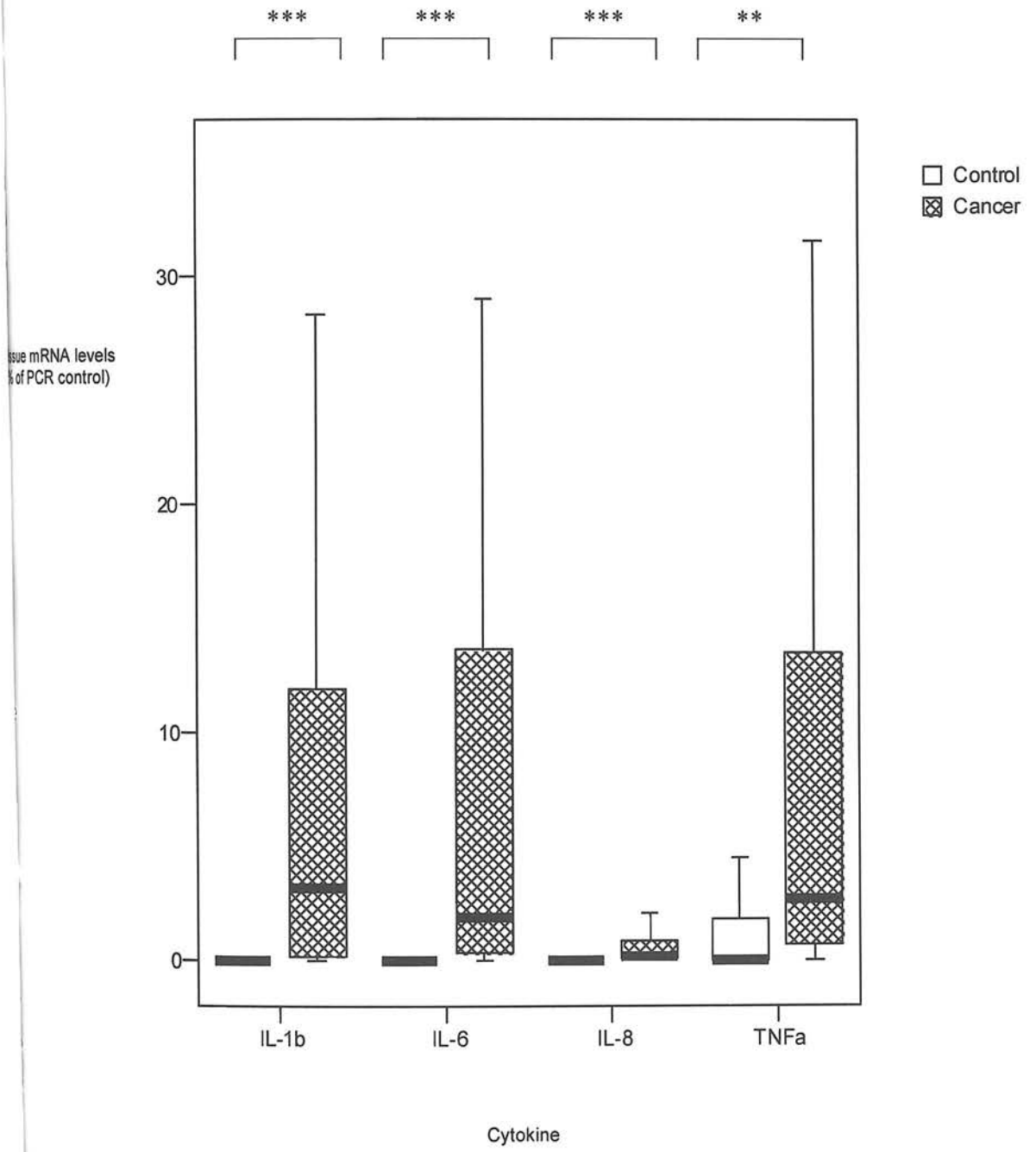
Cytokine protein concentrations were significantly higher in tumour tissue compared with concentrations in tissue from healthy controls: IL-1β 136 pg per mg of total protein (IQR 41-425), $p=0.007$; IL-6 3 pg/mg (IQR 0-46), $p<0.05$; IL-8 56 pg/mg (IQR 23-159), $p=0.007$; TNFα 7 pg/mg (IQR 1-26), $p<0.05$ (Table 5.2) (Figure 5.1B). Protein concentrations measured in benign tissue from cancer patients were also elevated when compared with healthy controls, but only IL-1β reached statistical significance ($p=0.041$). In addition, cytokine protein concentrations were significantly elevated in tumour tissue when compared with levels measured in non-neoplastic tissue from the same cancer patients.

Cytokine mRNA versus cytokine protein concentrations

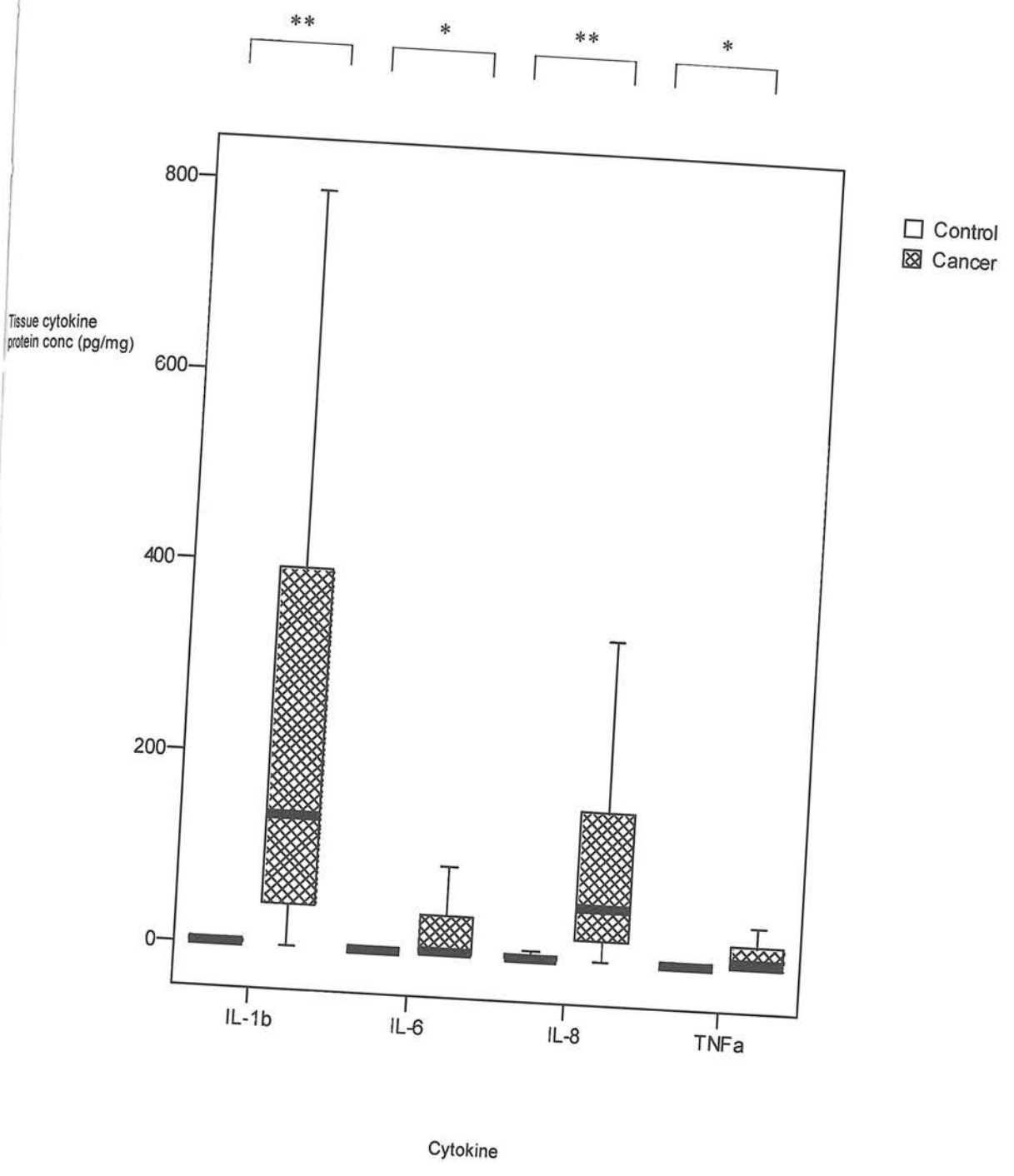
There was no correlation between tissue cytokine mRNA concentrations and cytokine tissue protein concentrations; IL-1β ($p=0.64$, $r=0.07$; Spearman rank), IL-6 ($p=0.46$, $r=-0.1$), IL-8 ($p=0.55$, $r=0.09$), TNFα ($p=0.90$, $r=0.02$). Increased mRNA concentrations were not associated with elevated tissue cytokine protein concentrations.

Figure 5.1 Comparison of cytokine concentrations of (A) mRNA and (B) protein between tissue from healthy controls and tumour tissue from patients with gastro-oesophageal cancer. The lines represent the median value, bars = inter-quartile range, error bars = extreme values. IL-1β = Interleukin-1β, TNFα = Tumour necrosis factor-α. * p<0.05, **p<0.01, ***p<0.001 (Mann-Whitney U Test)

(A)



(B)

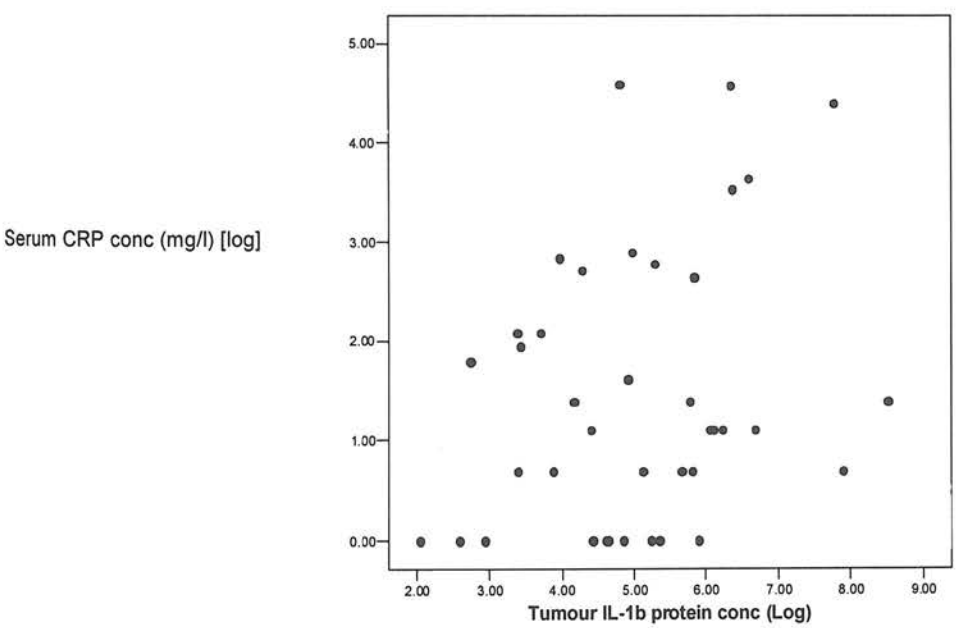

**
*
**
*
800
600
400
200
0
Tissue cytokine protein conc (pg/mg)
Control
Cancer
IL-1b
IL-6
IL-8
TNFa
Cytokine

Relationship between tissue cytokine concentrations and serum cytokine and acute phase protein concentrations

Tumour tissue IL-1β protein levels were positively correlated with serum CRP concentrations ($p=0.05$, $r = 0.31$; linear regression) and serum sTNF-R concentrations ($p=0.03$, $r = 0.36$) (Figure 5.2). There was also a trend towards a correlation between tumour IL-8 protein concentrations and serum sTNF-R concentrations, but this did not quite reach significance ($p=0.06$, $r= 0.32$). Neither IL-6 nor TNFα tissue protein concentrations were associated with systemic cytokine or acute phase protein concentrations.

There was no correlation between tissue cytokine mRNA concentrations and serum cytokine or serum acute phase protein concentrations (data not shown).

Figure 5.2 Tumour tissue IL-1β protein levels were positively correlated with serum CRP concentrations ($p=0.05$, $r = 0.31$; linear regression).

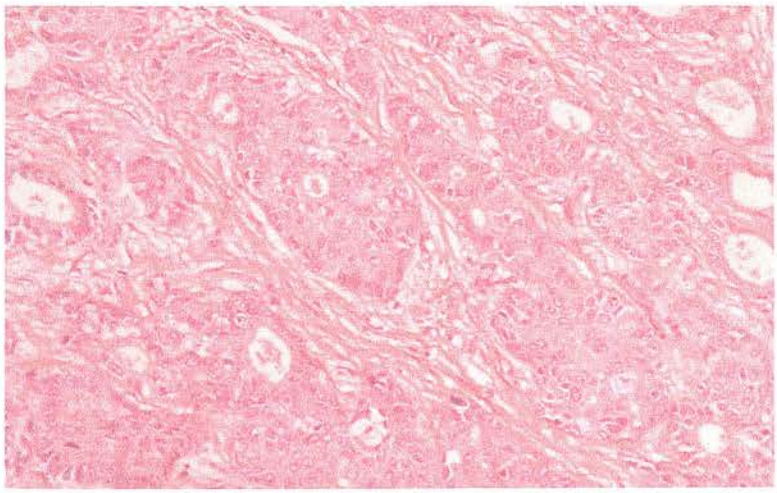


Histological Analysis

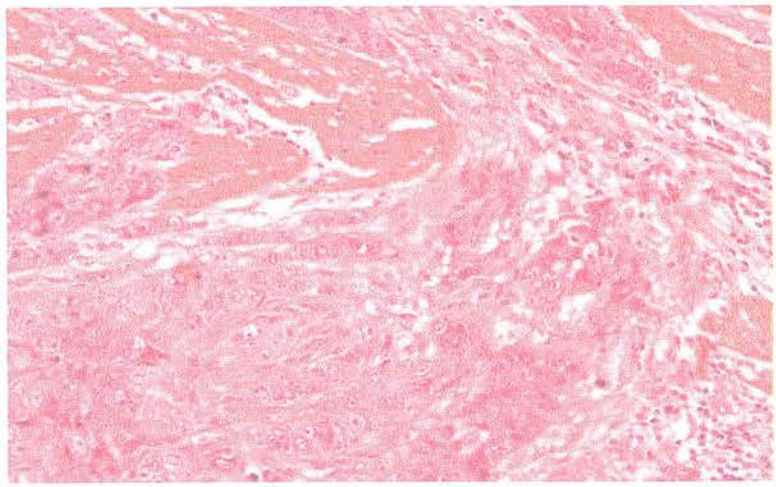
Slides from three patients recruited to the study could not be retrieved, therefore, 53 tumour sections were studied. Twenty-four (45%) tumour samples were classified as having scanty diffuse or patchy chronic inflammatory cells, 16 (30%) had a diffuse chronic inflammatory cellular infiltrate visible throughout the whole tumour, and the remaining 13 (25%) tumour sections had focal lymphoid aggregates only (Figure 5.3). Therefore, 40 (75%) tumours had evidence of an increased chronic inflammatory cell infiltrate. Tumour necrosis was evident in 13 (25%) samples, but there was no association between degree of inflammatory cell infiltrate and the presence of necrosis (p=0.92, Chi-square test). Similarly, a chronic inflammatory cellular infiltrate was not associated with Helicobacter pylori infection (p=0.67, Fisher's exact test; data not shown).

Figure 5.3 Representative photomicrographs taken from three patients with poorly differentiated adenocarcinoma of the oesophagus. Patient (A) demonstrates minimal/no inflammatory cell reaction. Patient (B) has a patchy chronic inflammatory cell infiltrate. Patient (C) shows a diffuse chronic inflammatory cellular infiltrate present throughout the tumour. Sections of tumour tissue were fixed with formalin and stained with haematoxylin and eosin. [Magnification x100]

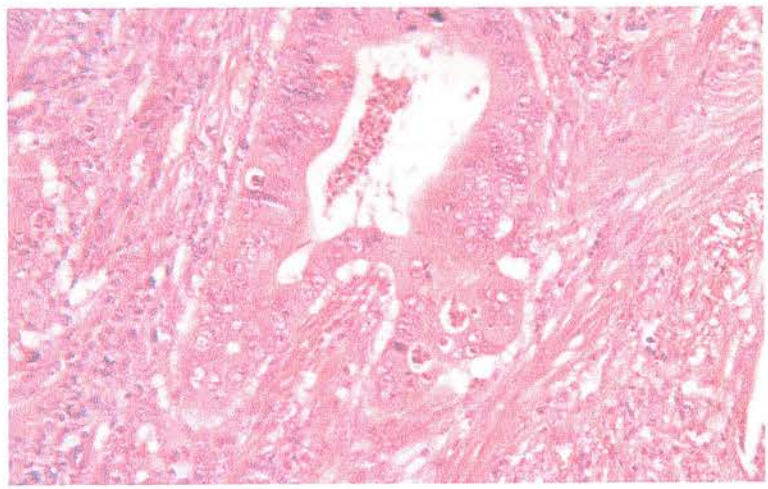
(A)



(B)



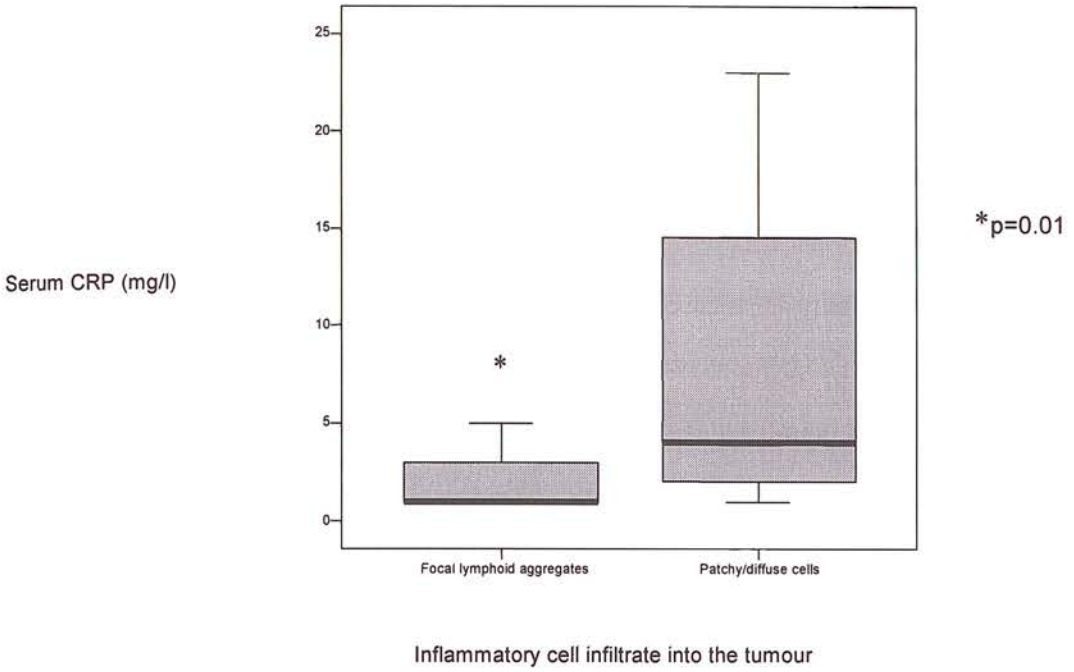
(C)



Relationship between an inflammatory cell infiltrate and markers of systemic inflammation

When compared with tumour sections possessing lymphoid aggregates alone, tissues with a diffuse or patchy inflammatory cellular infiltrate were associated with elevated serum CRP and sTNF-R concentrations ($p=0.01$ and $p=0.007$ respectively, Mann-Whitney U test) (Figure 5.4). There was no association between the presence of a chronic inflammatory cell infiltrate and serum cytokine concentrations.

Figure 5.4 A diffuse or patchy inflammatory cellular infiltrate was associated with elevated serum CRP concentrations (p=0.01, Mann-Whitney U test). Thick bar represents median, the box represents quartiles, and lines represent extreme values.



Relationship between an inflammatory cell infiltrate and tissue cytokine concentrations

There were no differences in tissue cytokine mRNA or protein concentrations between tumour tissues with a chronic inflammatory cell infiltrate and tumour samples without an inflammatory cell infiltrate.

Discussion

In the previous study (Chapter IV), we have shown that patients with gastro-oesophageal malignancy have elevated concentrations of serum acute phase proteins but similar concentrations of serum cytokine concentrations compared with a control population and 43% of patients have evidence of systemic inflammation at the time of diagnosis. In this study, we have shown that pro-inflammatory cytokine concentrations (mRNA and protein) are significantly elevated in tumour tissue (and adjacent non-neoplastic tissue) when compared with concentrations measured in tissue sampled from healthy controls. Moreover, tumour tissue IL-1β concentrations correlate with markers of systemic inflammation (CRP). In addition, a chronic inflammatory cellular infiltrate was identified in 75% of tumours and was also associated with markers of systemic inflammation (CRP), but was not associated with elevated tissue cytokine mRNA and protein concentrations.

Tissue cytokine concentrations

The data from Chapter IV suggests that circulating cytokine concentrations are an unreliable marker of systemic inflammation and a poor measure of tissue cytokine activity (Barber et al, 1999; Falconer et al, 1994). Moreover, these findings suggest that circulating cytokines may not be the key mediators of the acute phase response. Alternatively, local cytokine concentrations within target tissues may represent a better measure of systemic inflammatory cytokine activity. Host immune cells may become 'activated' by exposure to local mediators as they pass through the tumour mass. These activated peripheral blood mononuclear cells (PBMC's) may then release increased concentrations of pro-inflammatory cytokines within target tissues, for example, in the liver, thereby inducing an acute phase response. PBMC's from cancer patients with an elevated serum APPR demonstrate greater rates of pro-inflammatory cytokine (IL-6 and TNFα) production and these cells are then capable of stimulating increased CRP production by hepatocytes in co-culture systems (O'Riordain et al, 1999). The tumour itself may also be capable of constituently producing pro-inflammatory cytokines. Increased cytokine mRNA and protein concentrations have been measured within oesophageal tumour cells and CRP expression has even been detected within oesophageal

cancer cells (Yuan et al, 2000; Martignoni et al, 2005; Nozoe et al, 2003). In our study, pro-inflammatory cytokine mRNA and protein concentrations were either not detectable or found at very low levels in tissue collected from healthy controls. In contrast, cytokine mRNA and protein concentrations were measured at significantly higher concentrations in both non-neoplastic and tumour tissue collected from cancer patients. Moreover, concentrations of these cytokines were higher in the tumour tissue compared with the benign tissue sampled from the same patients. Why apparently normal tissue from patients with cancer should demonstrate increased tissue cytokine concentrations is unclear, but may represent a whole organ upregulation of pro-inflammatory activity where the effects of cytokines and other mediators may 'spill over' into adjacent tissues. Certainly other groups have identified similar findings. For example, Yuan and co-workers investigated IL-8 mRNA concentrations in tumour tissue and adjacent normal lung tissue in patients with non-small cell lung cancer and also found increased cytokine expression within the tumour tissue and adjacent normal tissue (Yuan et al, 2000). Other groups have similarly demonstrated increased tissue cytokine concentrations associated with progression along the metaplasia-dysplasia-carcinoma sequence in Barrett's oesophagus (Tselepis et al, 2002; Dvorakova et al, 2004).

In the present study median IL-1β concentrations were 10 – 100 fold higher than IL-6 in the tumour tissue and there was a weak but significant correlation between tumour tissue IL-1β concentration and serum CRP. There was a similar trend with IL-8. Both IL-1β and IL-8 are recognised as important cytokines in the generation of the systemic inflammatory response and it is possible that high tissue concentrations of these cytokines stimulate PBMC's as they pass through the tumour mass, which in turn act on target organs, such as the liver, to induce the synthesis of acute phase proteins that are associated with systemic inflammation (O'Riordain et al, 1999). Moreover, Martignoni and co-workers have suggested that IL-6 over-expression in cachectic pancreatic cancer patients is related to the ability of certain IL-6 producing tumours to sensitise PBMC's and induce IL-6 expression in PBMCs (Martignoni et al, 2005). In the latter study screening by DNA microassay analysis followed by quantitative PCR identified only IL-6 mRNA expression to be significantly increased in tumour samples of cachectic patients compared with non-cachectic patients or pancreas samples from normal controls. Immunohistochemistry suggested the source of IL-6 to be tumour cells

rather than host cells. The results of the present study, however, identify that at least in patients with gastro-oesophageal cancer IL-1β rather than IL-6 may be important as an initiator of the pro-inflammatory acute phase protein response. IL-6 may form a common final pathway via activated PBMC's. Interestingly, in the colon-26 murine model of cancer cachexia associated with systemic inflammation there appears to be a complex intra-tumoural amplification loop between IL-1β and IL-6, which can be down-regulated by IL-10 (Yasumoto et al, 1995; Fujiki et al, 1997).

In the present study we did not find any correlation between tissue cytokine mRNA concentrations and systemic cytokines or acute phase protein concentrations. Raddatz and co-workers did identify an association between tissue cytokine mRNA levels and systemic CRP protein concentrations in Crohn's disease (Raddatz et al, 2005). These differing results may be partly explained by the lack of correlation between tissue mRNA concentrations and protein concentrations in our study. While some groups have demonstrated a correlation between IL-1β and IL-6 mRNA and protein concentrations in an animal model of inflammatory joint disease, they also failed to show any correlation for TNFα mRNA and protein concentrations (Rioja et al, 2004). The difficulties of relating mRNA concentrations to protein concentrations has been extensively documented elsewhere, but it is also important to consider that real-time PCR is an exquisitely sensitive technique and that what we are detecting in some patients, although elevated, may have little or no functional significance as it may not be translated into protein. Cytokine protein concentrations are, therefore, likely to be a more robust measure of tissue cytokine activity than mRNA levels.

Inflammatory cell infiltrate

The presence of chronic inflammation (atrophic gastritis and oesophagitis) has been implicated in cancer development (Limburg et al, 2005). Both Barrett's metaplasia and Helicobacter-related gastritis are associated with a lymphocytic infiltrates (Biddlestone et al, 1998; Sung et al, 2000). The presence of an inflammatory cell infiltrate within tumour tissue and its relevance to tumour biology has been the subject of extensive current research. This topic has been described in detail in the General Introduction (Chapter II). Tumour-associated macrophages (TAM's) and tumour-infiltrating lymphocytes (TIL's) are commonly identified

within tumour tissue, and up to 60% of the tumour mass may be accounted for by host immune cells (van Ravenswaay-Claasen et al, 1992). The natural immune functions of these cells may be subverted by tumour-produced mediators, including cytokines. This creates a local environment favouring tumour growth and progression and attaining the tumour immune privilege (Chiou et al, 2005; Nakagomi et al, 1993; Curiel et al, 2004).

The present study aimed to investigate the contribution of an inflammatory cell infiltrate towards the generation of systemic inflammation. A chronic inflammatory cellular infiltrate was noted in 75% of tumour samples and was associated with elevated serum concentrations of CRP and sTNF-R. However, there were no differences in tissue cytokine concentrations (mRNA or protein) between tumours with a chronic inflammatory infiltrate and those without. This may suggest that the differential tissue cytokine expression is likely to be tumour-cell derived. Moreover, that IL-1β may be the key cytokine driving the systemic inflammatory response in patients with gastro-oesophageal cancer. Alternatively, these findings also support the theory that host immune cells are activated as they pass through the tumour mass and these cells then release elevated pro-inflammatory cytokine concentrations at the site of distant target tissues, for example inducing an APPR in the liver. In order to take this hypothesis further, attempts were made to measure cytokine concentrations in individual cell populations found within the tumour tissue, such as cancer cells, immune cells, and host stromal cells. To perform this task individual cell types need to be isolated. Laser capture microdissection (LCM) enables single cell types to be separated from multiple cell populations, thereby creating a 'pure' cell population for analysis (Emmert-Buck et al, 1996). This technique was attempted but had to be abandoned due to inconsistent results, which were related to poor RNA quality as a consequence of this technique. In addition, determination of cellular cytokine protein concentrations by the cytometric bead array system following LCM was not possible due to the low protein concentrations that were retrieved.

Conclusions

Systemic inflammation is present in 43% of patients with gastro-oesophageal cancer at the time of diagnosis. In this study, we have shown that pro-inflammatory cytokine concentrations (mRNA and protein) are significantly elevated in tumour tissue from patients with gastro-

oesophageal cancer when compared with concentrations measured in tissue sampled from healthy controls. Moreover, tumour tissue IL-1β concentrations are highly expressed and are positively associated with markers of systemic inflammation. In addition, a chronic inflammatory cellular infiltrate was identified in 75% of tumours and was also associated with elevated markers of systemic inflammation (CRP), but was not associated with elevated tissue cytokine mRNA and protein concentrations. This may suggest that the differential tissue cytokine expression is tumour-cell derived. Both a chronic inflammatory cell infiltrate and tumour tissue IL-1β over-expression are potential key initiators of systemic inflammation in patients with oesophagogastric cancer. Differential over-expression of IL-1β appears not to be related to the presence of a host cell chronic inflammatory infiltrate and may be host/tumour dependent.

To explore the influence of the host on the propensity to generate systemic inflammation in association with cancer, the next Chapter investigated the link between host cytokine genotype and the presence of systemic inflammation. Cytokine genotypes were compared with tumour tissue cytokine concentrations, markers of systemic inflammation, and with outcome in patients with gastro-oesophageal cancer.

CHAPTER VI

THE LINK BETWEEN HOST CYTOKINE GENOTYPE, TUMOUR TISSUE CYTOKINE CONCENTRATIONS, MARKERS OF SYSTEMIC INFLAMMATION, AND ADVERSE PROGNOSIS IN PATIENTS WITH GASTRO-OESOPHAGEAL CANCER

Deans DAC, Rose-Zerilli M, Wigmore SJ, Ross JA, Howell WM, Jackson AA, Grimble RF, Fearon KCH
Host cytokine genotype is related to adverse prognosis and systemic inflammation in gastro-oesophageal cancer.
Annals of Surgical Oncology 2007; 14(2): 329-339

ABSTRACT

This Chapter examined the role of host cytokine genotype in the generation of systemic inflammation. The pattern of cytokine genotype expression in a cohort of patients with gastro-oesophageal cancer was documented and the link between host genotype, tumour tissue cytokine concentrations, markers of systemic inflammation, and adverse prognosis investigated.

Two hundred and three patients underwent SNP genotyping by TaqMan allelic discrimination genotyping. A control population was also genotyped (266 healthy volunteers). Blood was collected from every patient for determination of serum CRP and serum cytokine concentrations by ELISA. Tumour tissue was collected from 53 patients at the time of surgery and tissue cytokine mRNA concentrations were measured by real-time PCR and tissue protein concentrations were determined by a cytometric bead array system.

Distribution of the cytokine genotypes was similar between patients and controls. The IL-6 –174 CC and IL-10 –1082 GG genotypes were associated with elevated serum CRP ($p=0.03$, $p=0.01$ respectively; Mann-Whitney U test) and sTNF-R ($p=0.015$, $p=0.02$) concentrations. In addition, the IL-6 –174 CC was associated with elevated IL-6, IL-10 and IL-12 tumour tissue concentrations. Both these genotypes were also associated with reduced survival duration ($p=0.004$, $p=0.047$; log-rank test). TNFα AA genotype was also associated with reduced survival duration on univariate ($p=0.020$) and multivariate analysis ($p=0.006$, Cox's multivariate model), but not with inflammatory markers. No other cytokine polymorphisms were associated with systemic/tissue inflammatory markers or prognosis.

The host genotype may influence levels of pro-inflammatory cytokine production in host immune cells or cancer cells or both, which, in turn, may influence the generation of systemic inflammation in cancer patients. Furthermore, cytokine genotypes are associated with adverse prognosis among patients with gastro-oesophageal cancer that may act in association with, but not entirely with, an inflammatory mediated mechanism.

INTRODUCTION

The previous Chapters identified that patients with gastro-oesophageal cancer have elevated concentrations of serum acute phase proteins and 43% have evidence of systemic inflammation at the time of diagnosis (Chapter IV). In addition, pro-inflammatory cytokine concentrations within tumour tissue were significantly elevated and tumour IL-1β concentrations correlated with systemic markers of inflammation (Chapter V). Furthermore, a chronic inflammatory cell infiltrate was found in 75% of tumours and this finding was also associated with elevated markers of systemic inflammation, but was not associated with differences in tumour tissue cytokine concentrations (Chapter V). Therefore, both a chronic inflammatory cell infiltrate and tumour tissue IL-1β over-expression are potential key initiators of systemic inflammation in patients with oesophagogastric cancer.

It is unclear why some patients develop a systemic inflammatory response in association with cancer while others do not. Possible explanations include tumour characteristics, where a particular tumour phenotype may produce pro-inflammatory cytokines and other mediators that may incite an inflammatory response. Alternatively, the host immune cells may be responsible for the primary stimulus for systemic inflammation. Either way the interactions between host and tumour cells are crucial to the development of cancer-associated inflammation. This Chapter examined the role of host cytokine genotype in the generation of systemic inflammation. The key hypothesis being that cytokine genotype may influence the ability of the host to generate a systemic inflammatory reaction in association with cancer. In this Chapter the pattern of cytokine genotype expression in a cohort of patients with gastro-oesophageal cancer is documented and the potential link between host genotype, tumour tissue cytokine concentrations, markers of systemic inflammation, and adverse prognosis was investigated.

Variations in genotype for a number of cytokines have been associated with changes in both serum cytokine and serum acute phase protein concentrations and prognosis among patients with cancer. *IL-1β* polymorphisms have previously been shown to influence the generation of systemic inflammation and survival in patients with pancreatic cancer (Barber et al, 2000).

The possession of the less common allele 2 was associated with increased levels of serum CRP concentrations, increased production of IL-1β by peripheral blood mononuclear cells, and reduced overall survival.

A single nucleotide polymorphism (SNP) at the –308 position of the *TNFα* gene lies within the promoter region and the A allele has been associated with higher levels of TNFα production *in vitro* following stimulation with endotoxin (Louis et al, 1998). Variation at the –308 locus has been associated with conflicting levels of TNFα production both in *in vitro* studies and among patients with sepsis (Mira et al, 1999; Stuber et al, 1995). Other work, again on pancreatic cancer patients, found no link between genotype and serum soluble TNF receptor (sTNF-R) concentrations or serum CRP concentrations (Barber et al, 1999b). There was a trend for reduced survival duration associated with possession of the AA genotype, but this did not reach statistical significance (p=0.13). Another group identified that possession of the A allele was associated with elevated serum sTNF-R concentrations in patients with non-Hodgkin's lymphoma and that was associated with reduced overall survival (Juszczynski et al, 2002).

A SNP at the –252 locus of the lymphotoxin α (*LTα*) [tumour necrosis factor β] gene has also been shown to modify levels of TNF production. Septic surgical patients homozygous for the AA genotype had significantly higher mortality than those patients who were homozygous for GG (Stuber et al, 1996). In patients with cancer, homozygotes had a more favourable prognosis than heterozygotes in advanced lung cancer and patients possessing the type 1 allele were shown to have poorer survival in oesophageal cancer (Shimura et al, 1994; O'Mahony et al, 1998).

A SNP at –174 in the *IL-6* promoter region has been associated with increased serum IL-6 concentrations and worse outcome among cardiac surgery patients (Burzotta et al, 2001). Survival data for cancer patients is conflicting. The possession of the C allele has been associated with earlier stage disease and better outcome (independent of stage) in women with ovarian cancer and breast cancer (Hefler et al, 2003; DeMichele et al, 2003). However, the same genotype has also been associated with adverse survival in another group of patients with breast cancer (Iacopetta et al, 2004).

IL-10 is generally regarded as an anti-inflammatory cytokine important in the attenuation of the inflammatory response. The *IL-10* gene polymorphism at position –1082 lies within the promoter region and the AA genotype is generally thought to be associated with reduced levels of IL-10 production, but this is contradicted by one study (Tagore et al, 1999; Huizinga et al, 2000). The high *IL-10* producer genotype (GG) has been linked with more advanced stage among patients with gastric cancer (Wu et al, 2003).

With a view to understanding more clearly the role of host genotype in the genesis of tumour-related systemic inflammation this study examined the relationship between cytokine polymorphisms (*IL-1β -511, IL-6 -174, IL-10 -1082, TNFα -308, and LTα +252*), tumour tissue cytokine concentrations, markers of systemic inflammation, and adverse prognosis in patients with gastro-oesophageal cancer.

MATERIALS AND METHODS

Study Patients

Patients diagnosed with gastric or oesophageal cancer between March 2002 and May 2004 within Lothian and Borders regions were invited to take part in the study. This was the same patient cohort as previously described in Chapter IV. All patients were eligible for genotyping and measurement of serum inflammatory markers (serum cytokines and acute phase proteins). Only those patients who underwent surgical resection were eligible for determination of tissue cytokine concentrations (see Chapter V). Duration of survival, defined as time from histological diagnosis to death, was recorded for all patients.

Cytokine Genotyping

Study patients were genotyped by TaqMan allelic discrimination genotyping as described in Chapter III. In addition to the patient group, blood samples were also collected from healthy controls for genotyping. The control group used in this study comprised 266 British Caucasian bone marrow and solid organ donors, collected via the Histocompatibility & Immunogenetics

Laboratory, Southampton University Hospitals. The mean age of these controls (140 males, 126 females) was 39 years at the time of blood collection (age range 3-69). All cytokine genotyping results in this control group have been published previously (Howell et al, 2005; Smith et al, 2004).

Determination of serum cytokine and serum CRP concentrations

CRP concentrations were measured as a representative of the acute phase response and as a surrogate marker for the presence of systemic inflammation. Serum CRP and serum cytokine concentrations were measured by ELISA as previously described in Chapter III.

Determination of tissue cytokine mRNA and protein concentrations

Samples of tumour tissue were collected from patients at the time of surgical resection and tissue cytokine mRNA and protein concentrations were measured by quantitative PCR and cytometric bead array as described in Chapters III and V.

Histological Analysis

Representative sections of tumour tissue were prepared from each patient who underwent surgical resection. Specimens were fixed with formalin and stained with haematoxylin and eosin. The extent of chronic inflammatory cell infiltrate was assessed by a Consultant pathologist as described in Chapter V. Samples were classified as having a scanty diffuse chronic inflammatory cell infiltrate, a diffuse chronic inflammatory cellular infiltrate present throughout the whole tissue, or focal lymphoid aggregates only.

Statistical Analysis

Comparisons between groups of continuous variables were made by the Mann-Whitney U test. Categorical variables were compared by the Chi-squared test. Correlations between continuous variables were assessed by the Spearman rank correlation coefficient. Survival between groups was analysed by the log-rank test and Cox's proportional hazard's model was used for multivariate analysis. A P value ≤ 0.05 was considered statistically significant. Significant P values were corrected for multiple comparisons using Bonferroni correction.

Genotype expression and patient characteristics

Patient demographics are shown in Table 6.1. 203 patients were genotyped. Cytokine genotype was not associated with patient age or sex or any other demographic characteristics. Neither was there any association with any pathological characteristics, such as tumour stage, histological subtype or tumour grade. In particular, the IL-1β polymorphism was not associated with an increased risk of distal gastric cancer, which has been reported previously (Lee et al, 2003) (data not shown).

Table 6.1 Study patient demographics (n=203)

		Number [%]
Age (years) *		71 (62-78)
Sex	Male	134 [66]
	Female	69 [34]
Tumour Site	Oesophageal	91 [45]
	Oesophago-gastric junction	37 [18]
	Gastric	75 [37]
Histology	Adenocarcinoma	172 [85]
	Squamous cell carcinoma	26 [13]
	Indeterminate	5 [2]
Grade	Well differentiated	5 [2]
	Moderately differentiated	69 [34]
	Poorly differentiated	129 [64]
UICC Stage	1	23 [12]
	2	33 [16]
	3	76 [37]
	4	71 [35]
Treatment undertaken	Surgery with curative intent	91 [45]
	Pre-operative chemotherapy/Surgery	22 [11]
	Chemoradiotherapy with curative intent	7 [3]
	Palliative Chemotherapy	24 [12]
	Palliative Radiotherapy	6 [3]
	Stent/dilatation/laser/symptomatic	53 [26]

*Values are median (interquartile range).

Prevalence of genotype expression

The prevalence of cytokine genotypes is presented in Table 6.2. There was a significant difference between cases and controls with the IL-1β -511 CC (p=0.01, OR = 1.664 (95% CI = 1.137-2.434)) and CT (p=0.03, OR = 0.657 (95% CI = 0.453-0.955) genotypes, but this did not remain so when corrected for multiple comparisons, making this finding most likely occurring due to chance. There were no significant differences between cancer patients and healthy controls in the distribution of any of the other genotypes. All genotype frequencies in both the patient and control groups were distributed in accordance with the Hardy-Weinberg equilibrium except for the IL-6 patient group (p=0.05), the TNFα control group (p=0.04) and the LTα control group (p=0.002).

Table 6.2 Allelic distribution of cytokine genotypes among the study population. Significant P values were corrected for multiple comparisons using Bonferroni correction (values in parenthesises).

Cytokine	Allele	Study Patients n (%)	Controls n (%)	P value*	OR† (95% CI)
IL-1β -511	CC	89 (45.4)	87 (33.3)	0.01 (0.06)	1.664 (1.137-2.434)
	CT	81 (41.3)	135 (51.7)	0.03 (0.15)	0.657 (0.453-0.955)
	TT	26 (13.3)	39 (14.9)	n/s	Ref§
	HWE‡	0.27	0.25		
IL-6 –174	GG	71 (36.0)	79 (35.3)	n/s	1.034 (0.694-1.541)
	GC	83 (42.1)	101 (45.1)	n/s	0.887 (0.603-1.304)
	CC	43 (21.8)	44 (19.6)	n/s	Ref
	HWE	0.05	0.26		
IL-10 –1082	GG	54 (27.0)	57 (25.6)	n/s	1.077 (0.699-1.659)
	AG	93 (46.5)	120 (53.8)	n/s	0.746 (0.509-1.093)
	AA	53 (26.5)	46 (20.6)	n/s	Ref
	HWE	0.32	0.24		
TNFα -308	GG	124 (62.0)	146 (68.2)	n/s	0.760 (0.507-1.139)
	AG	61 (30.5)	56 (26.2)	n/s	1.238 (0.808-1.898)
	AA	15 (7.5)	12 (5.6)	n/s	Ref
	HWE	0.06	0.04		
LTα +252	AA	82 (42.1)	92 (41.8)	n/s	1.010 (0.684-1.491)
	AG	84 (43.1)	83 (37.7)	n/s	1.249 (0.844-1.850)
	GG	29 (14.9)	45 (20.5)	n/s	Ref
	HWE	0.33	0.002		

† Odds Ratio. ‡ P value for Hardy-Weinberg Equilibrium (HWE) (Chi-squared test). § Reference Genotype.

Link between cytokine genotype and markers of systemic inflammation

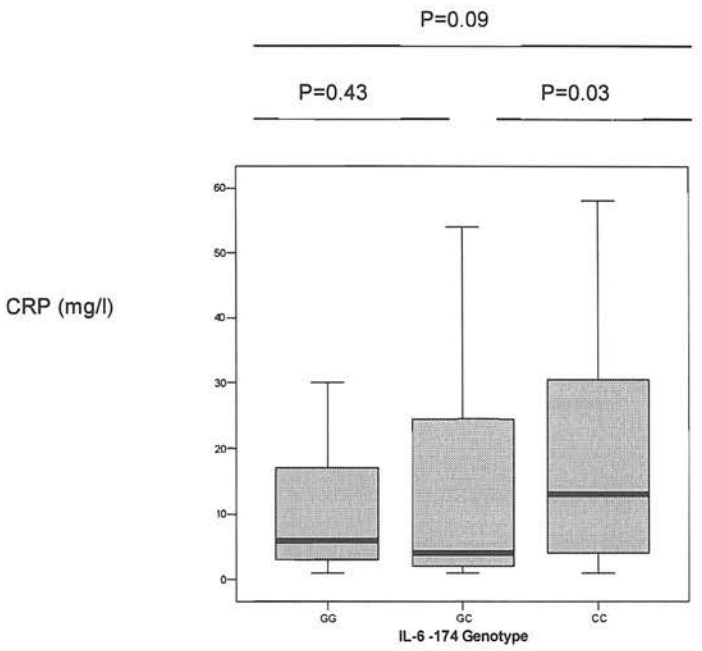
Eighty-two (41%) patients had evidence of systemic inflammation (CRP >10mg/l). The median serum concentration of IL-6 was 9.1pg/ml (interquartile range 0-88pg/ml), IL-8 median concentration 0 pg/ml (interquartile range 0-118 pg/ml), and for sTNF-R the median concentration was 3.3ng/ml (interquartile range 1.9-5.8ng/ml). IL-1β and IL-10 were only detectable in 4 (2%) and 10 (5%) patient's serum respectively.

The IL-6 CC homozygous genotype was associated with significantly elevated serum CRP concentrations when compared with GC and GG genotypes (median 13mg/l [range 4-35mg/l] versus median 6mg/l [range 2-22mg/l]) (p=0.03, Mann-Whitney U test) (Figure 6.1A). This genotype was also associated with elevated serum sTNF-R concentrations (p=0.015; Mann-Whitney U test).

The GG homozygous IL-10 genotype was also associated with elevated serum CRP concentrations (median 16mg/l [range 3-34mg/l]) when compared with the AA genotype (median 6mg/l [range 3-13mg/l]) (p=0.02, Mann-Whitney U test) and the AG genotype (median 5mg/l [range 2-24mg/l]) (p=0.03) (Figure 6.1B). The GG genotype was also associated with elevated serum sTNF-R concentrations (p=0.05; Mann-Whitney U test). Possession of both of these genotypes (IL-6 CC and IL-10 GG) was associated with increasing serum CRP concentrations (p=0.013, Chi square test) (Figure 6.2). None of the other cytokine genotypes were associated with serum CRP or serum cytokine concentrations.

Figure 6.1 Serum C-reactive protein (CRP) concentrations presented by (A) interleukin-6 (IL-6) genotype (GG = 71 patients, GC = 83 patients, CC = 43 patients) and (B) interleukin-10 (IL-10) genotype (AA = 53 patients, AG = 93 patients, GG = 54 patients). The lines represent the median value, bars = inter-quartile range, error bars = extreme values. [Mann-Whitney U Test]

A)



B)

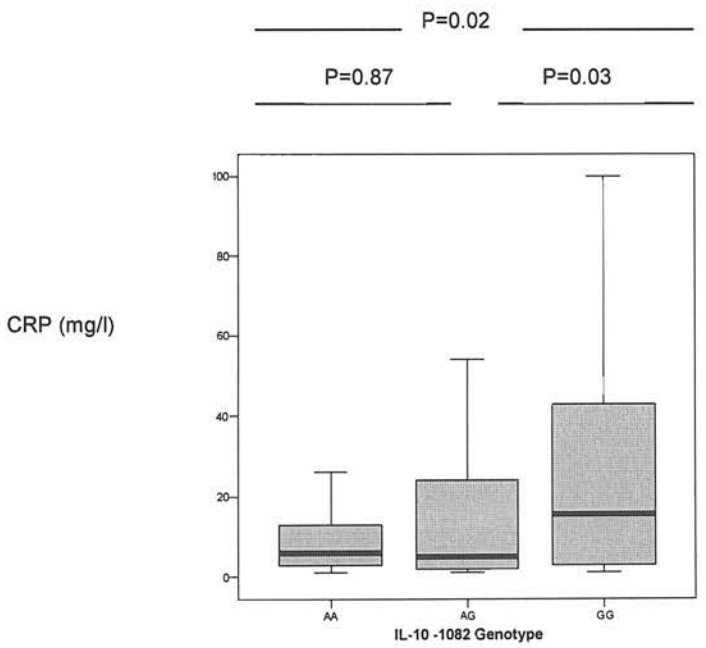
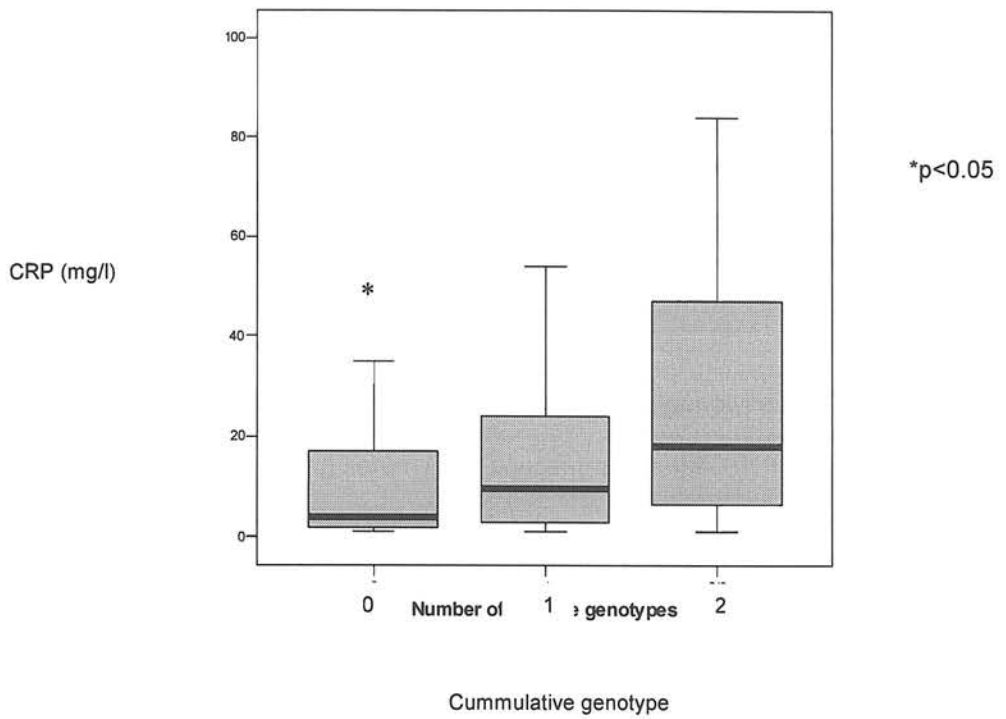


Figure 6.2 Possession of one or both of the following genotypes, IL-6 CC and IL-10 GG, was associated with increasing serum CRP concentrations (p=0.013, Chi squared test).



Link between cytokine genotype and markers of tissue inflammation

(a) Tissue cytokine mRNA and protein concentrations

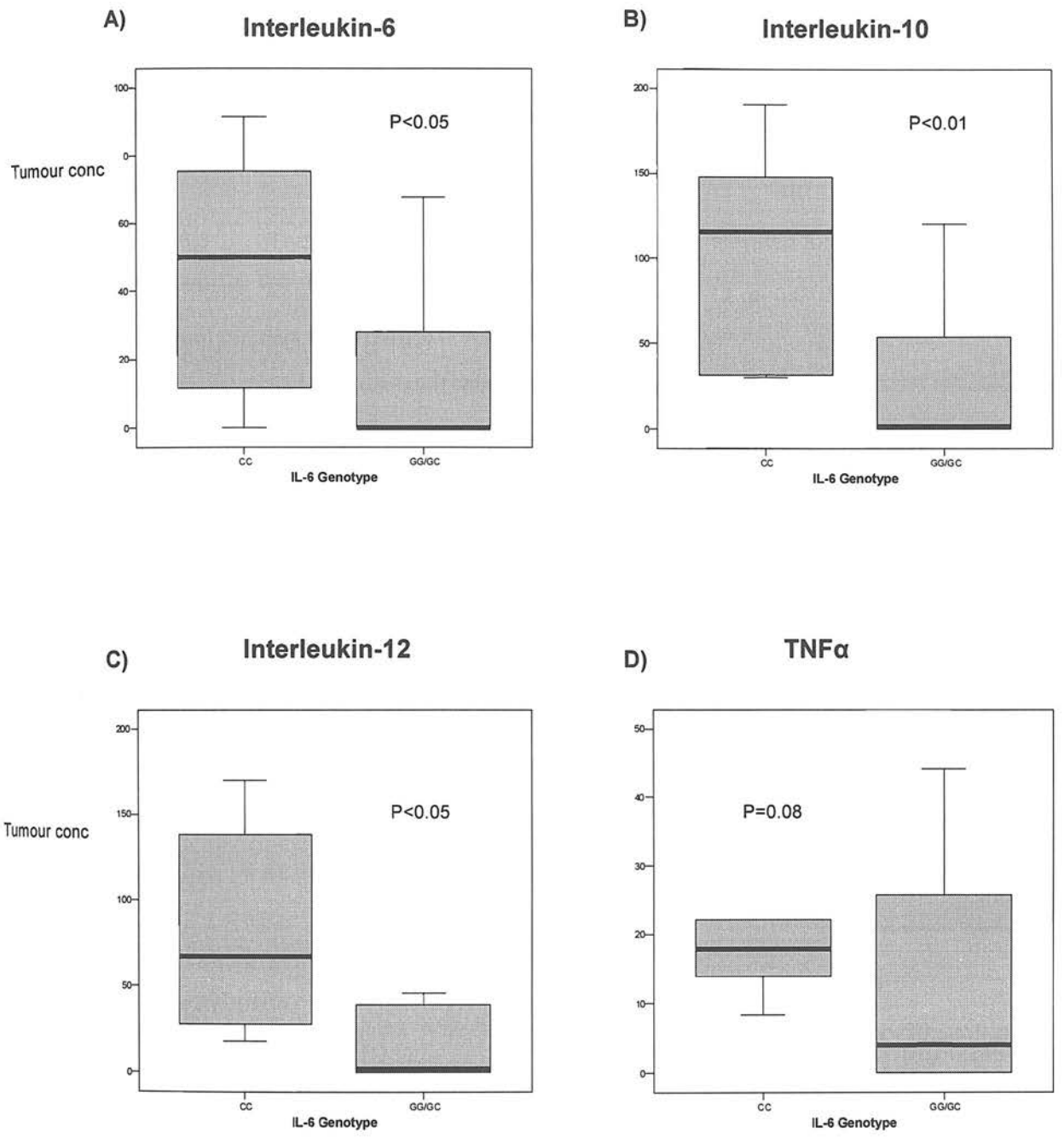
The IL-6 CC genotype was associated with elevated tumour tissue IL-6 (p=0.029; Mann-Whitney U test), IL-10 (p=0.009), and IL-12 (p=0.012) protein concentrations (Table 6.3) (Figure 6.3). There was also a trend for elevated tissue TNFα concentrations, but this did not quite reach statistical significance (p=0.08). Additionally, the IL-1β AA genotype was linked with reduced tumour IL-8 concentrations when compared with the GG genotype (p=0.021) and the AG genotype (p=0.042). None of the other genotypes were associated with tissue cytokine protein or mRNA concentrations.

Table 6.3 The association between host IL-6 genotype and tumour tissue cytokine concentrations measured by cytometric bead array (pg/mg total protein). The IL-6 CC genotype was associated with elevated tumour tissue cytokine concentrations.

Tumour Cytokine (pg/mg total protein)	IL-6 -174 Genotype		
	CC	CG/GG	P value*
IL-1β	270 (121-298)	123 (30-438)	0.34
IL-6	50 (9-79)	0 (0-30)	0.029
IL-8	106 (22-164)	54 (19-204)	0.62
IL-10	115 (31-158)	2 (0-69)	0.009
IL-12	67 (25-146)	2 (0-43)	0.012
TNFα	18 (13-27)	4 (0-27)	0.08

Values are median (interquartile range). *Mann-Whitney U test.

Figure 6.3 The IL-6 CC genotype was associated with elevated tumour tissue cytokine concentrations. A) IL-6 (p=0.029); B) IL-10 (p=0.009); C) IL-12 (p=0.012); and D) TNFα (p=0.08). [Mann-Whitney U Test.]



(b) Histological analysis

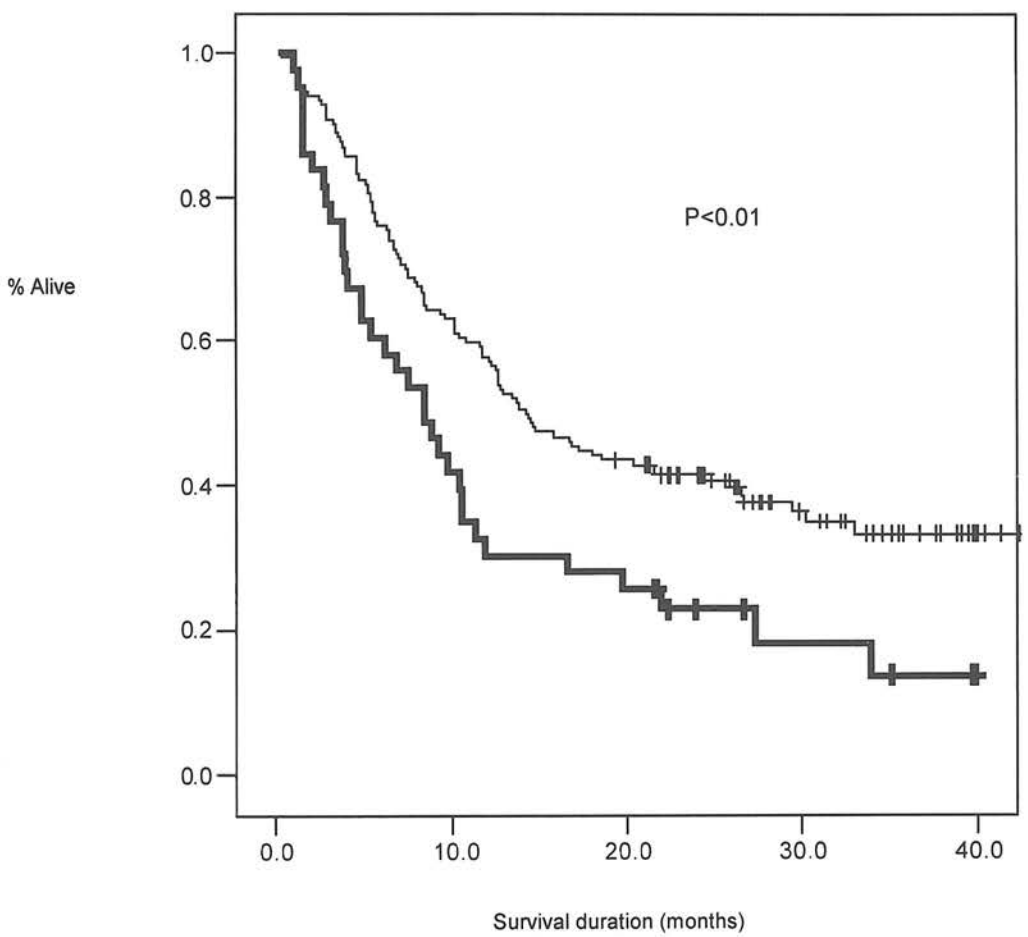
Tissue sections were available for 53 of the 203 patients genotyped (see Chapter V). 24 (45%) tumour samples were classified as having scanty diffuse or patchy chronic inflammatory cells. 16 (30%) had a diffuse chronic inflammatory cellular infiltrate visible throughout the whole tumour. The remaining 13 (25%) tumour sections had focal lymphoid aggregates only. There was no association between the extent of inflammatory cell infiltrate into the tumour tissue and any of the cytokine genotypes.

Association between cytokine genotype and survival duration

Survival of patients by genotype is presented in Figures 6.4 and 6.5. Possession of the CC genotype for IL-6 was associated with reduced survival duration when compared with GG or GC genotype (median survival 8.4 months versus 14.1 months) (p=0.004; Log-rank test) (Figure 6.4A). The homozygous genotype GG for IL-10 was associated with reduced survival compared with AA/AG genotypes (median survival 10.2 months versus 12.8 months, p=0.047) (Figure 6.4B). Similarly, AA homozygosity for the TNFα genotype was associated with adverse prognosis (median survival 6.4 months versus 13.4 months, p=0.020) (Figure 6.4C). Furthermore, the cumulative possession of more than one of these genotypes was associated with reduced survival (no alleles, median survival 16.8 months; 1 allele, median survival 8.5 months; 2 or 3 alleles, median survival 8.8 months; p=0.001) (Figure 6.5). Genotypes for IL-1β and LTα were not associated with prognosis.

Figure 6.4 Kaplan-Meier survival plots presented by (A) interleukin-6 (IL-6) genotype (CC median survival 8.4 months [heavy line], GC/GG median survival 14.1 months [light line]) (p=0.004; Log-rank test) and (B) interleukin-10 (IL-10) genotype (GG median survival 10.2 months [heavy line], AA/AG median survival 12.8 months [light line]) (p=0.047) and (C) tumour necrosis factor α (TNFα) genotype (AA median survival 6.4 months [heavy line], AG/GG median survival 13.4 months [light line]) (p=0.020).

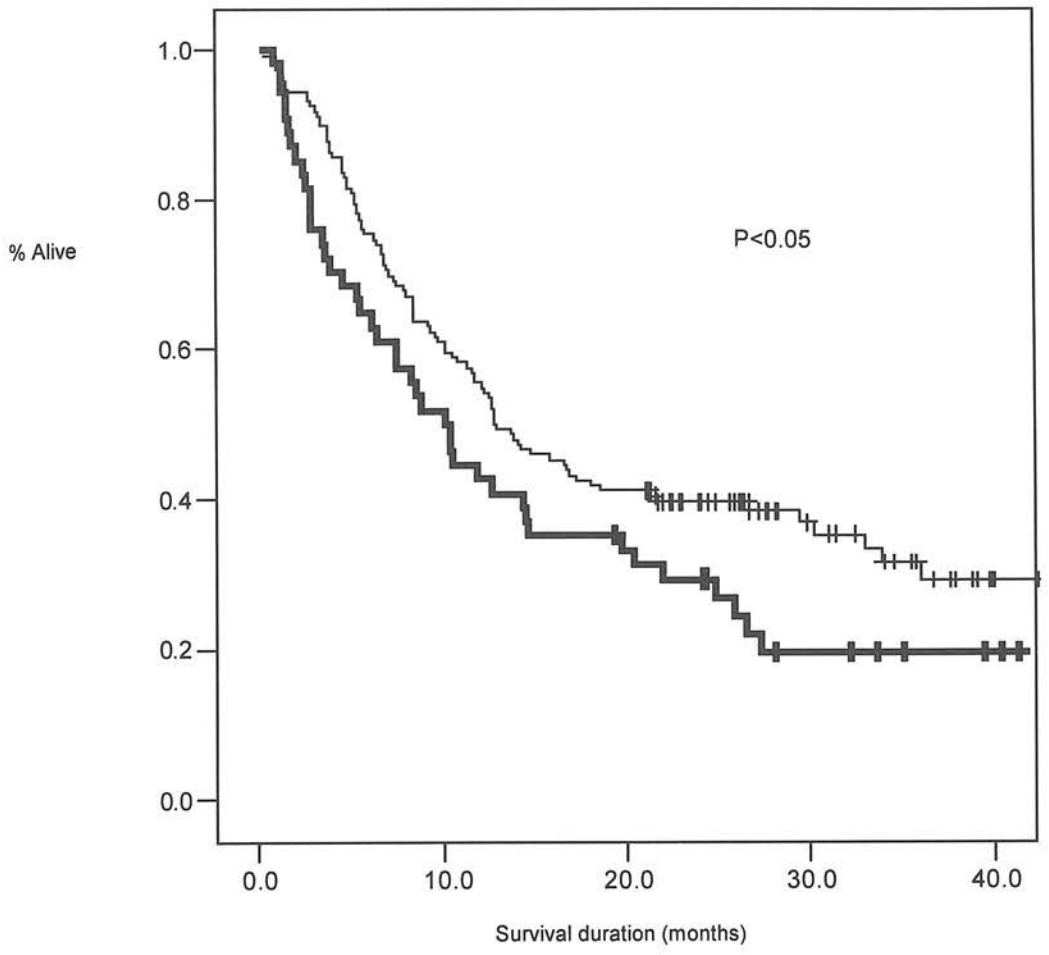
A)



Number at risk (Genotype):

CC	43	18	11	4	0
CG/GG	154	97	66	26	4

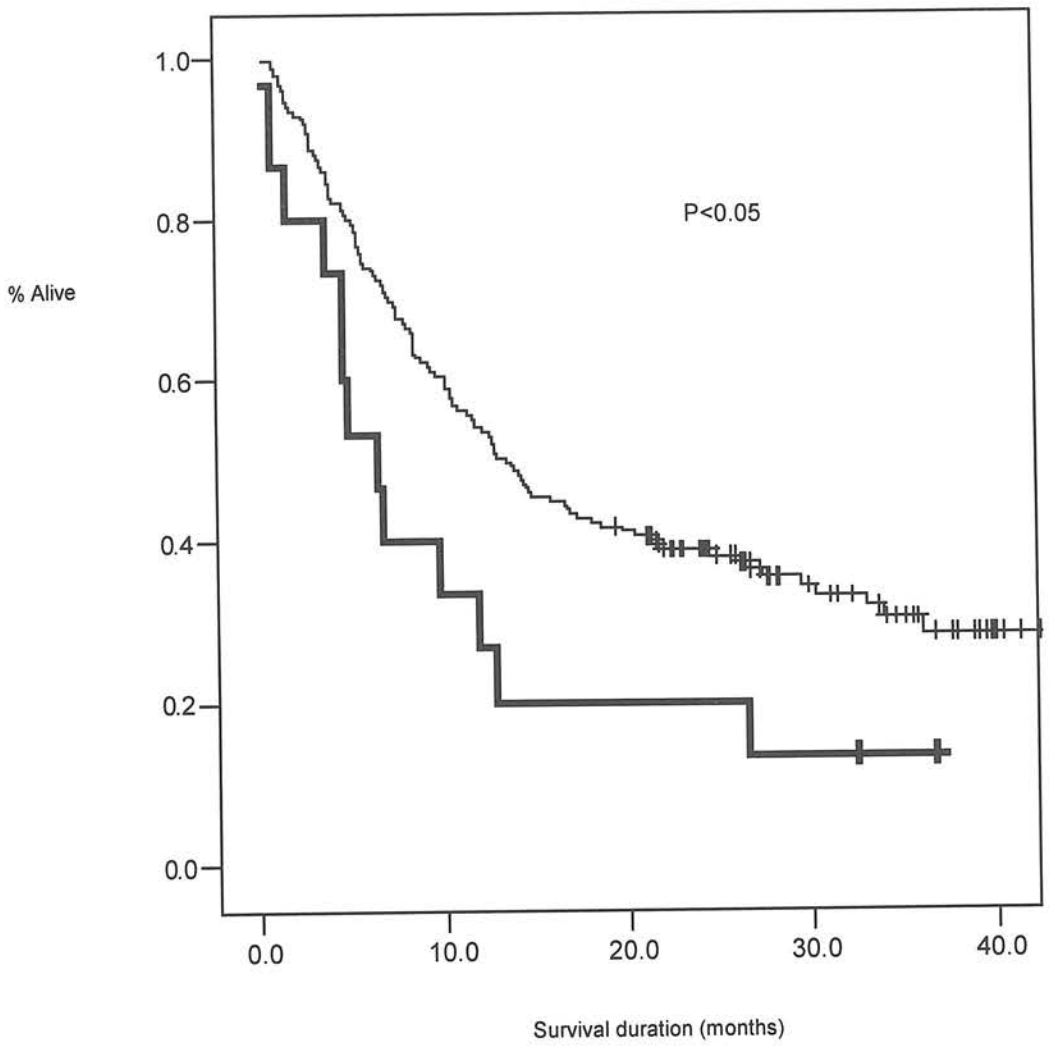
B)



Number at risk (Genotype):

GG	54	28	17	7	2
AG/AA	146	89	60	23	2

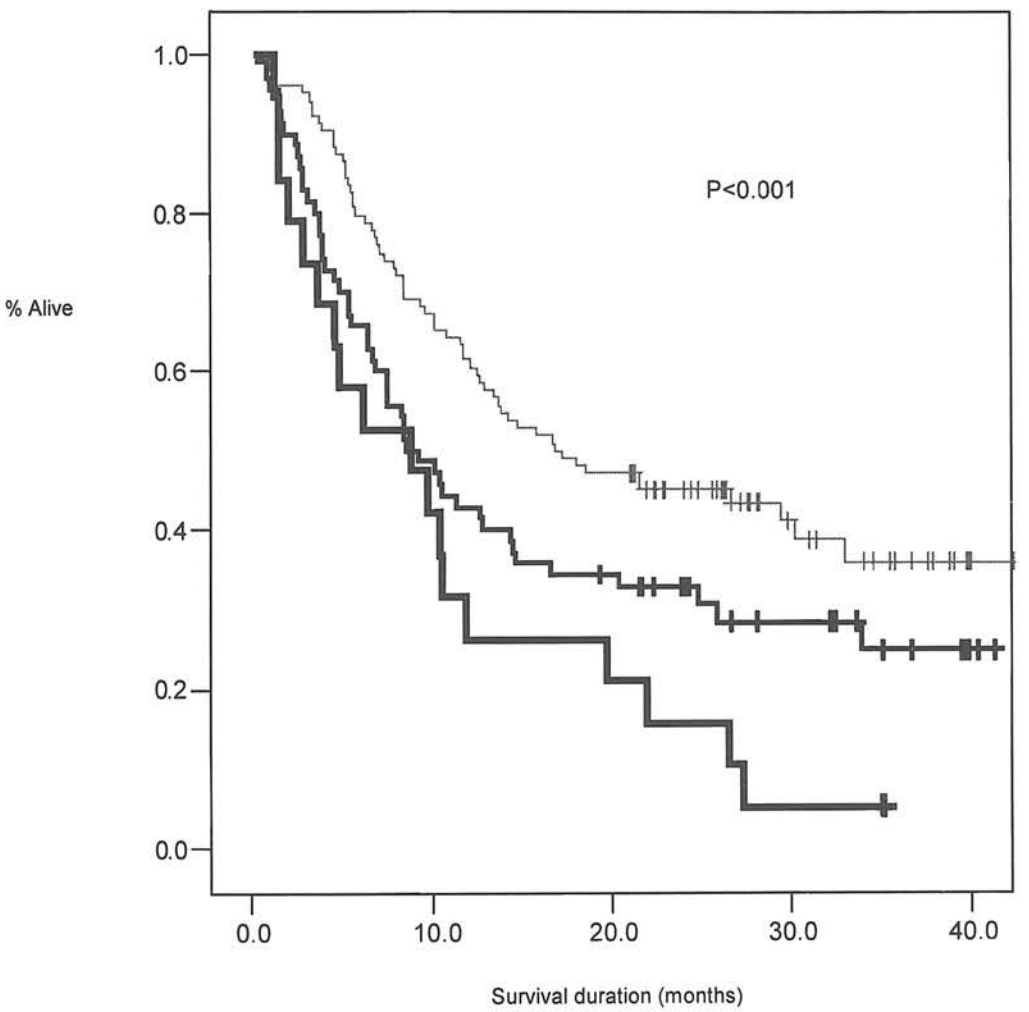
C)



Number at risk (Genotype):

AA	15	5	3	2	0
AG/GG	185	112	75	29	4

Figure 6.5 Kaplan-Meier survival plot stratified for the cumulative possession of interleukin-6 (IL-6) CC genotype, interleukin-10 (IL-10) GG genotype and tumour necrosis factor α (TNFα) AA genotype. Possession of no alleles (light line), median survival 16.8 months; 1 allele (medium line), median survival 8.5 months; 2 or 3 alleles (heavy line), median survival 8.8 months (p=0.001; Log-rank test).



Number at risk (Genotype):

0 Alleles	104	70	49	17	2
1 Allele	70	34	23	11	2
2/3 Alleles	19	8	4	1	0

Multivariate survival analysis

Multivariate analysis identified the AA genotype for TNFα as an independent adverse prognostic indicator when tested with age, sex, stage of disease, grade of tumour and serum CRP concentration (p=0.006, hazard ratio 2.5; Cox's proportional hazard's model) (Table 6.4). Increasing age, male sex, advanced disease stage, elevated serum CRP concentration, and the TNFα AA genotype were all independently associated with poor prognosis. Genotypes for IL-6 and IL-10 were not independent prognostic indicators (p=0.48 and p=0.18 respectively).

Table 6.4 Multivariate survival analysis of 203 patients with gastro-oesophageal cancer using Cox's proportional hazard's model. A hazard ratio greater than 1 represents an increased risk of death. Increasing age, male sex, advanced disease stage, elevated serum CRP concentration, and the TNFα AA genotype were all independently associated with poor prognosis.

Variable	P value	Hazard Ratio	95% CI
Age	0.007	1.2	1.0-1.3
Sex	0.04	1.8	1.0-3.0
Stage of disease	<0.001	2.4	1.8-3.2
Grade of tumour	0.88	1.0	0.7-1.6
Serum CRP	0.002	1.1	1.0-1.2
TNFα AA genotype	0.006	2.5	1.3-4.9

DISCUSSION

This Chapter has investigated the influence of host cytokine genotype on serum markers of systemic inflammation and tumour tissue cytokine concentrations. IL-6 –174 CC and IL-10 –1082 GG genotypes were associated with elevated markers of systemic inflammation (serum CRP and sTNF-R concentrations) and the IL-6 –174 CC genotype was also associated with elevated tumour tissue cytokine concentrations (IL-6, IL-10 and IL-12). These two genotypes were also linked with adverse prognosis. In addition, the TNFα -308 AA genotype was also linked with adverse outcome and was identified as an independent prognostic indicator, but this genotype was not associated with markers of systemic inflammation. These findings suggest that the cytokine genotype of the host may influence the ability of the patient to generate a systemic inflammatory response in association with gastro-oesophageal cancer. In addition, cytokine genotype may influence prognosis in these patients.

Genotype prevalence in the study population

The distributions of genotypes were similar between cancer patients and controls in this study and frequencies were similar to those previously published in studies of similar populations (Howell et al, 2005; Smith et al, 2004). Although we did identify an association between gastro-oesophageal cancer and the IL-1β -511 CC genotype, this relationship lost its significance following correction for multiple comparisons. El-Omar et al have proposed an association between IL-1β polymorphisms and an increased risk of gastric cancer among patients with Helicobacter pylori infection (El-Omar et al, 2000). We found no such association on sub-group analysis and other groups have similarly failed to demonstrate such an association (Lee et al, 2003; Kato et al, 2001).

Cytokine genotype and serum/tissue markers of inflammation

The IL-6 CC genotype was associated with elevated serum concentrations of CRP and sTNF-R. A previous study of healthy volunteers identified the presence of the -174C allele to be associated with higher baseline CRP levels (Westerberg et al, 2004). In cancer patients the rates of production of IL-6 can be linked to markers of systemic inflammation such as CRP.

Although our study did not identify any association between IL-6 genotype and serum IL-6 concentrations, we did find an association with the CC genotype and tumour tissue cytokine concentrations (including IL-6). It is possible that the CC genotype may be associated with elevated IL-6 production, which acts at the tissue level to promote an acute phase response. The similar association between IL-6 genotype and sTNF-R may reflect the more stable nature of the receptor molecule compared with the other cytokines, and in this regard sTNF-R may behave more as a marker of systemic inflammation. The positive association between the IL-6 CC genotype and tumour tissue cytokine concentrations occurred without apparent differences in the level of chronic inflammatory cellular infiltrate into the tissues. It is therefore possible that this genotype is associated with elevated tumour cell production of these cytokines, which, in turn, can be linked with markers of systemic inflammation (CRP).

We also found an association between the GG IL-10 genotype and elevated serum CRP and sTNF-R concentrations. The –1082 AA polymorphism is generally thought to be associated with reduced levels of IL-10 production (Tagore et al, 1999). One would therefore expect the GG genotype to be associated with increased levels of IL-10 production. An association between increased levels of an anti-inflammatory cytokine and elevated concentrations of acute phase proteins may initially appear contradictory, but may simply reflect the increased counter-regulatory activity of this important anti-inflammatory mediator in response to the presence of systemic inflammation.

We found no association between IL-1β, TNFα, or LTα genotypes and CRP concentrations. In pancreatic cancer patients an association between allele 2 IL-1β genotype and elevated serum CRP levels has been documented, but in the present study there was no such association between the SNP at position –511 and CRP levels (Barber et al, 2000). Similarly, there was no association between TNFα polymorphisms and CRP, a finding also in support of previous work on pancreatic cancer patients (Barber et al, 1999b). Data relating to the –308 polymorphism and levels of TNFα production remain contradictory, however polymorphisms at this locus do not appear to influence serum CRP levels. Serum CRP concentrations were measured in a group of patients who had undergone cardiac surgery and this study did not find any differences between CRP levels and TNFα –308 genotypes

(Gander et al, 2004). Another group similarly failed to demonstrate differences in CRP concentrations by –308 genotype among smokers (El-Omar et al, 2000).

This study failed to show any association between cytokine genotype and serum cytokine concentrations (except for sTNF-R). This lack of association between serum cytokine concentrations and serum acute phase protein concentrations, tissue cytokine concentrations, and now cytokine genotypes simply reinforces the feeling that circulating cytokines have little functional relevance in systemic inflammation. It would seem that local tissue cytokine concentrations are the key determinant. IL-1β genotype has been linked with levels of IL-1β production by peripheral blood mononuclear cells (PBMC) in pancreatic cancer patients, but not with serum IL-1β concentrations (Barber et al, 2000). PBMC production of IL-1β may better reflect tissue IL-1β levels rather than circulating concentrations.

Cytokine genotype and prognosis

The IL-6 –174 CC and the IL-10 –1082 GG genotypes were associated with reduced survival duration. The association between systemic inflammation and adverse prognosis among cancer patients has been well documented and in this study we have shown these two genotypes to be associated with elevated acute phase protein (CRP) concentrations. It is therefore possible that the adverse prognosis associated with these polymorphisms is related to the presence of systemic inflammation. This is supported by multivariate analysis where these genotypes lost their significance as prognostic indicators when CRP was co-analysed. The CC IL-6 genotype has been linked with adverse prognosis among breast cancer patients, where possession of the CC polymorphism was associated with higher grade tumours and worse overall survival (Iacopetta et al, 2004). The GG genotype for IL-10 –1082 has previously been linked with advanced stage and reduced survival in gastric cancer patients (Wu et al, 2003).

We also found the AA TNFα -308 genotype to be related to adverse prognosis. In contrast to the IL-6 and IL-10 genotypes, we found no association between TNFα polymorphisms and systemic inflammation. It is therefore less likely that the reduced survival associated with this cytokine is related entirely to the generation of an inflammatory response. In addition, the AA genotype was an independent prognostic indicator on multivariate

analysis, independent of CRP concentration, stage and other clinico-pathological characteristics that have previously been associated with adverse prognosis. The AA genotype has previously been associated with reduced survival in pancreatic cancer patients and other groups have similarly found the possession of the A allele to be linked with adverse outcome in patients with non-Hodgkin's lymphoma (Barber et al, 1999b, Juszczynski et al, 2002). However, the AA genotype was only identified in 8% of patients, making it less useful in the clinical setting. The association between systemic inflammation and prognosis will be explored in detail in Chapter IX.

Conclusions

IL-6 CC and IL-10 GG genotypes are associated with markers of systemic inflammation in patients with gastro-oesophageal cancer. In addition, the IL-6 CC genotype is linked with elevated tumour tissue cytokine concentrations, but not with an increased chronic inflammatory cell infiltrate. In the previous Chapter, elevated tumour tissue cytokine concentrations have been identified in patients with systemic inflammation and tissue concentrations of IL-1β have been correlated with serum CRP concentrations. It is likely that the host genotype may influence levels of pro-inflammatory cytokine production in host immune cells or cancer cells or both. These increased cytokine levels can be measured in the tumour tissue and are associated with elevated serum acute phase protein concentrations and this translates into the generation of systemic inflammation. In addition, these genotypes are markers of adverse outcome in patients with gastro-oesophageal malignancy, possibly through the association with systemic inflammation. The association between systemic inflammation and prognosis will be explored further in Chapter IX.

The TNFα AA genotype was also linked with adverse prognosis and this association was independent of systemic inflammation. Therefore, there is a pro-inflammatory cytokine haplotype (IL-6 CC, IL-10 GG and TNFα AA) that is associated with adverse prognosis among patients with gastro-oesophageal cancer that may act in association with, but not entirely with, an inflammatory mediated mechanism.

The previous Chapters have so far investigated the role of cytokines in the aetiology of systemic inflammation in patients with gastro-oesophageal cancer. The following two

Chapters (VII and VIII) will investigate the role of tumour-derived mediators in the aetiology of systemic inflammation in these patients. The two mediators that will be studied are parathyroid hormone-related peptide (PTHrP) and proteolysis-inducing factor (PIF).

CHAPTER VII

SERUM PARATHYROID HORMONE-RELATED PEPTIDE (PTHrP) AS A POTENTIAL MEDIATOR OF SYSTEMIC INFLAMMATION AND IT'S RELATIONSHIP TO ADVERSE PROGNOSIS AND ADVERSE NUTRITIONAL STATUS IN PATIENTS WITH GASTRO-OESOPHAGEAL CANCER

Deans DAC, Black J, Paterson-Brown S, Wigmore SJ, Ross JA, Fearon KCH.
Serum parathyroid hormone-related peptide (PTHrP) contributes to systemic inflammation and is associated with adverse prognosis in gastro-oesophageal cancer
Cancer 2005; 103(9): 1810-1818

ABSTRACT

Serum concentrations of parathyroid hormone-related peptide (PTHrP) have been linked with elevated markers of systemic inflammation, cachexia and adverse outcome in patients with cancer. This Chapter investigated PTHrP expression in a cohort of patients with gastro-oesophageal malignancy and examined the role of PTHrP as a potential mediator of systemic inflammation. Any relationship with adverse prognosis and adverse nutritional status in these patients was also investigated.

Blood was collected from patients at the time of diagnosis for determination of PTHrP (radio-immunoassay), calcium, and serum cytokines and acute phase proteins (ELISA) concentrations. Nutritional assessment was undertaken at the same time as serum collection. Patients underwent routine staging and treatment and survival duration was recorded. Around one-third of patients had an additional serum PTHrP measured approximately three months following the initial measurement.

151 patients were studied. Six (4%) patients were hypercalcaemic. 17% patients had elevated serum PTHrP levels at diagnosis and 49% patients had elevated levels at three months, irrespective of treatments undertaken. There was no association between PTHrP and serum calcium concentrations. An elevated serum PTHrP concentration was associated with markers of systemic inflammation (elevated concentrations of serum CRP and sTNF-R and reduced concentrations of albumin and transferrin). Elevated PTHrP concentrations were also linked with reduced survival duration, but this did not reach significance on longer follow-up ($p=0.23$). PTHrP concentrations measured later in the course of the disease (3 months) correlated significantly with markers of adverse nutritional status.

These findings suggest that PTHrP may play a role in the generation of systemic inflammation in patients with gastro-oesophageal malignancy. Therefore, mediators other than pro-inflammatory cytokines, such as PTHrP, may contribute to the generation of systemic inflammation among some patients with cancer. The relationship between systemic inflammation and cachexia/decreased survival may provide a possible indirect role for PTHrP in these processes.

INTRODUCTION

In order to investigate further the aetiology of systemic inflammation in patients with gastro-oesophageal cancer attention is now directed towards the potential role of tumour-derived factors. Both parathyroid hormone-related peptide (PTHrP) and proteolysis-inducing factor (PIF) have been linked with elevated markers of systemic inflammation in animal and human studies (Ogata, 2000; Takahashi et al, 2003; Pollock et al, 1996; Funk et al, 1997; Funk et al, 1998). These mediators have also been associated with cachexia and adverse outcome in patients with cancer (Iguchi et al, 2001; Todorov et al, 1996; Wigmore et al, 2000; Cariuk et al, 1997; Cabal-Manzano et al, 2001). PIF was investigated in Chapter VIII. This Chapter investigated the role of PTHrP as a potential mediator of systemic inflammation in the cohort of patients with gastro-oesophageal malignancy and examined any relationship with adverse prognosis and adverse nutritional status in these patients.

Parathyroid hormone-related peptide (PTHrP)

PTHrP is a tumour-derived circulating factor that has been associated with hypercalcaemia of malignancy, affecting up to 20% of patients during the advanced stages of cancer (Twycross, 1997; Suva et al, 1987). Elevated serum PTHrP has been reported in 50-90% of hypercalcaemic cancer patients (Truong et al, 2003). PTHrP mimics the actions of parathyroid hormone on the kidneys and bone to increase serum calcium concentration. More recently, PTHrP has been proposed as a growth factor whose activity is thought to promote tumour growth, particularly aiding the formation of osteolytic bone metastases (Guise, 1997).

Association with markers of inflammation

PTHrP may contribute to the pro-inflammatory cytokine cascade and several studies have identified elevated serum PTHrP concentrations in association with elevated serum cytokine concentration in cancer patients (Ogata, 2000; Takahashi et al, 2003). PTHrP has been shown to stimulate IL-6 production from osteoblasts *in vivo* (Pollock et al, 1996). Funk *et al* investigated the effects of endotoxaemia on PTHrP synthesis within hepatocytes. Near-lethal dose of endotoxin resulted in marked induction of PTHrP mRNA levels and PTHrP protein

synthesis (Funk et al, 1997). In addition, administration of PTHrP to mice increased serum acute phase protein levels. This group also found that synovial cells from patients with rheumatoid arthritis produced increased levels of PTHrP, and these levels were further enhanced by addition of IL-1β and TNFα (Funk et al, 1998).

PTHrP expression in gastro-oesophageal cancer

Among patients with oesophageal cancer, elevated serum PTHrP has been described in association with hypercalcaemia and PTHrP mRNA and protein have also been demonstrated within cancer cells (Jais et al, 1997). A large series from Japan, which included 382 patients with oesophageal cancer identified hypercalcaemia in 1.3% of patients at the time of diagnosis (Tachmori et al, 1991). The incidence of hypercalcaemia increased to 38% in patients who were monitored within 2 months of death and this study reported a close association between hypercalcaemia and PTHrP levels. Similarly, elevated PTHrP production has been reported in association with gastric cancer (Abdeen et al, 1995; Engelich et al, 2000). One study identified PTHrP expression within tumours cells in 71 out of 92 patients with gastric adenocarcinoma, none of whom had hypercalcaemia (Alipov et al, 1997). These authors also noted that PTHrP production was associated with poorly differentiated tumours.

Association with cancer cachexia

PTHrP has also been proposed as a pro-cachectic factor. PTHrP is produced by a human lung cancer model (HARA-B) and, when implanted into mice, results in a significant reduction in body weight and tissue mass, an effect that is reversed by addition of an antibody to PTHrP (Iguchi et al, 2001). This model was associated with elevated serum calcium levels and it is not clear whether the observed cachexia was due to the systemic effects induced by hypercalcaemia, as food intake in the weight-losing mice was lower than controls. The relationship of PTHrP to adverse nutritional status needs further investigation.

Link with prognosis in cancer

Recent interest in PTHrP has been directed towards its role in prognosis. A study of 76 hypercalcaemic patients with several types of cancer with advanced stage found elevated

serum PTHrP in 54% (Truong et al, 2003). Increased PTHrP was an independent adverse prognostic indicator for patients less than 65 years of age but had no prognostic role in older patients. Others studies have found no association between elevated serum PTHrP and reduced survival (Lee et al, 1997). The detection of PTHrP within tumours themselves and its role in prognosis has similarly found differing conclusions. Patients with breast cancer that produce PTHrP were found to have a favourable prognosis in one study, but this observation was not corroborated by others (Henderson et al, 2001; Kissin et al, 1993). Administration of a PTHrP antibody to mice bearing PTHrP secreting tumours not only resulted in a reduction of serum calcium levels but also improved survival duration in the treated mice (Sato et al, 1993). The role of PTHrP in determining prognosis remains unclear.

PTHrP as a potential growth factor

The detection of PTHrP in tumours without associated hypercalcaemia has raised the possibility that PTHrP may have a role in addition to calcium homeostasis, such as the regulation of cellular proliferation and differentiation. Epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) have been implicated in the progression of gastric cancers and these growth factors have been found to up-regulate PTHrP gene expression in some cell lines (Kiriyama et al, 1993). Rats implanted with prostate cancer cells that over-produce PTHrP had significantly enhanced tumour growth and tumour size compared with controls (Dougherty et al, 1999). The gene encoding PTHrP is a downstream target for the proto-oncogenes K-ras and src tyrosine kinase (Li and Drucker, 1994; Kamai et al, 2001). While src expression is common in premalignant epithelium of Barrett's oesophagus (Kumble et al, 1997), c-K-ras expression is rare (Arber et al, 2000). Whether the expression of src in gastro-esophageal cancer correlates with increased expression of PTHrP and hypercalcemia is unknown but may provide a mechanism for the functional activation of tumour-derived PTHrP in this tumour type. Whether PTHrP expression alone is sufficient to cause hypercalcemia is also unclear, but a requirement for further modification might explain why PTHrP may be produced without associated hypercalcaemia.

This Chapter will, therefore, investigate the role of PTHrP as a potential mediator of systemic inflammation in the cohort of patients with gastro-oesophageal malignancy and examine any relationship with adverse prognosis and adverse nutritional status in these patients.

MATERIALS AND METHODS

Study population and sample collection

The study population was the same patient cohort as described in previous Chapters. Blood samples were collected at the time of recruitment to the study and before any treatment was commenced. A second blood sample was collected from a subgroup of patients following initiation of treatment after a mean time interval of 95 days (range 41-151 days) from the time of the first sample collection. No patients had symptoms related to possible hypercalcaemia and none had bone metastases identified following computerised tomography staging. None were receiving treatment either to supplement or reduce calcium levels. Renal function was assessed by measuring serum creatinine concentration and values were within the normal range for all patients. Clinical and pathological information was recorded for each patient, including treatment modality, tumour grade and histological subtype. Duration of survival, defined as time from histological diagnosis to death, was recorded for all patients.

Nutritional assessment and performance status scoring

Nutritional assessment included calculation of body mass index (BMI) and performing anthropometry measurements (Chapter III). Muscle wasting was not sufficiently severe to require correction for muscle bulk. Performance status was assessed using the Karnofsky index (Karnofsky and Burchenal, 1949).

Measurement of serum PTHrP and serum calcium concentrations

Serum cPTHrP concentrations were measured by radio-immunoassay (Daiichi, Tokyo, Japan) as previously described (Chapter III). Using this assay, serum PTHrP levels in healthy

volunteers were 13.8 - 55.3 pmol/l for males and 13.9 - 54.0 pmol/l for females (Fukunaga, 1992). No female patients had a cPTHrP serum concentration between 54.0 - 55.3 pmol/l, and therefore for simplicity serum levels above 55.3 pmol/l were considered above the normal range for both sexes. Serum total calcium was measured by an automated analyser (Clinical Biochemistry Department, Edinburgh Royal Infirmary, UK). The normal range for corrected calcium in our laboratory is 2.12 – 2.62 mmol/l.

Measurement of serum cytokine and serum acute phase protein concentrations

Serum acute phase protein concentrations and serum cytokine concentrations were measured by ELISA as described in Chapter III.

Statistical analysis

Correlation between serum cPTHrP and serum calcium concentrations was tested by linear regression analysis. Analysis of serum cPTHrP and continuous variables was tested by the Mann-Whitney U test. Analysis of serum cPTHrP and categorical data was undertaken with chi-square test and Fisher's exact test. Log-rank analysis was used to determine prognostic value for univariate analysis and Cox's proportional hazard's model was used for multivariate analysis. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Study patient demographics

Patient demographics are shown in Table 7.1. 151 patients were recruited to this study. Sixty (40%) patients underwent surgical resection, 14 of these received pre-operative chemotherapy. Three patients, all of whom had squamous cell carcinoma of the oesophagus, received chemo-irradiation with curative intent. The remaining 88 patients received a range of palliative treatments, such as palliative chemotherapy, palliative radiotherapy, or insertion of a stent or endoscopic laser therapy. Performance status was assessed using the Karnofsky index. The median score was 90 (interquartile range 70-100) and the value correlated

strongly with stage (p<0.0001) and mortality (p<0.0001) (data not shown). The mean follow-up period was 31 months (range: 22 months – 42 months). At the end of this follow-up period 103 (68%) patients had died.

Table 7.1 Demographics for the whole study population (n=151). Demographics are also shown for the subgroup of patients who provided a second blood sample after a mean time interval of 95 days (n=51). [+]Values are median (interquartile range).

		Whole Group (n = 151)	**Sub-group (n = 51)**
		Number [%]	
Age (years) [+]		72 (63-78)	68 (61-75)
Sex	Male	95 [63]	36 [71]
	Female	56 [37]	15 [29]
Tumour Site	Oesophageal	64 [42]	30 [59]
	Oesophago-gastric junction (OGJ)	28 [18]	10 [20]
	Gastric	59 [39]	11 [22]
Histology	Adenocarcinoma	132 [87]	47 [92]
	Squamous cell carcinoma	16 [11]	4 [8]
	Indeterminate	1 [1]	-
	Small cell carcinoma	2 [1]	-
Grade	Well differentiated	-	-
	Moderately differentiated	37 [25]	21 [41]
	Poorly differentiated	114 [76]	30 [59]
UICC Stage	1	13 [9]	10 [20]
	2	24 [16]	11 [22]
	3	54 [36]	22 [43]
	4	60 [40]	8 [16]
Treatment undertaken	Surgery with curative intent	46 [31]	30 [59]
	Pre-operative chemotherapy + Surgery	14 [9]	13 [25]
	Chemoradiotherapy with curative intent	3 [2]	-
	Palliative Chemotherapy	17 [11]	-
	Palliative Radiotherapy	4 [3]	-
	Stent/dilatation/laser/symptomatic	67 [44]	8 [16]
Survival	Alive	48 [32]	32 [63]
	Dead	103 [68]	19 [37]

Distribution of serum cPTHrP and calcium concentrations in the study population

The mean serum level of cPTHrP was 39.4 pmol/l (range 8.6 - 100.0 pmol/l; S.D. 17.9) (Figure 7.1). Twenty-six (17%) patients had elevated cPTHrP serum levels. The mean serum calcium concentration was 2.29 mmol/l (range 1.67 – 2.79 mmol/l; S.D. 0.17). Twelve (8%) patients were hypocalcaemic and 6 (4%) were hypercalcaemic. There was no correlation between serum cPTHrP and corrected calcium levels (linear regression; r=0.03, p = 0.72) (Figure 7.2). Patients with elevated serum cPTHrP did not have associated elevated serum calcium levels (p=0.63, Mann-Whitney U Test).

Figure 7.1 Histograms representing the distribution of serum cPTHrP concentrations within the study population at the time of diagnosis.

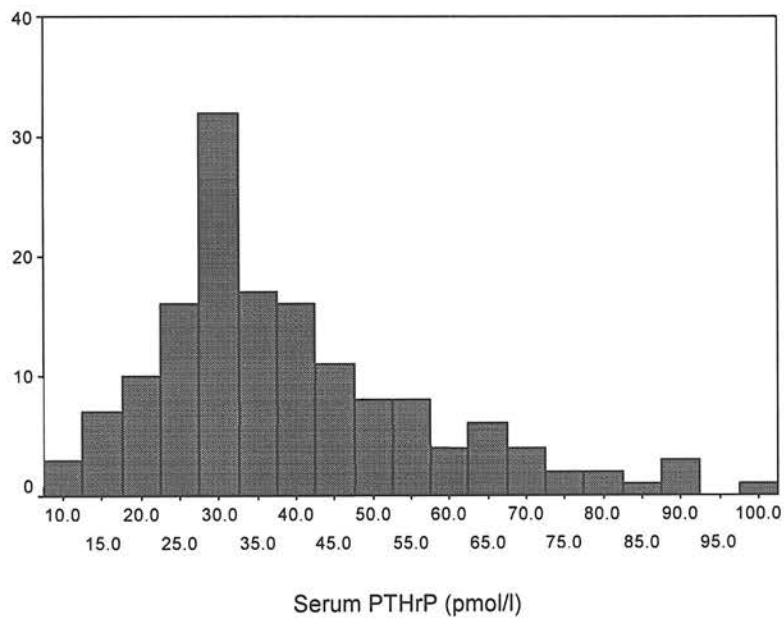
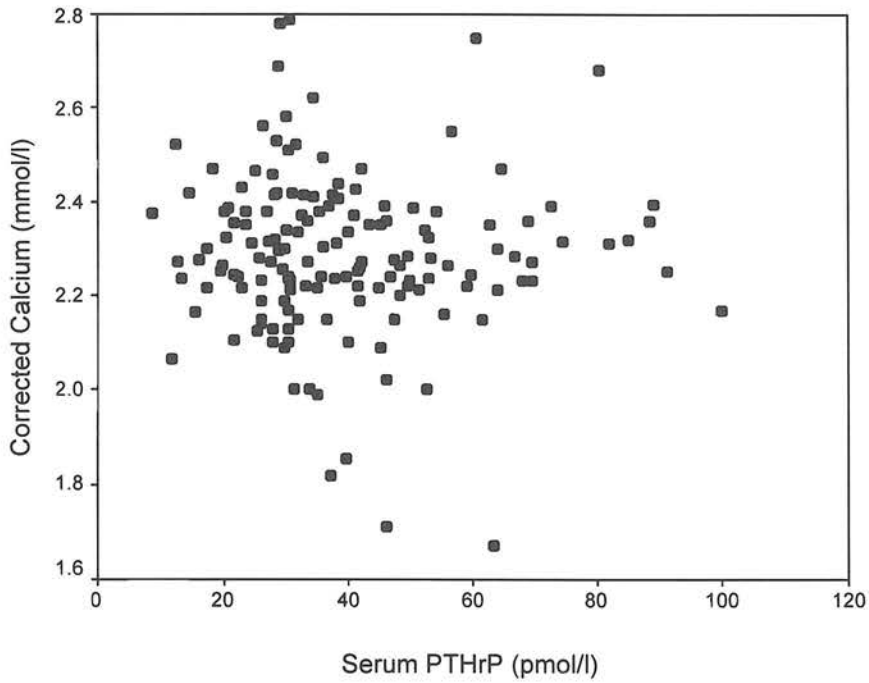


Figure 7.2 Scatter plot demonstrating the lack of correlation between serum calcium and serum PTHrP concentrations (r =0.03, p=0.72; linear regression analysis).



Association between serum PTHrP concentrations and demographic and clinical data

cPTHrP levels and patient/tumour characteristics are outlined in Table 7.2. There was no association between serum cPTHrP concentration and demographic or pathological data, including tumour location, histology or stage. Although an association with cPTHrP and tumour grade did not reach significance (p=0.24), poorly differentiated tumours were more likely to be associated with elevated cPTHrP levels. However, serum calcium concentrations were significantly higher in those patients with squamous histology (p=0.005) and in females (p=0.01; both Mann-Whitney U test) (data not shown).

Table 7.2 Serum cPTHrP levels and patient characteristics

		Serum PTHrP (pmol/l)		
		Normal (n=125)	Elevated (n=26)	P value
Age (years)[+]		72 (63-78)	75 (62-83)	0.264*
Sex	Male	80	15	0.702#
	Female	45	11	
Tumour Site	Oesophageal	52	12	
	Oesophago-gastric junction	24	4	0.604#
	Gastric	49	10	
Histology	Adenocarcinoma	109	23	
	Squamous cell carcinoma	13	3	1.00°
	Indeterminate	1	0	
	Small cell carcinoma	2	0	
Grade	Well differentiated	0	0	
	Moderately differentiated	33	4	0.243#
	Poorly differentiated	92	22	
UICC Stage	1	12	1	
	2	20	4	0.949°
	3	44	10	
	4	49	11	
Karnofsky score	≤ 50	6	3	
	60	9	4	
	70	15	3	
	80	20	3	0.194*
	90	33	2	
	100	34	7	
	Not recorded	8	4	

[+]Median (interquartile range). * Mann-Whitney U Test. #Chi-square test. °Fisher's exact test

Association between serum cPTHrP concentrations and markers of systemic inflammation

The relationship between serum cPTHrP concentrations and serum cytokine and acute phase protein concentrations are shown in Table 7.3. An elevated serum cPTHrP concentration was associated with elevated serum sTNF-R and CRP concentrations (p=0.008 and p=0.06, respectively) and with reduced concentrations of albumin and transferrin (p=0.02, p=0.009) (Figure 7.3). There was no association between serum cPTHrP concentrations and serum cytokine concentrations.

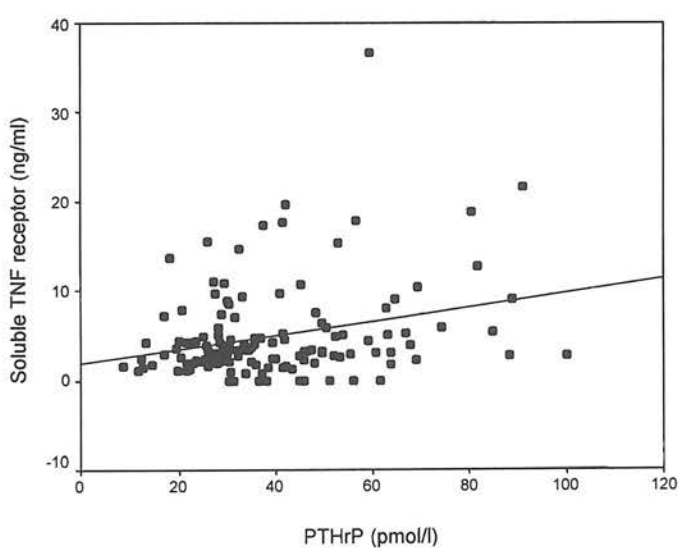
Table 7.3 Comparison of serum cPTHrP concentrations with serum cytokine and acute phase protein concentrations.

	Serum PTHrP (pmol/l)		
	Normal	**Elevated**	***P value****
CRP (mg/l)	6.5 (2.3-23.8)	17 (4.5-60.8)	**0.06**
Transferrin (mg/l)	1.8 (1.4-2.2)	1.5 (1.1-1.8)	**0.009**
Haptoglobin (mg/l)	2094 (1521-2829)	2205 (1113-2639)	0.46
Albumin (g/l)	40.0 (36.0-43.0)	37.0 (34.0-40.5)	**0.02**
α1-Antichymotrypsin (mg/l)	402 (337-520)	451 (326-601)	0.47
IL-1β (pg/ml)	0	0	0.34
IL-6 (pg/ml)	8.9 (0-86.6)	0 (0-51.6)	0.46
IL-8 (pg/ml)	0 (0-147.0)	0 (0-126.2)	0.81
IL-10 (pg/ml)	0	0	0.54
sTNF-R (ng/ml)	3.2 (1.9-4.9)	5.3 (2.9-10.4)	**0.008**

CRP= C-reactive protein. sTNF-R=soluble tumour necrosis factor receptor. Median (interquartile range)

*Mann-Whitney U Test.

Figure 7.3 A scatter plot demonstrating the correlation between serum cPTHrP concentrations and serum soluble TNF receptor (sTNF-R) concentrations (p=0.008, r=0.26).



Association between serum cPTHrP concentrations and nutritional status

Measurements of nutritional status are shown in Table 7.4. Measurements of nutritional variables were similar between patients with elevated serum cPTHrP concentrations and those with concentrations within the normal range, except for triceps skinfold measurements, which were higher in the group with elevated cPTHrP (p=0.04).

Table 7.4 Comparison of serum cPTHrP concentrations measured at the time of diagnosis with nutritional variables.

Nutritional variable	Serum PTHrP (pmol/l)		
	Normal	Elevated	*P value**
BMI at diagnosis	24.1 (21.2-28.0)	25.3 (22.3-28.8)	0.53
Weight loss (%)	7.6 (1.9-14.9)	6.7 (1.9-12.9)	0.73
Mid-arm circumference (cm)	27.0 (24-30)	28.0 (26.5-32.0)	0.14
Triceps skinfold (mm)	11.5 (8.0-15.0)	14.5 (9.0-18.0)	0.04
Arm muscle circumference (cm)	23.4 (20.8-25.2)	23.9 (21.7-26.2)	0.42

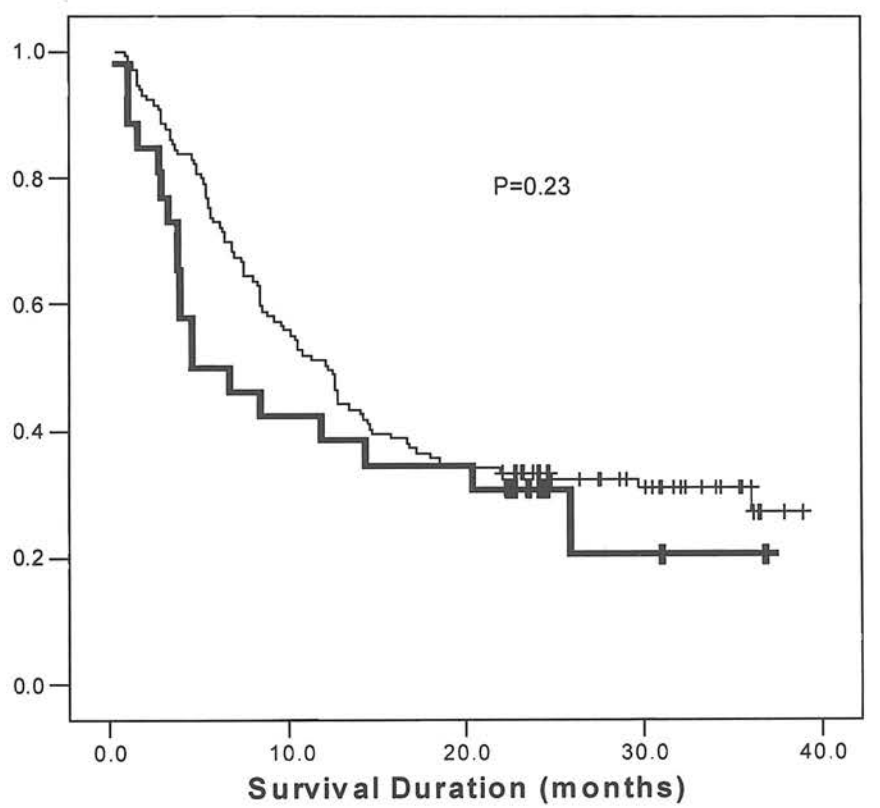
BMI = body mass index. Median (interquartile range). *Mann-Whitney U Test

Association between serum cPTHrP concentrations and survival duration

Initial analysis of the data after a mean follow-up period of 12 months identified an elevated serum cPTHrP concentration at the time of diagnosis to be associated with adverse prognosis. Twelve out of 26 (46%) patients with elevated cPTHrP had died at the time the survival data was censored compared with 39 out of 125 (31%) patients with normal cPTHrP levels (p=0.038, log-rank test). However, on multivariate survival analysis, when age, sex, stage and grade of tumour were included in Cox's proportional hazards model, in addition to serum cPTHrP, elevated cPTHrP was not an independent predictor of survival (p=0.45).

These results were published in the original description in *Cancer*. Subsequent survival analysis with a longer duration of follow-up (mean follow-up 31 months) only identifies a trend between an elevated serum cPTHrP concentration and adverse outcome (p=0.23, log-rank test) (Figure 7.4).

Figure 7.4 Kaplan-Meier curve comparing survival between patients with normal serum cPTHrP concentrations (light line) and elevated serum cPTHrP concentrations (heavy line). Initial analysis at 12 months follow-up identified an elevated serum cPTHrP concentration as an adverse prognostic variable. However, after a longer period of follow-up (31 months) the survival difference between the two groups was no longer significant (p=0.23; log-rank test).



Number at risk:					
Normal PTHrP	125	72	44	24	0
Elevated PTHrP	26	11	9	2	0

Longitudinal study of serum cPTHrP concentrations

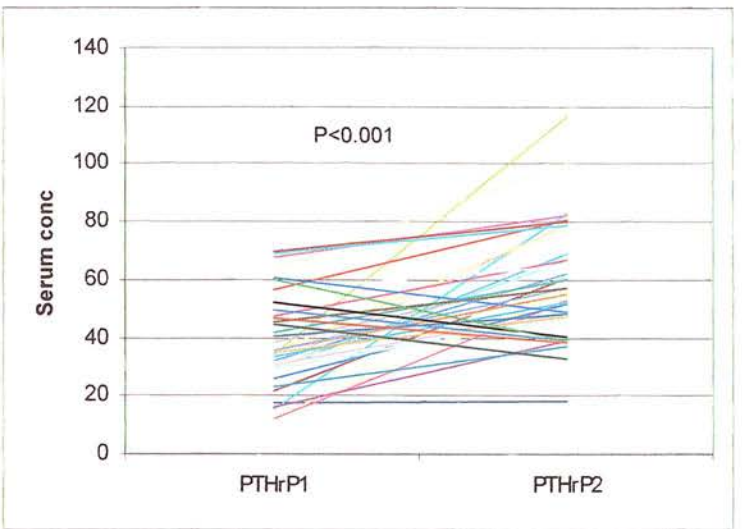
A second blood sample was collected from 51 patients after a mean time interval of 95 days (range 41-151 days) from the time of the first measurement (see Table 7.1). Between the first and second measurements 30 (59%) patients had undergone surgical resection, 13 (25%) patients had received pre-operative chemotherapy but had not yet undergone surgery, and 8 (16%) patients had been palliated by non-surgical methods (for example, stenting). These measurements were performed a minimum of 92 days following surgery and a minimum 41 days following completion of pre-operative chemotherapy.

The median serum PTHrP 2 concentration was 54.9 pmol/l (inter-quartile range 47.7-64.9 pmol/l) (Table 7.5). 25 (49%) patients had an elevated cPTHrP concentration at the second measurement compared with 17% of patients who had an elevated cPTHrP concentration at the time of diagnosis. The mean serum calcium concentration was 2.29 mmol/l (range 1.82 – 2.68 mmol/l; S.D. 0.15). As before, there was no correlation between serum cPTHrP and serum calcium concentrations (p=0.97; linear regression). Only 1 (2%) patient had hypercalcaemia at 3 months (and this patient had a normal cPTHrP concentration).

Table 7.5 Serial measurements of serum cPTHrP concentrations stratified according to intervening therapeutic intervention. PTHrP 1 = serum PTHrP concentration at diagnosis. PTHrP 2 = serum PTHrP concentration measured an average 95 days later.

Treatment modality	PTHrP 1 (pmol/l)	PTHrP 2 (pmol/l)	95% CI of the difference	P value
Surgery (n=30)	34.9 (30.2-56.1)	56.9 (48.6-69.8)	13.4 to 32.3	<0.001
Pre-op chemo (n=13)	35.3 (29.9-45.8)	48.2 (38.9-58.8)	1.0 to 25.9	0.066
Palliative (n=8)	30.4 (19.4-48.7)	55.9 (42.7-76.3)	3.2 to 41.6	0.028
Whole group (n=51)	35.3 (28.2-48.1)	54.9 (47.7-64.9)	13.7 to 27.2	<0.001
% Elevated^	17	49	-	-

Figure 7.5 Changes in serum PTHrP concentrations over approximately 3 months for individual patients with gastro-oesophageal cancer (n=51) [p<0.001, 95% CI 13.7 to 27.2; Paired T test].



Median serum PTHrP concentrations increased in all three treatment groups between measurement 1 and measurement 2 (Table 7.5) (Figure 7.5). The greatest increase was seen in patients who had undergone surgical resection (p<0.001; Paired T test) or who had been treated by palliative modalities alone (p=0.028). A similar, but non-significant, increase in serum PTHrP concentrations also occurred in patients who received pre-operative chemotherapy (p=0.066).

Association between serum PTHrP 2 concentrations and markers of systemic inflammation and survival duration

Blood was collected for determination of CRP and albumin concentrations at the same time that measurements of PTHrP 2 were undertaken. In contrast, to elevated concentrations of cPTHrP measured at the time of diagnosis, elevated serum PTHrP 2 concentrations were not associated with increased serum CRP concentrations (p=0.89; Mann-Whitney U test) or reduced serum albumin concentrations (p=0.66). There was no association between serum

PTHrP 2 concentrations and any demographic or clinical data. Similarly, there was no association between PTHrP 2 concentration and survival duration (p=0.81; log rank test).

Association between serum cPTHrP 2 concentrations and nutritional status

Serum cPTHrP concentrations measured after approximately 3 months (PTHrP 2) were associated with adverse nutritional status, but those measured at the time of diagnosis were not (Table 7.6). Elevated serum cPTHrP 2 concentrations were associated with increased rates of weight loss (p=0.043; Mann-Whitney U test), reduced mid-arm circumference measurements (p=0.015), reduced arm-muscle measurements (p=0.031), and lower BMI (p=0.005). There were no differences in dysphagia scores (p=0.16; chi-square), serum calcium concentrations (p=0.61; Mann-Whitney U test), or dietary intake (p=0.14; chi square) between the groups.

Table 7.6 Nutritional variables and serum cPTHrP levels measured at the time of diagnosis and after an approximate time interval of 3 months in the same cohort of patients. PTHrP 1 = serum concentration at the time of diagnosis, PTHrP 2 = serum concentration 3 months following diagnosis.

Nutritional variable	Serum PTHrP conc (pmol/l)					
	PTHrP 1 (n=51)			PTHrP 2 (n=51)		
	Normal (80%)	Elevated (20%)	P value*	Normal (51%)	Elevated (49%)	P value*
BMI	27.5 (22.6-31.2)	25.8 (23.3-29.7)	0.84	27.6 (23.8-31.0)	23.1 (19.4-26.3)	0.005
Weight loss (%)	3.4 (0.1-11.0)	1.3 (0-4.5)	0.24	7.7 (2.9-12.4)	11.1 (8.5-20.2)	0.043
Mid-arm circumference (cm)	30.0 (26.0-31.4)	29.3 (25.8-32.0)	0.84	28.5 (25.0-31.1)	25.8 (23.5-28.0)	0.015
Triceps skinfold (mm)	12.0 (9.0-17.0)	18.0 (10.3-22.5)	0.10	10.5 (8.0-19.0)	10.8 (7.3-15.9)	0.56
Arm muscle circumference (cm)	24.7 (21.9-27.2)	25.0 (20.0-26.3)	0.40	24.7 (21.0-26.4)	22.2 (20.2-24.6)	0.031

DISCUSSION

In this study 17% of patients with gastro-oesophageal malignancy demonstrated elevated serum PTHrP concentrations at the time of diagnosis without evidence of hypercalcaemia. An elevated serum PTHrP concentration was associated with markers of systemic inflammation (elevated concentrations of serum CRP and sTNF-R and reduced concentrations of albumin and transferrin). There was also a trend of association between elevated PTHrP concentrations and adverse prognosis. Serum PTHrP concentrations increased with progression of disease and after a mean time interval of three months following diagnosis an elevated PTHrP concentration was detected in 49% of patients. Increased PTHrP concentrations occurred irrespsectve of treatment modality and without evidence of hypercalcaemia. Furthermore, these follow-up measurements were linked with markers of adverse nutritional status.

In the present study only 6 (4%) patients were hypercalcaemic and this is in agreement with a large Japanese study where 1.3% of oesophageal cancer patients had elevated serum calcium at the time of diagnosis (Tachimori et al, 1991). Patients with gastric or oesophageal cancers experience hypercalcaemia infrequently and bone metastases are uncommon. Despite normal plasma calcium levels, 26 (17%) patients in the present study had elevated serum cPTHrP at the time of diagnosis. Increased levels of cPTHrP were equally likely irrespective of tumour origin or histological type. Similarly, in there was no association between advanced stage and elevated cPTHrP levels, an observation previously reported in other studies. Although not statistically significant in this series, the present data did suggest a trend between increased cPTHrP production and poor tumour differentiation. A study involving a much larger series of patients would be required to explore this potential association. A possible link between tumour grade and over production of cPTHrP has been reported (Alipov et al, 1997).

We also noted that serum PTHrP concentrations appear to increase with disease progression. At the time of diagnosis 17% of patients had elevated serum PTHrP compared with 49% of patients 3 months later. This apparent increase in serum PTHrP concentration appears not to be influenced by therapeutic interventions, occurring with equal likelihood

among patients who have had potentially curative surgery and those who have received palliative treatments alone The rise in serum concentrations may represent residual disease in these patients', however; we found no association between an elevated serum PTHrP 2 concentration and prognosis.

Forty-four percent of patients studied demonstrated an elevated systemic acute phase protein response at diagnosis. Concentrations of CRP and sTNF-R were higher whilst albumin and transferrin (both negative acute phase reactants) were lower in patients with elevated PTHrP. These data suggest a possible further enhancement of systemic inflammation in association with elevated PTHrP. Plasma levels of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 were not significantly different between the two groups.

We found no correlation between serum calcium levels and cPTHrP. This raises the possibility of an additional role of cPTHrP acting as a growth factor potentiating tumour progression. We found no clear association between elevated cPTHrP and disease stage. However, those patients with elevated serum levels of cPTHrP had worse prognosis, which was initially significant on univariate analysis, but not on multivariate testing, and lost its' statistical significance with a longer period of follow-up. The association between elevated serum cPTHrP and systemic inflammation may contribute to the adverse prognosis noted in this patient group.

Several tumour models have suggested that PTHrP is associated with adverse nutritional status (Iguchi et al, 2001). No differences in nutritional status were observed between patients with or without an elevated cPTHrP measured at diagnosis, except for triceps skinfold thickness. Patients with elevated cPTHrP were found to have increased triceps measurements compared with patients with normal serum levels of cPTHrP ($p=0.04$). This finding is likely to represent sample error and be a consequence of the higher proportion of females, who have a greater triceps skinfold thickness per kilogram bodyweight than males, among the patients with elevated cPTHrP. The similarity in nutritional status between the two patient groups may be explained by the fact that the sample population had normal serum calcium levels. It is possible that the correlation noted elsewhere between elevated PTHrP and weight loss is attributable to the systemic effects of hypercalcaemia (eg anorexia). In patients with normal serum calcium PTHrP may not have any independent effect on

nutritional status. However, elevated serum PTHrP concentrations measured an average three months following diagnosis were associated with adverse nutritional status in the absence of hypercalcaemia and without apparent differences in dysphagia scores or levels of dietary intake. These findings support the animal studies where elevated PTHrP concentrations in mice were associated with a significant reduction in body weight and tissue mass, an effect that is reversed by addition of an antibody to PTHrP (Iguchi et al, 2001). It is possible that the elevated serum PTHrP concentrations are increasing serum calcium concentrations among these patients resulting in 'sub-clinical' hypercalcaemia, which, in turn, may induce anorexia and result in weight loss.

Conclusions

An elevated serum PTHrP concentration was identified in 17% of patients with gastro-oesophageal cancer at the time of diagnosis and in the absence of hypercalcaemia. Elevated concentrations were positively associated with markers of systemic inflammation. An elevated serum cPTHrP was also associated with adverse prognosis, but did not predict outcome independent of other covariates. PTHrP concentrations increased with disease progression and were not influenced by therapeutic interventions. Approximately three months following diagnosis an elevated serum PTHrP concentration was detected in 49% of patients, compared with 17% of the same patient cohort when measured at the time of diagnosis. These elevated PTHrP concentrations measured later in the course of the disease were linked with adverse nutritional status in the absence of hypercalcaemia. These findings suggest that PTHrP may play a role in the generation of systemic inflammation in patients with gastro-oesophageal malignancy. Therefore, mediators other than pro-inflammatory cytokines, such as PTHrP, may contribute to the generation of systemic inflammation in some patients with cancer. The exact mechanisms remain unclear. The relationship between systemic inflammation and cachexia/decreased survival may provide a possible indirect role for PTHrP in these processes. These associations were investigated in later Chapters (Chapters IX and X). The next Chapter investigated the potential role of another tumour-derived mediator, proteolysis-inducing factor (PIF), in the aetiology of systemic inflammation in patients with gastro-oesophageal cancer.

CHAPTER VIII

PROTEOLYSIS-INDUCING FACTOR EXPRESSION IN PATIENTS WITH GASTRO-OESOPHAGEAL CANCER AND ITS RELATIONSHIP WITH SYSTEMIC INFLAMMATION, NUTRITIONAL STATUS AND PROGNOSIS

Deans DAC, Wigmore SJ, Gilmour H, Tisdale MJ, Fearon KCH, Ross JA
Expression of the proteolysis-inducing factor core peptide mRNA is up-regulated in both tumour and adjacent normal tissue in gastro-oesophageal malignancy.
British Journal of Cancer 2006; 94(5): 731-736

ABSTRACT

This Chapter continues the investigation into the potential role of tumour-derived mediators in the aetiology of systemic inflammation in patients with gastro-oesophageal cancer. Proteolysis-inducing factor (PIF) is a potential tumour-derived mediator that has been identified in human cancer patients and may be linked with markers of systemic inflammation, cachexia and outcome. In this study, PIF gene expression was investigated in tumour and benign tissue from patients with gastro-oesophageal cancer and in biopsies from healthy volunteers. Urinary PIF expression was also investigated and levels of expression were compared with markers of systemic inflammation, nutritional status, and prognosis.

Tumour tissue and adjacent benign tissue were collected from patients with gastro-oesophageal cancer (n=46). Tissue samples were also collected from healthy volunteers (n=11). PIF mRNA expression was quantified by real-time PCR. The presence of urinary PIF was determined by Western blot in 51 patients. Blood was collected for measurement of serum cytokine and acute phase protein concentrations by ELISA. Clinical and pathological information along with nutritional status was collected prospectively.

PIF mRNA was detected in 27 (59%) tumour samples and 31 (67%) adjacent benign tissue samples. Four (36%) gastro-oesophageal biopsies from healthy controls also expressed PIF mRNA. Expression was higher in tumour tissue ($p=0.031$) and benign tissue ($p=0.022$) from cancer patients compared with healthy controls. PIF mRNA concentrations did not correlate with weight loss, markers of systemic inflammation, or prognosis. Urinary PIF was detected in 26 (51%) patients and was associated with increased weight loss ($p=0.004$) and reduced anthropometry measurements without differences in dietary intake or dysphagia scores. There was a trend between detection of PIF in patients' urine and reduced serum concentrations of the negative acute phase protein albumin ($p=0.11$). The presence of PIF in patients' urine was weakly associated with elevated serum CRP concentrations ($p=0.06$), but was not associated with adverse survival.

PIF-CP mRNA expression was up-regulated in both tumour and adjacent normal tissue in gastro-oesophageal malignancy, but was also detected in normal tissue from healthy volunteers. Levels of gene expression did not correlate with clinical variables and may not

reflect levels of the functional protein. In contrast, the glycosylated PIF protein detected in patients' urine was associated with systemic inflammation and nutritional depletion. The presence of this functionally active glycoprotein may, therefore, contribute in a minor way to the aetiology of systemic inflammation in patients with cancer as well as influencing cachexia/prognosis.

INTRODUCTION

Whilst systemic inflammation may be mainly driven by pro-inflammatory cytokines, other tumour-derived mediators may also contribute to the generation of systemic inflammation in patients with cancer. Moreover, the metabolic abnormalities associated with cancer may be due to other factors, which may have partial effects via systemic inflammation, or other direct effects, for example stimulating proteolysis. This Chapter will investigate proteolysis-inducing factor (PIF) expression in patients with gastro-oesophageal malignancy and compare patterns of expression with markers of systemic inflammation, nutritional status and prognosis.

PIF is a potential tumour-derived mediator that has been identified in human subjects and may upregulate other mediators/markers of systemic inflammation. PIF may also be a direct mediator of muscle proteolysis and hence a major contributory factor to cachexia.

Potential role in cancer cachexia

Initially isolated from a murine cachexia tumour model (MAC16), a human homologue of PIF was subsequently identified in human urine from weight losing cancer patients, but not from weight-stable cancer patients or patients with weight loss secondary to benign disease (Todorov et al, 1996; Wigmore et al, 2000; Cariuk et al, 1997). PIF has been named as such because it has been shown to induce skeletal muscle proteolysis both in vitro and in vivo acting through the ubiquitin proteolytic pathway (Lorite et al, 1997; Lorite et al, 2001; Todorov et al, 1997). The molecule demonstrates a high degree of glycosylation, consisting of a

peptide core of 11-14 kDa and carbohydrate residues contributing to the estimated total molecular size of 24 kDa (Todorov et al, 1997). The glycosylation appears essential for its proteolytic activities as neither the peptide core alone nor de-glycosylated native PIF have any affect on weight loss in mice (Todorov et al, 1996).

Some gastrointestinal cancers and cancer cell lines have been shown to produce glycosylated PIF protein (Cabal-Manzano et al, 2001). Glycosylated PIF has been detected within tumour cells by immunohistochemistry and in the urine of the same subjects by Western blot. The presence of glycosylated PIF in tumour cells was also associated with increased weight loss among these patients. PIF protein has also been identified from the human melanoma cell line (G361) (Todorov et al, 1999) and the pancreatic cell line (MIA PaCa2) (unpublished data).

Human-derived PIF has been designated human cachexia-associated protein (HCAP) by early identification of the human gene sequence (Genebank accession number AR053250) and patent (US 5834192) (Akerblom and Murray, 1998) and later by a group who investigated its expression in prostate cancer patients (Wang et al, 2003). HCAP mRNA was detected in 13 out of 15 radical prostatectomy specimens and 7 out of 9 bone metastases, but was not detectable in normal prostate or in any adjacent non-malignant prostate tissue from prostatectomy samples. In-situ hybridisation confirmed that HCAP mRNA was only detectable in cancer cells and not in surrounding stromal cells or benign prostate tissue. HCAP mRNA expression was also detected from prostate cancer cell lines. When two of these cell lines (PC-3M and LuCaP35) were implanted into mice there was a strong correlation between HCAP mRNA expression from the cancer cells and weight loss among the mice (Wang et al, 2003).

Association with markers of inflammation

In hepatocytes glycosylated PIF has been shown to stimulate production of the cytokines IL-6 and IL-8 and the acute phase protein CRP via induction of transcription factors NF-kB and STAT3 (Watchorm et al, 2001). PIF may, therefore, contribute to the inflammatory state observed in conjunction with cancer cachexia in addition to its proteolytic function, and could

conceivably also play a role in the generation of a pro-inflammatory state out-with the context of cancer.

Molecular homology and possible roles in malignant disease

A BLAST search of the human genome using the PIF-CP cDNA sequence (Genebank accession number AY590150) identified 3 products that arise from the single gene locus on 12q3.1; dermcidin, neuronal survival-promoting peptide, and a candidate breast cancer oncogene. Dermcidin (DCD) is a novel peptide that was identified from human sweat and was shown to possess antimicrobial properties (Schittek et al, 2001). Unlike the PIF molecule, DCD appears to be unglycosylated. DCD mRNA was detected in normal skin, benign naevi and malignant melanoma cells by RT-PCR. Screening of adult and fetal tissue panels did not identify DCD mRNA in any other tissue. The authors therefore concluded that DCD was exclusive to skin and associated appendages.

Another PIF homologue has been identified following its isolation from the culture medium of neuronal cells grown under oxidative stress (Cunningham et al, 1998). In vivo studies suggest that this protein confers survival benefits to hypoxic neuronal cells and the molecule has been termed neuronal diffusible survival-promoting peptide (DSEP).

The third peptide, which has been mapped to the PIF gene locus, was identified using SAGE in breast cancer tissue (Porter et al, 2003). The gene was only expressed in a subset of invasive breast carcinomas and their lymph node metastases. In-situ hybridisation studies located the mRNA to cancer cells only with no expression found within stromal cells. Northern blot analysis of 75 human adult and fetal tissues detected RNA expression only in the pons and paracentral gyrus of the brain. The protein was identified in 10% of invasive breast cancers, 2 out of 64 pancreatic cancers, and normal sweat glands. Receptors were found in the brain and on tumour cells that were producing the protein, suggesting an autocrine/paracrine mode of action. When the cDNA sequence was introduced into a breast cancer cell line (21NT), these cells had accelerated growth and were more resistant to oxidative stress and hypoglycaemia. The authors have suggested that this gene may function as an oncogene in breast cancer with survival promoting properties.

More recently, a group of investigators probed a panel of normal human tissue cDNA for PIF-CP using real-time PCR and found absent or very low levels of expression (Monitto et al, 2004). In an analysis of unpaired breast tissue samples minimal PIF expression in normal breast tissue was detected. However, significantly elevated PIF expression within breast tumours was observed. Mice implanted with tumours transfected with a PIF vector were found to produce increased levels of PIF mRNA and protein but the protein was not glycosylated and these mice did not develop wasting. However, the tumours from these mice were significantly larger than controls, supporting a previous hypothesis that PIF may confer cell survival properties (Wang et al, 2003).

This Chapter will investigate PIF expression in urine and tumour tissue collected from patients with gastro-oesophageal cancer. Patterns of expression will be compared with markers of systemic inflammation, nutritional status and prognosis in these patients.

MATERIALS AND METHODS

Study population: patients and controls

All patients diagnosed with gastric or oesophageal cancer between June 2002 and March 2004 within Lothian and Borders regions were invited to take part in the study. No patients were excluded. Samples of blood and urine were collected at the time of diagnosis. Representative samples of tumour tissue were collected from patients who underwent surgical resection, as described previously. Full clinical and pathological information was recorded for each patient, including treatment modality undertaken. Duration of survival, defined as time from histological diagnosis to death, was recorded for all patients.

Gastric or oesophageal biopsies were collected from 11 healthy volunteers at the time of endoscopic examination. In all instances both the macroscopic and microscopic assessments were considered normal (as described in Chapter V).

Nutritional Assessment

All patients underwent nutritional assessment, including calculation of body mass index (BMI) and anthropometry measurements. Dietary intake was determined by interview based on food intake over the previous 3 days and a proportion of the patients completed food diaries. Dysphagia was scored using the system first described by Knyrim (Knyrim et al, 1993).

Serum cytokine and acute phase protein concentrations

Serum cytokine and acute phase protein concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (Chapter III).

Quantification of tissue PIF-core peptide mRNA

Tissue was obtained from patients who underwent surgical resection. A section of tumour tissue and a sample of benign mucosa from the same organ were collected from each patient. Tissue was also collected from healthy controls. Tissue samples were homogenised and PIF-core peptide mRNA was quantified by real-time PCR.The methodology is described in detail in Chapter III.

Identification of urinary PIF

Patients provided urine samples at the time of diagnosis. Detection of urinary PIF was initially attempted by Western blot by following the published methodology and then by mass spectrometry, but we were unable to reliably detect urinary PIF by either technique (see Chapter III for an extensive discussion). However, urinary PIF was successfully detected in patient samples at Professor Tisdale's laboratory, Aston University, Birmingham. Urine samples from 51 patients with a range of weight loss were investigated for PIF expression by Western blot. Results were interpreted by an investigator who was blinded to the weight loss and other clinical data.

Immunohistochemistry

Immunohistochemistry was attempted using the monoclonal antibody that was involved in the original identification of PIF and also using the commercially marketed 'cachectic factor

antibody' (Alpha Diagnostic, San Antonio, USA). Both antibodies produced widespread non-specific binding, despite extensive protocol modifications. In the case of the monoclonal antibody the lack of specificity was considered to be due to antibody binding to carbohydrate moieties on other molecules, both intracellular and on the cell surface (including CD 59). The polyclonal antibody (Alpha Diagnostic, San Antonio, USA) prepared against the core peptide of PIF was not sufficiently specific for use. Therefore, immunohistochemistry provided no meaningful results.

Statistical analysis

Comparisons between groups of continuous variables were made by the Mann-Whitney U test. Categorical variables were compared by the Chi-squared test or Fisher's exact test. Comparisons between tumour tissue PIF-core peptide gene expression and benign tissue expression were tested by linear regression analysis following natural logarithmic transformation of the data. Survival between groups was analysed by the log-rank test. Probabilities ≤ 0.05 were considered significant.

RESULTS

Study population

134 subjects were recruited to this study and urine and blood samples were collected from each patient. Tissue samples were additionally collected from 46 of these patients who underwent surgical resection. The demographics for those patients from whom tissue samples were obtained are shown in Table 8.1.

Table 8.1 Tissue samples were collected from 46 patients who underwent surgical resection. Patient demographics are outlined below. *Median (inter-quartile range).

		Number [%]
Age (years)		65 (58-75)
Sex	Male	32 [70]
	Female	14 [30]
Tumour Site	Oesophageal	22 [48]
	Oesophago-gastric junction	9 [20]
	Gastric	15 [33]
Histology	Adenocarcinoma	43 [94]
	Squamous cell carcinoma	3 [6]
Grade	Well differentiated	4 [9]
	Moderately differentiated	20 [44]
	Poorly differentiated	22 [48]
UICC Stage	1	14 [30]
	2	12 [26]
	3	15 [33]
	4	5 [11]
Treatment undertaken	Oesophagectomy alone	20 [43]
	Gastrectomy alone	15 [33]
	Pre-operative Chemotherapy + surgery	11 [24]
Status	Alive	35 [76]
	Dead	11 [24]

1. Expression of PIF-core peptide mRNA in tissues

Cancer cell lines

A number of cell lines were investigated for PIF expression using conventional (non-quantitative) PCR. This was necessary to identify a positive control for the patient analysis and to confirm that our primer sequences could identify PIF gene expression. PIF mRNA expression was confirmed in the pancreatic cell line MIA PaCa2 and this was later used as the positive control for the patient studies. We also confirmed the previous finding that the melanoma cell line G361 produced PIF (Todorov et al, 1999) and expression was also identified in a number of hepatic cell lines (Table 8.2).

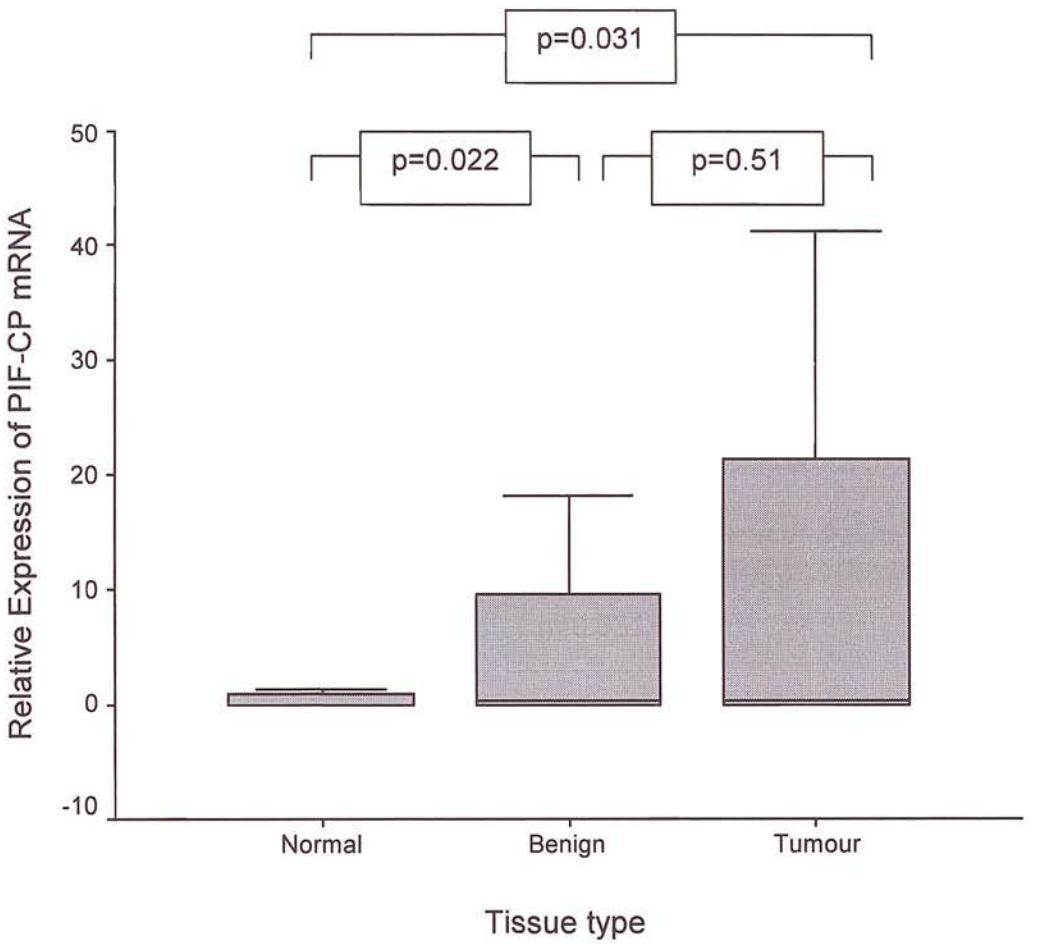
Table 8.2 PIF-core peptide mRNA expression in a number of cell lines.

PIF mRNA detected	No PIF mRNA identified
Pancreatic	Breast
MIA PaCa2	MCF 7
Hepatic	Hepatic
SK-Hep	Primary hepatocytes
Hep G2	
Huh7	
Melanoma	Monocytic
G361	THP 1

PIF–CP mRNA expression in healthy controls

PIF-CP mRNA was detected in gastro-esophageal biopsy tissue from 4 (36%) healthy controls. Of these positive tissue samples, 3 were gastric biopsies and 1 was oesophageal tissue. Although PIF-CP mRNA levels were detectable, the levels of expression were significantly lower when compared with levels of expression in tumour tissue ($p=0.031$, Mann-Whitney U Test) and adjacent benign tissue ($p=0.022$) taken from cancer patients (Figure 8.2).

Figure 8.2 A comparison of relative expression of PIF-CP mRNA in tissue from healthy controls (normal), tumour tissue, and benign tissue collected from cancer patients (benign). The lines represent the median value, bars = inter-quartile range, error bars = extreme values. [Mann-Whitney U Test]



PIF–CP mRNA expression in tissue from cancer patients

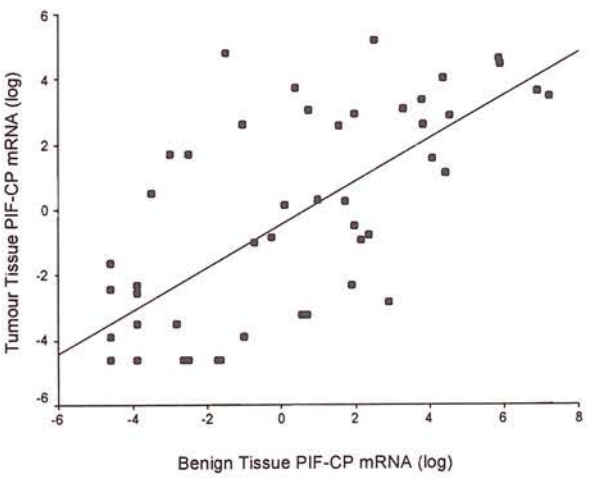
PIF-CP mRNA was detected in 27 (59%) tumour samples and 31 (67%) adjacent benign tissue samples. In 24 (52%) patients PIF mRNA was detected in both the tumour tissue and adjacent benign tissue collected from the same patient (Table 8.3). There was a strong correlation between paired mRNA concentrations in tumour tissue and mRNA concentrations in adjacent benign tissue ($p<0.0001$, $r=0.73$; linear regression) (Figure 8.3). Although levels of

PIF gene expression tended to be higher in tumour tissue compared with levels measured in non-neoplastic tissue, this did not reach statistical significance (p=0.51, Mann-Whitney U Test) (Figure 8.2). In 12 (26%) patients PIF-CP mRNA was not detected in either the tumour or adjacent benign tissue. For the remaining 10 patients, PIF-CP mRNA was detected in either tumour tissue only (n=3) or adjacent benign tissue only (n=7). There was no difference in level of gene expression between patients who received pre-operative chemotherapy and those who did not (tumour tissue, p=0.58; benign tissue, p=0.72: Mann-Whitney U Test).

Table 8.3 Tissue expression of PIF-CP mRNA in paired tumour and benign tissue.

Paired tumour tissue and benign tissue expression	Benign tissue expression only
24 (52%)	7 (15%)
Tumour tissue expression only	**Neither tumour tissue nor benign tissue expression**
3 (7%)	12 (26%)

Figure 8.3 Correlation between paired tumour tissue PIF-CP mRNA concentrations and benign tissue PIF-CP mRNA concentrations (p<0.0001, r = 0.73; linear regression). All values underwent natural logarithmic transformation.



Association between tissue PIF gene expression and markers of systemic inflammation

Nine (20%) of the 46 patients who provided tissue samples had evidence of systemic inflammation (serum CRP >10 mg/l). There was no association between levels of PIF-CP mRNA in either tumour tissue (p=0.89) or adjacent benign tissue (p=0.81) and elevated serum CRP concentrations (Mann-Whitney U test). Neither was there any association with any of the other acute phase protein concentrations or serum cytokine concentrations (data not shown).

Association between tissue PIF gene expression and nutritional status

Tumour tissue PIF-CP mRNA did not correlate with weight loss (p=0.37; linear regression), mid-arm circumference (p=0.10), triceps skinfold thickness (p=0.37), or arm-muscle circumference (p=0.14). Similarly, benign tissue mRNA concentrations did not correlate with any nutritional variable. Patients in whom PIF-CP mRNA was measurable in both tumour and adjacent benign tissues did not have adverse nutritional status when compared with patients without detectable PIF-CP mRNA in either tissue type (Table 8.4). There remained no correlation when the data were analysed according to dysphagia scores (data not shown).

Table 8.4 Tissue PIF-CP mRNA expression and nutritional variables. Nutritional variables were similar between patients in whom PIF-CP mRNA was measurable in both tumour tissue and benign tissue and patients who had no detectable mRNA (Mann-Whitney U Test).

	PIF-CP mRNA detected in both tumour tissue and benign tissue (n=24)	PIF-CP mRNA not detectable in either tumour tissue or benign tissue (n=12)	P value
Weight loss (%*)	3.3 (0.4-9.5)	5.3 (0-12.2)	0.93
MAC (percentile group*)	10-25 (5-50)	10-25 (5-50)	0.96
Triceps (percentile group*)	25-50 (10-50)	25-50 (25-50)	0.37
AMC (percentile group*)	10-25 (5-50)	25-50 (5-50)	0.96

*Median (interquartile range). MAC = mid-arm circumference, Triceps = triceps skinfold thickness, AMC = arm muscle circumference

Association between tissue PIF gene expression and prognosis

PIF-CP mRNA in either tumour tissue (p=0.64) or benign tissue (p=0.51) was not associated with adverse prognosis (Mann-Whitney U test). Similarly, those patients with detectable mRNA in both tumour and benign tissue did not have adverse survival compared with patients no detectable PIF-CP gene in either tissue type (p=0.79; Chi-square analysis). In addition, there was no association between mRNA levels and age, sex, tumour position, histology, degree of differentiation, or stage (data not shown).

2. Urinary PIF expression

Urine samples were collected from 134 patients and included the 46 patients who provided tissue samples and who are described above. We were unable to reliably and consistently detect urinary PIF to our satisfaction in our laboratory (see Materials and Methods Chapter for a detailed discussion). However, 51 of these patients with a range of weight loss were investigated for PIF expression by Professor M Tisdale's team at Aston University. Blots were interpreted by an investigator who was blinded to weight loss and other data.

PIF was detected in 26 (51%) patient's urine. The presence of urinary PIF was not associated with patient's age (p=0.84; Mann-Whitney U test), sex (p=0.61; Chi-square test), tumour position (p=0.27; Chi-square test), histological type (p=0.58; Chi-square test), or stage of disease (p=0.79; Chi-square test). There was a weak association between poorly differentiated tumours and PIF detected in patient's urine but this did not reach significance in this study (p=0.12; Chi-square test).

Association between urinary PIF status and markers of systemic inflammation

The presence of urinary PIF was inversely associated with serum concentrations of the negative acute phase protein albumin (p=0.011) and there was a trend towards a positive association between urinary PIF and elevated serum CRP concentrations (p=0.061; Chi-

square test). However, urinary PIF was not associated with any other serum cytokine or acute phase protein concentrations (Mann-Whitney U test – data not shown).

Association between urinary PIF status and nutritional status

Table 8.5 compares urinary PIF status with nutritional variables. Patients who had PIF detected in their urine had lost a median 13.4% (inter-quartile range 7.7-21.3%) of their pre-morbid body weight by the time of diagnosis, compared with PIF negative patients; median weight loss 4.4% (range 1.6-10.6%) (p=0.004; Mann-Whitney U test). One patient who was positive for PIF had lost 32.3% of their pre-illness body weight by time of diagnosis (Figure 8.4). PIF production was also associated with reduced mid-arm circumference (p=0.057) and arm muscle circumference (p=0.016), but not triceps skinfold thickness (p=0.433). This suggests a preferential loss of lean tissue. These differences in nutritional variables between PIF positive and PIF negative patients were independent of dysphagia scores (p=0.29), but there was a weak, but non-significant, association between PIF detectable in the urine and reduced dietary intake (p=0.062).

Figure 8.4 Percentage weight loss at the time of diagnosis according to the presence or absence of urinary PIF. Patients who had PIF detected in their urine had lost a median 13.4% of their pre-morbid body weight by the time of diagnosis, compared with PIF negative patients; median weight loss 4.4% (p=0.004; Mann-Whitney U test). The lines represent the median value, bars = inter-quartile range, error bars = extreme values.

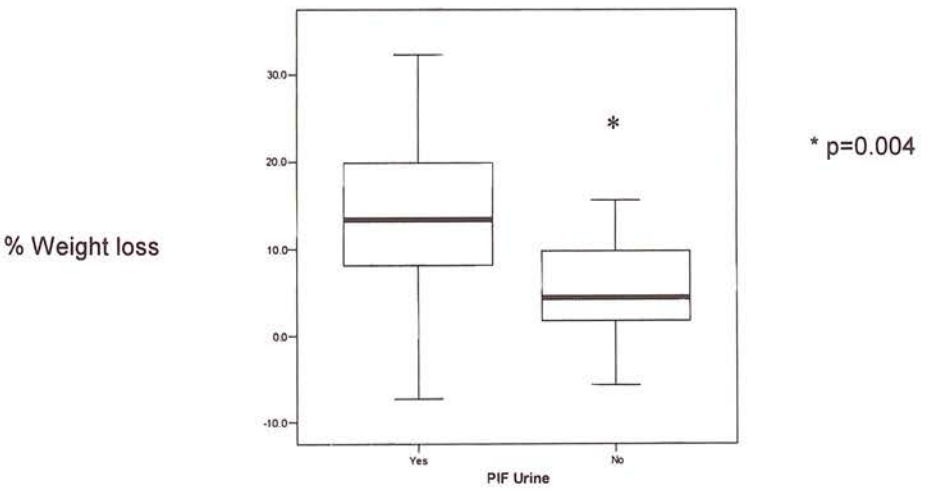


Table 8.5 Nutritional variables and urinary PIF status. The presence of urinary PIF was associated with increased total weight loss, reduction in mid-arm circumference and arm muscle circumference, and reduced serum albumin concentrations (Mann-Whitney U Test). Absolute values were normalised into percentile groups before analysis.

	PIF Positive (n=26)	PIF Negative (n=25)	P value[+]
Pre-illness BMI	25.3 (22.6-32.0)	28.2 (24.7-31.1)	0.24
BMI at diagnosis	21.7 (18.9-29.4)	26.7 (23.1-29.2)	0.06
% weight loss	13.4 (7.7-21.3)	4.4 (1.6-10.6)	0.004
Mid-arm circumference (percentile group)	7.5 (5-25)	25 (5-50)	0.06
Triceps skinfold thickness (percentile group)	25 (5-50)	25 (25-63)	0.43
Arm muscle circumference (percentile group)	5 (5-14)	10 (5-50)	0.02
Dietary intake*			
1	8	13	0.06
2	13	11	
3	5	1	
Dysphagia score			
0	10	9	0.29
1	3	8	
2	6	7	
3	6	1	
4	1	0	

All values are median (inter-quartile range). BMI = Body mass Index. *Dietary intake: 1=normal, 2=reduced, 3=poor #Dysphagia score: 0=normal, 1=solid dysphagia, 2=softened foods, 3=liquidized foods, 4=total dysphagia.

Association between urinary PIF status and prognosis

The presence of urinary PIF was not associated with adverse survival: PIF present, median survival 10.6 months; PIF absent, median survival 10.4 months (p=0.35; Log-rank test).

3. Relationship between tissue mRNA and urinary PIF expression

Nineteen patients were investigated for both PIF mRNA tissue expression and urinary PIF expression. The presence of urinary PIF was not associated with elevated PIF mRNA

concentrations in either tumour tissue (p=0.59; Mann-Whitney U test) or benign tissue (p=0.38).

DISCUSSION

This Chapter has shown that PIF gene expression is up-regulated in both tumour tissue and adjacent non-neoplastic tissue in patients with gastro-oesophageal malignancy. PIF mRNA was detected in 59% of tumour samples and 67% of benign tissues sampled from cancer patients, compared with 36% of tissues from healthy controls. However, the apparent increased rates of gene expression in these tissues may have little functional consequence on the host as levels of PIF mRNA are not associated with markers of systemic inflammation, nutritional status or prognosis. In contrast, urinary PIF protein was detected in 51% of patients with cancer and was associated with adverse nutritional variables and was weakly associated with systemic inflammation (reduced serum albumin concentrations and elevated CRP concentrations). There was no correlation between PIF mRNA expression in the tissues and detection of PIF protein in the urine.

Previous studies have suggested that PIF-CP mRNA is absent or expressed at only minimal levels in normal human tissues and found at significantly elevated levels in some tumours and various cancer cell lines. However, we have detected PIF-CP mRNA in benign tissue in the majority of patients with gastro-oesophageal cancer (67%), and these levels are equivalent to levels detected within adjacent tumour tissues. In addition, we detected PIF-CP mRNA in biopsy tissue from healthy controls, although at significantly lower levels of expression and also in a number of cell lines. In a previous study PIF-CP (HCAP) mRNA appeared to be limited to cancer cells and their metastases (Wang et al, 2003). The data in the present thesis suggests that PIF-CP gene expression is not limited to neoplastic tissue and that the PIF-CP gene may also be active in normal tissues. Furthermore, the level of gene expression appears to be similar in tumour tissue and adjacent benign tissue, but lower in normal tissue from healthy patients. Therefore, in some individuals with cancer there may

be up-regulation of the PIF-CP gene not only in tumour tissue but also within the whole organ or even in distant tissues.

The relevance of PIF-CP gene up-regulation remains obscure. Although PIF-CP has been identified as a putative cell survival factor we found no association between gene expression and prognosis, stage or grade of tumour. Therefore, the present study is unable to confirm a tumour survival-promoting role for this gene in our study. However, there was a weak association between urinary PIF and poor tumour grade, but not with advanced disease stage or prognosis. Mice implanted with a tumour carrying the PIF-CP gene demonstrated increased rate of tumour growth compared with controls (Monitto et al, 2004). The present study did not evaluate tumour volume, but we did not identify any differences in tumour stage or grade between patients with elevated mRNA levels and those with undetectable mRNA levels.

Urinary PIF was detected in 51% of patients and was associated with increased rates of total body weight loss (13% versus 4%) and reduced mid-arm circumference and arm muscle circumference values, but not triceps skin-fold thickness measurements. This suggests a preferential loss of lean body mass. These differences in nutritional assessment occurred independently of dysphagia scores and without a significantly reduced level of food intake. Previously, we have found an association between weight loss and urinary PIF among pancreatic cancer patients (Todorov et al, 1996; Wigmore et al, 2000; Cariuk et al, 1997). However, another group have recently investigated urinary PIF expression in patients with metastatic gastro-oesophageal cancer and found similar levels of expression (56%), but they found no association with weight loss (Jatoi et al, 2006). They concluded that no association was apparent due to widespread weight loss secondary to the mechanical effects of the tumour. However, in that study almost half the patients were weight stable or had lost less than 5% body weight making that explanation less likely. It is also possible, given the difficulties experienced in this study, that what was identified as PIF was in fact another molecule, perhaps CD59.

Tissue PIF-CP mRNA expression was not associated with weight loss or any adverse nutritional variable. In the detection of urinary PIF we are of course identifying the glycosylated PIF protein, whereas in the tissue study we have measured mRNA for the core

peptide and so this finding is perhaps unsurprising. Only a small fraction of the total peptide core would be expected to be glycosylated, and since PIF is only present at 1 part in 10^8 of the total protein, expression of the peptide core would not be expected to be rate-limiting for PIF formation, rather the expression of the glycosidases, their rate of catalysis and substrate availability (Todorov et al, 1996). Whether variation in glycosylation of PIF occurs and whether this influences biological activity is unknown. Antibodies raised against the core peptide do not reliably detect urinary PIF.

Systemic inflammation was identified in 20% of patients recruited to the tissue study and was not associated with PIF-core tissue mRNA concentrations. However, urinary PIF was associated with reduced serum albumin concentrations and was weakly associated with elevated serum CRP concentrations. *In vitro* work has demonstrated that hepatocytes produce increased levels of IL-6, IL-8 and CRP in response to PIF stimulation, thought to be mediated through transcription factors NFκB and STAT3 (Watchorn et al, 2001). PIF may play only a minor role in the complex interaction between pro-inflammatory mediators and a direct relationship between PIF and acute phase proteins may not be identified out-with the *in vitro* cell model.

It has been suggested that PIF is an important protein during embryological development and becomes quiescent during adult life (Watchorn et al, 2001). Neoplastic transformation would then allow the re-expression of the gene in adulthood (similar to carcinoembryonic antigen). We have found PIF-CP mRNA expression in normal healthy tissue albeit at reduced levels compared with tumour tissue and benign tissue from cancer patients and so this explanation is perhaps implausible for PIF-CP but may be important in re-expression of the glycosyltransferases. It is also important to consider that real-time PCR is an exquisitely sensitive technique and that what we are detecting in some patients, although elevated, may have little or no functional significance as it may not be translated into protein.

It is possible that the gene for the PIF-CP may confer a survival advantage to a tumour, promoting tumour growth and spread, although this study was unable to confirm this. Neither tissue mRNA expression nor urinary PIF were linked with adverse prognosis. Alternatively, the PIF-CP gene may be expressed to some degree in all tissue types and its diverse functions are due to post-transcriptional and post-translational modifications. The high

degree of glycosylation of the PIF molecule may be due to aberrant glycosylation by tumour cells and this glycosylation may confer the proteolytic activity associated with PIF (Todorov et al, 1997). This aberrantly processed molecule could not be distinguished from any conventional versions by determination of the mRNA expression or indeed perhaps by the currently used monoclonal antibody.

There was no association between levels of PIF core peptide mRNA expression and PIF glycoprotein detectable in the urine. The difficulties of relating mRNA concentrations to protein concentrations has been extensively documented elsewhere, and it is also important to consider that real-time PCR is an exquisitely sensitive technique and that what we are detecting in some patients, although elevated, may have little or no functional significance as it may not be translated into protein. In addition, what we are detecting in the urine is a carbohydrate moiety bound to the peptide core. Detection of urinary PIF therefore is utterly dependent upon appropriate glycosylation of the peptide.

In our laboratory we were unable to identify urinary PIF with certainty. We were able to demonstrate using mass spectrometry that the monoclonal antibody may bind non-specifically to carbohydrate moieties bound to molecules other than PIF (for example, CD 59). In addition, our results were inconsistent and not reproducible. Subsequent attempts to identify the PIF molecule from immuno-precipitates generated by the antibody have so far been unsuccessful even using deglycosylation strategies and mass spectrometry/MALDI-TOF. This topic is discussed in detail in Chapter III. The urinary PIF data presented in this study was performed by Professor Tisdale's team at Aston University. This group has a longstanding experience in performing this assay with consistent and reproducible results, which were interpreted by an investigator who was blinded to the clinical data. Nevertheless, given the non-specific binding observed in our immunohistochemistry studies questions remain as to what exactly the antibody is binding to within patients' urine.

Conclusions

PIF mRNA is expressed in healthy tissue and is found at significantly elevated levels within tumour tissue and adjacent benign tissue from cancer patients. However, measuring mRNA expression is unreliable as an indicator of glycosylated PIF protein expression and, therefore,

has limited value in determining its role in the aetiology of systemic inflammation and outcome in patients with cancer. Post-translational modification of PIF may be a key step in determining the biological role of PIF in patients with cancer. In contrast, urinary PIF expression is associated with adverse nutritional status and is weakly associated with markers of systemic inflammation. The association between urinary PIF expression and adverse nutritional status may be mediated through the concurrent association with systemic inflammation. Alternatively, PIF may affect the nutritional status of the host via more direct pathways, such as stimulating skeletal muscle proteolysis through up-regulation of the ubiquitin proteolytic pathway. However, there are difficulties in reliably detecting PIF in urine and attention needs to be directed at developing robust and reproducible quantitative assessments of glycosylated PIF protein.

PIF gene expression is up-regulated in tissues from cancer patients, but levels of expression do not relate to levels of the glycosylated peptide by Western blot in patients' urine. In contrast, urinary PIF is associated with markers of systemic inflammation and adverse nutritional status. Once more, this association may be a consequence of the clinical sequelae of systemic inflammation or may be mediated through more direct mechanisms relating to other actions of these tumour-derived mediators.

Systemic inflammation is commonly identified in patients with gastro-oesophageal cancer. Pro-inflammatory cytokines are key in the development of systemic inflammation and tumour tissue cytokine concentrations (especially IL-1β) and the presence of a chronic inflammatory cell infiltrate have been identified as important mediators in this process. Cytokine genotype also influences the ability of the host to generate a systemic inflammatory response in these circumstances. In addition to cytokines, other tumour-derived factors, such as PTHrP and PIF, are also involved in the aetiology of systemic inflammation and may also have additional direct effects on nutritional status and prognosis in patients with cancer. The next two Chapters investigated the association between systemic inflammation and adverse prognosis in patients with gastro-oesophageal cancer and explored the role of cachexia as an aetiological factor in the link between systemic inflammation and adverse prognosis.

CHAPTER IX

THE CLINICAL SIGNIFICANCE OF THE ASSOCIATION BETWEEN SYSTEMIC INFLAMMATION AND ADVERSE PROGNOSIS IN PATIENTS WITH GASTRO-OESOPHAGEAL CANCER:

A DETAILED ANALYSIS OF PATIENT OUTCOME AT DIFFERENT STAGES OF DISEASE AND UNDERGOING DIFFERENT TREATMENT OPTIONS

ABSTRACT

The thesis thus far has investigated the aetiology of systemic inflammation in patients with gastro-oesophageal malignancy. This Chapter examined the association between systemic inflammation and adverse prognosis in these patients. The link between serum cytokine and serum acute phase protein concentrations and survival duration was investigated in a cohort of patients with gastro-oesophageal cancer. This association was tested in a multivariate survival model against established prognostic variables. In addition, the prognostic value of tumour tissue cytokine concentrations and a chronic inflammatory cell infiltrate was determined.

A consecutive series of 220 patients with gastro-oesophageal cancer were recruited at the time of diagnosis. Demographic, clinical and pathological information was collected propectively. Blood was collected for determination of serum cytokine and serum acute phase protein concentrations by ELISA. Cytokine concentrations were also measured in tumour tissue collected from patients undergoing surgical resection and the degree of chronic inflammatory cellular infiltrate was assessed in the specimens. Survival duration was recorded for each patient.

Patients were followed-up for an average 32 months (range 18-45 months) and at the time of censoring the data 147 (67%) patients had died. Serum acute phase protein concentrations, but not serum cytokine concentrations, were associated with survival duration at diagnosis. CRP concentrations were identified as the best individual marker of adverse outcome. The magnitude of the systemic inflammatory response also had prognostic value. An increase in serum CRP concentration from a value of 1 mg/l to 10 mg/l was associated with an almost 50% increase in likelihood of death. Conversely, CRP concentration measured after initiation of treatment was not associated with prognosis. Patient age, Karnofsky performance score, stage of disease, and tumour grade were also identified as prognostic indicators on univariate analysis. Multivariate analysis identified stage of disease ($p<0.001$), Karnofsky score ($p<0.001$) and serum CRP concentrations ($p=0.012$) as independent prognostic indicators. Tumour tissue cytokine concentrations were not associated with prognosis. However, a chronic inflammatory cell infiltrate into the tumour tissue was linked with adverse survival.

The presence of systemic inflammation is an ominous prognostic marker in patients with gastro-oesophageal cancer. The precise links between systemic inflammation and adverse prognosis are not clear. The generation of a systemic inflammatory response may reflect either tumour phenotype with increased malignant potential or a host response that leaves the individual susceptible to metastatic spread. Equally there may be an indirect link via cachexia.

INTRODUCTION

The previous Chapters in this thesis have investigated the aetiology of systemic inflammation in patients with gastro-oesophageal malignancy. In Chapter IV the prevalence of systemic inflammation in patients with gastro-oesophageal cancer was investigated. This Chapter examined the clinical significance of the association between systemic inflammation and adverse prognosis in these patients.

The relationship between serum cytokine concentrations and prognosis in cancer patients remains unclear. Elevated serum cytokine concentrations are generally regarded as markers of poor prognosis, but conflicting results have been found in pancreatic cancer, lung cancer and many other (Falconer et al, 1994; Martignoni et al, 2005; Songur et al,2004; Tas et al, 2005). This may be due to the poor correlation between serum cytokine concentrations and markers of systemic inflammation (see Chapter IV). The prognostic value of serum cytokine concentrations in gastric and oesophageal cancer remains equally contradictory.

In contrast, concentrations of serum acute phase proteins demonstrate more stability within the systemic compartment. Concentrations of serum positive acute phase proteins are elevated in patients with gastro-oesophageal cancer and systemic inflammation is evident in 43% of patients at the time of diagnosis (Chapter IV). Concentrations of acute phase proteins have been linked with reduced quality of life scores, reduced performance status, and reduced response rates to chemo/radiotherapy, and overall worse outcomes in patients with cancer (Gabay and Kushner, 1999; DeWys et al, 1980; Barber et al, 1999). An APPR has been shown to be an independent adverse prognostic indicator in the majority of cancers,

including pancreatic, lung, breast, melanoma, lymphoma, ovarian, renal, and gastrointestinal tumours (Rashid et al, 1982; Nozoe et al, 2001; Falconer et al, 1995; Alexandrakis et al, 2003). In patients with gastric cancer the presence of systemic inflammation has been associated with a markedly reduced median survival (9 versus 53 weeks, $p<0.001$) (Rashid et al, 1982). Similarly, two Japanese studies have identified a shortened survival in oesophageal cancer patients with an elevated serum C-reactive protein (CRP) at the time of diagnosis (Nozoe et al, 2001; Shimada et al, 2003). More recently a group from the UK has similarly identified elevated serum CRP and reduced serum albumin concentrations as independent prognostic indicators among patients with inoperable gastro-oesophageal cancer (Crumley et al, 2006). Detection of CRP within oesophageal cancer cells themselves has also been identified as an independent prognostic indicator on multivariate analysis (Nozoe et al, 2003). In addition, a large study of patients with colorectal, breast, gastric and bronchogenic cancers demonstrated a negative relationship between the magnitude of the systemic inflammatory response and survival duration (McMillan et al, 2001). Serum concentrations of acute phase proteins have also been linked with disease progression and the development of recurrent disease (Falconer et al, 1995; Barber et al, 1999; McMillan et al, 2003; Fujita et al, 1999; Jamieson et al, 2005).

The presence of an inflammatory infiltrate within tumours and its relevance to prognosis has been investigated in a number of cancer types. Tumour-associated macrophages (TAM's) have been associated with reduced disease-free survival among lung, head and neck, and endometrial cancer (Ohno et al, 2004; Marcus et al, 2004; Chen et al, 2005). In contrast, increased numbers of TAM's, eosinophils, mast cells and lymphocytes have been linked with improved survival in colorectal cancer (Svennevig et al, 1984; Jass, 1986; Nielsen et al, 1999). The prognostic significance of tumour-associated inflammatory cells is less clear in gastro-oesophageal cancer. An increased TAM infiltrate was associated with more advanced disease stage in patients with gastric cancer in one study, whereas other studies have suggested a more favourable prognosis associated with a more pronounced macrophage infiltration (Heidl et al, 1987; Ohno et al, 2003; Tsujitani et al, 1987). Similarly, increasing tumour-infiltrating lymphocyte (TIL's) count has been linked with decreased risk of death from gastric cancer in one study, but associated with an adverse prognosis in another

(Grogg et al, 2003; Setala et al, 1996). Studies relating to oesophageal cancer are equally contradictory (Koide et al, 2004; Ma et al, 1999). Adverse outcome relating to an apparent increased immune response to the tumour may at first appear contradictory, however, possible explanations for adverse outcome may include increased recruitment of inflammatory cells by the tumour to aid tumour progression. Tumour-infiltrating immune cells are influenced by locally produced tumour-derived mediators. Immune cells may produce free radicals and other mediators, such as degradation enzymes, in response to these mediators that may contribute to DNA damage and regulate cellular proliferation and invasion (Harrison et al, 2000; Kim et al, 2000; Kaiser et al, 1999; Ernst, 1999). In addition, these TAM's and TIL's may contribute to the generation of systemic inflammation in patients with cancer and the systemic sequelae that follow, for example, alterations in metabolism and wasting, may contribute to accelerated demise.

This Chapter investigated the clinical significance of the association between systemic inflammation and adverse prognosis in patients with gastro-oesophageal cancer. A detailed analysis of patients' outcome at different stages of disease and undergoing different treatment options was performed.

MATERIAL AND METHODS

Study population

The sample population was the same as that studied in Chapter IV. In summary, patients diagnosed with gastric or oesophageal cancer within the Lothian and Borders regions between March 2002 and June 2004 were eligible for inclusion into the study. No patients were excluded and subjects were recruited to the study within two weeks of diagnosis. Patients were staged as per Department policy and underwent treatments following discussion at the multidisciplinary team meeting (MDT). Demographic, clinical, and

pathological data were prospectively collected on each patient. Performance status was assessed using the Karnofsky index (Karnofsky and Burchenal, 1949). Survival duration, defined as the time from tissue diagnosis to death, was recorded for every patient.

Measurement of serum acute phase protein and cytokine concentrations

Blood was collected from every patient at the time of diagnosis and following initiation of treatments. Serum cytokine and acute phase protein concentrations were determined by ELISA, as previously described in Chapters III and IV.

Measurement of tissue inflammation

Sections of tumour tissue were collected from patients who underwent surgical resection. Cytokine concentrations were measured by cytometric bead array in homogenised tissue samples. The degree of chronic inflammatory cell infiltrate into the tissues was assessed by a Consultant Pathologist. Both these methodologies are described in Chapters III and V.

Statistical analysis

The prognostic significance of continuous variables was tested using Cox's univariate analysis and categorical data was analysed by the log-rank test. Survival curves were plotted using the Kaplan-Meier method. Multivariate survival analysis was performed using Cox's proportional hazards model. The accuracy of variables at predicting death was determined by receiver operating characteristics (ROC) curves.

RESULTS

Study patients

The patient population was the same as that studied in Chapter IV. Group demographics are presented in Table 4.1 (Chapter IV). In summary, 220 patients were studied and were followed-up for an average 32 months and minimum of 18 months (range 18-45 months). At the time of censoring the data 147 (67%) patients had died. Three patients died in the post-operative period and 1 patient died from injuries sustained following a fall. Information

obtained from death certificates indicated that all other deaths were disease related. Overall median survival was 12.6 months.

1. Association between clinical/pathological variables and prognosis

Patient demographics and clinical variables

The relationship between clinical and pathological variables and prognosis are shown in Table 9.1. Increasing age at the time of diagnosis (p=0.015; Cox's univariate analysis) and reduced Karnofsky performance scores (p<0.001; Cox's univariate analysis) were associated with reduced prognosis. Neither sex (p=0.909; log-rank test) nor smoking status (p=0.310; log-rank test) were associated with survival duration. Results of sub-group analysis by tumour position (oesophageal versus OG junction versus gastric) gave similar results and, therefore, only results for the whole patient group are shown.

Table 9.1 Relationship of clinical and pathological variables with prognosis.

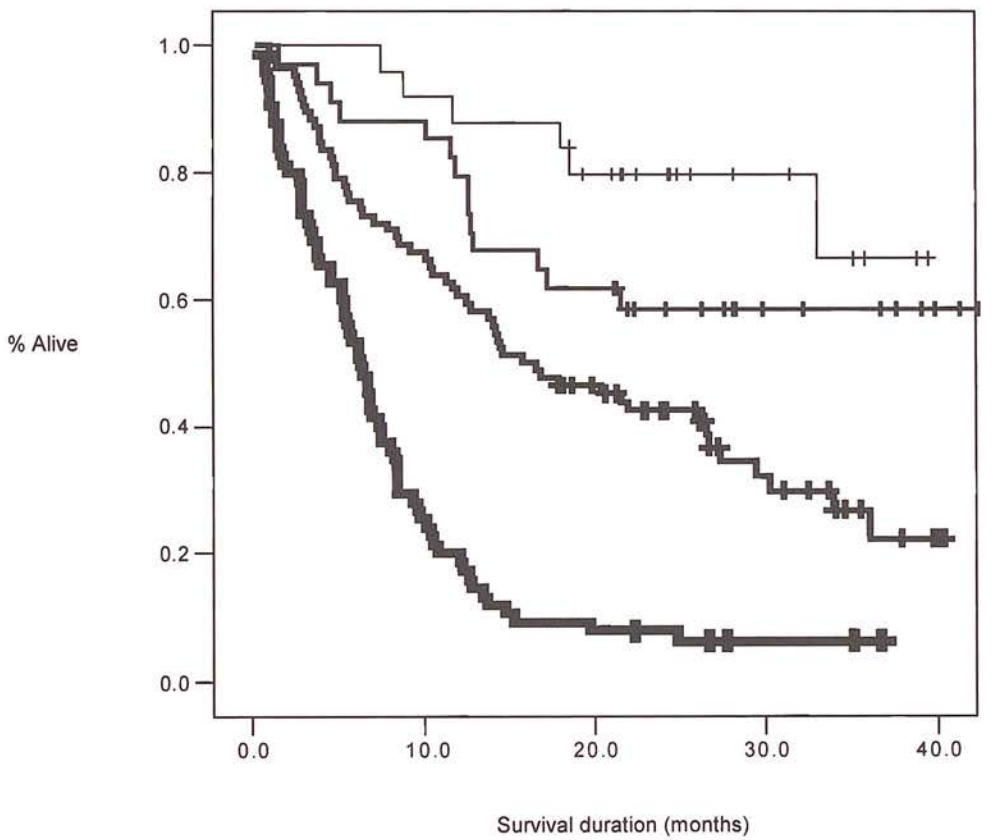
		NUMBER [%]	P VALUE
Age (years) [+]		71 (62-78)	0.015[¶]
Sex	Male	145 [66]	0.909[¥]
	Female	75 [34]	
Smoker	Current	63 [29]	0.310[¥]
	Ex-smoker	68 [30]	
	Never	89 [41]	
Karnofsky score	30	4 [2]*	<0.001[¶]
	40	1 [1]	
	50	5 [2]	
	60	17 [8]	
	70	25 [12]	
	80	36 [17]	
	90	50 [24]	
	100	70 [34]	
Tumour Site	Oesophageal	101 [46]	0.595[¥]
	Oesophago-gastric junction	40 [18]	
	Gastric	79 [36]	
Histology	Adenocarcinoma	185 [84]	0.771[¥]
	Squamous cell carcinoma	30 [14]	
	Small cell/Indeterminate	5 [2]	
Grade	Well differentiated	3 [2]*	0.009[¥]
	Moderately differentiated	63 [34]	
	Poorly differentiated	118 [64]	
Helicobacter pylori	Present	13 [12]*	0.678[¥]
	Absent	95 [88]	
UICC Stage	1	25 [11]	<0.001[¥]
	2	34 [16]	
	3	86 [39]	
	4	75 [34]	

[+]Values are median (interquartile range). [¶] Cox's univariate analysis. [¥] Log-rank test.

Pathological characteristics

Stage of disease was associated with survival duration (Figure 9.1). Stage I was associated with a median survival >40 months, stage II: median survival >40 months, stage III: median survival 15.7 months, and stage IV: median survival 6.3 months ($p<0.001$; log-rank test). Tumour grade also influenced survival; moderately differentiated tumours had a median survival of 21.5 months versus poorly differentiated tumours, median survival 10.4 months ($p=0.009$; log-rank test). Overall prognosis was similar irrespective of the site of the primary tumour (oesophageal versus OG junction versus gastric) ($p=0.595$; log-rank test). Similarly, survival duration was not associated with tumour histology ($p=0.771$ for the whole patient group and $p=0.675$ for the oesophageal cancer group alone) or Helicobacter pylori status ($p=0.687$ for the whole patient group and $p=0.623$ for the gastric cancer group alone).

Figure 9.1 Kaplan-Meier survival curve analysing survival duration according to final disease stage. Stage I [light line]: median survival >40 months, stage II [medium line]: median survival >40 months, stage III [heavy line]: median survival 15.7 months, stage IV [heaviest line]: median survival 6.3 months [p<0.001; log-rank test].



Number at risk:					
Stage I	25	23	17	7	0
Stage II	34	30	21	8	2
Stage III	86	58	36	14	2
Stage IV	75	19	6	2	0

2. Association between markers of systemic inflammation and prognosis

Serum cytokine and acute phase protein concentrations and survival duration

Serum concentrations of positive acute phase proteins were negatively associated with survival duration: CRP ($p<0.001$, Cox's univariate analysis) and α1-antichymotrypsin ($p<0.001$) (Table 9.2). Conversely, concentrations of negative acute phase reactants were positively associated with survival duration: albumin ($p<0.001$) and transferrin ($p=0.004$). Serum haptoglobin concentrations were linked with reduced survival, but this association did not reach statistical significance ($p=0.062$). Serum sTNF-R concentrations were also negatively associated with survival duration ($p=0.001$), but none of the other serum cytokines were associated with prognosis: IL-6 ($p=0.876$) and IL-8 ($p=0.415$).

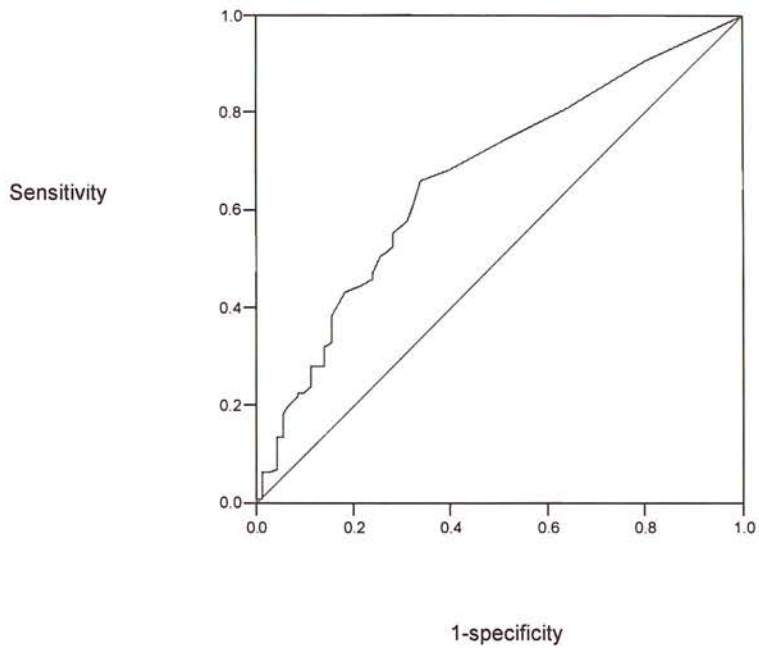
Serum CRP concentrations were identified as the best predictors of prognosis among all the acute phase proteins and cytokines measured, as determined by receiver operating characteristics (ROC) curves [area under the curve = 0.67, $p<0.001$] (Table 9.2) (Figure 9.2).

Table 9.2 Relationship between serum concentrations of acute phase proteins and cytokines with survival duration.

	Cox's univariate analysis			Receiver Operating Characteristics (ROC) curve		
	HR	95% CI	P value	AUC	95% CI	P value
CRP	1.01	1.00-1.01	<0.001	0.67	0.59-0.74	<0.001
ACT	1.00	1.00-1.00	<0.001	0.57	0.48-0.65	0.123
Haptoglobin	1.00	1.00-1.00	0.062	0.57	0.48-0.66	0.104
Albumin	0.94	0.91-0.97	<0.001	0.60	0.52-0.67	0.021
Transferrin	1.00	1.00-1.00	0.004	0.65	0.58-0.73	0.001
IL-6	1.00	1.00-1.00	0.876	0.51	0.42-0.59	0.883
IL-8	1.00	1.00-1.00	0.415	0.55	0.46-0.63	0.310
sTNF-R	1.04	1.02-1.06	0.001	0.64	0.56-0.72	0.002

HR = Hazard ratio, AUC = Area under the curve

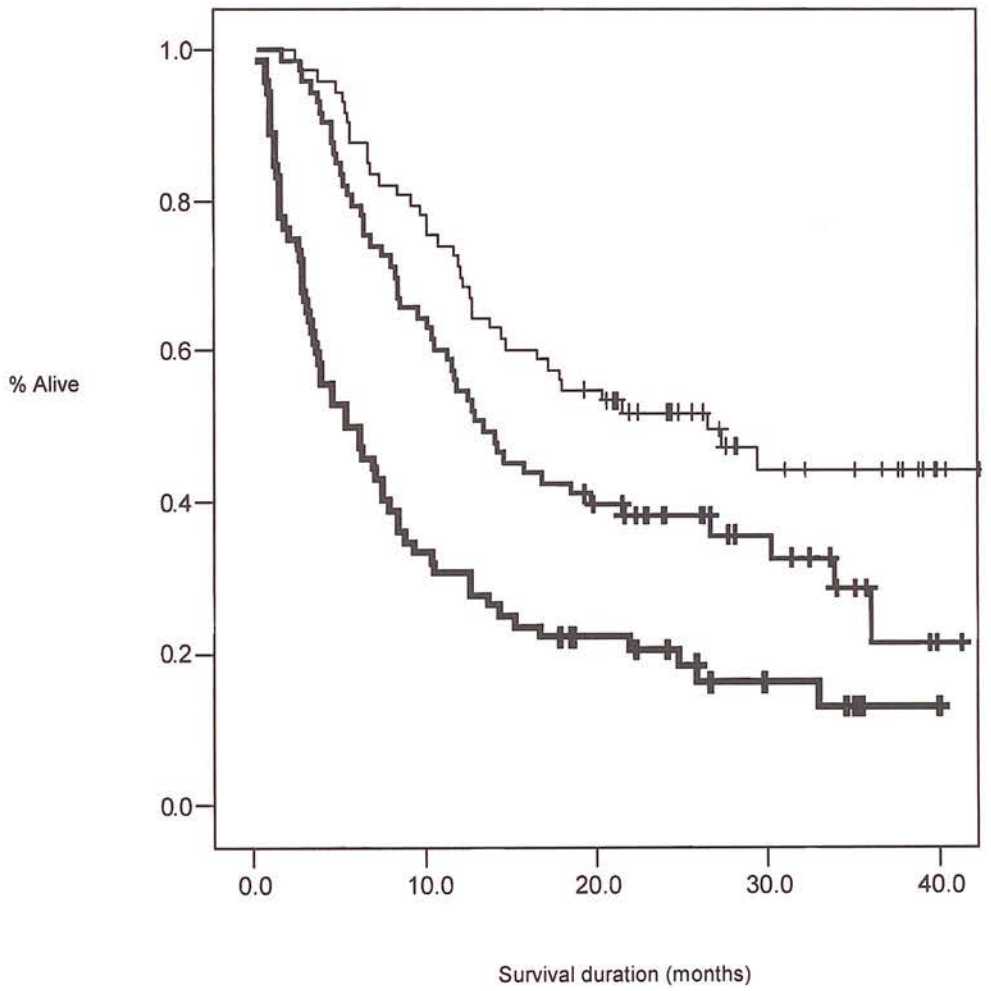
Figure 9.2 Serum CRP concentrations were identified as the best predictors of prognosis, as determined by receiver operating characteristics (ROC) curves [area under the curve = 0.67, p<0.001].



Magnitude of markers of systemic inflammation and survival duration

Increasing serum CRP concentrations were associated with significantly reduced survival duration (Figure 9.3). When analysed by tertiles, the lowest CRP group had a median serum concentration of 2 mg/l (inter-quartile range 1-2 mg/l) and a median survival of 26.5 months, the mid-tertile group had a median serum concentration of 7 mg/l (inter-quartile range 5-11 mg/l) and a median survival of 13.4 months, and the highest group had a median serum concentration of 43 mg/l (inter-quartile range 25-80 mg/l) and a median survival of 5.3 months [p<0.001, log-rank test].

Figure 9.3 Kaplan-Meier survival curve analysing survival duration according to CRP by tertile group. Tertile 1 [light line]: median CRP concentration = 2 mg/l (median survival 26.5 months), tertile 2 [medium line]: median CRP concentration = 7 mg/l (median survival 13.4 months), tertile 3 [heavy line]: median CRP concentration = 43 mg/l (median survival 5.3 months) [p<0.001; log-rank test].



Number at risk:					
Tertile 1	73	57	39	14	2
Tertile 2	73	47	27	12	1
Tertile 3	72	24	13	5	0

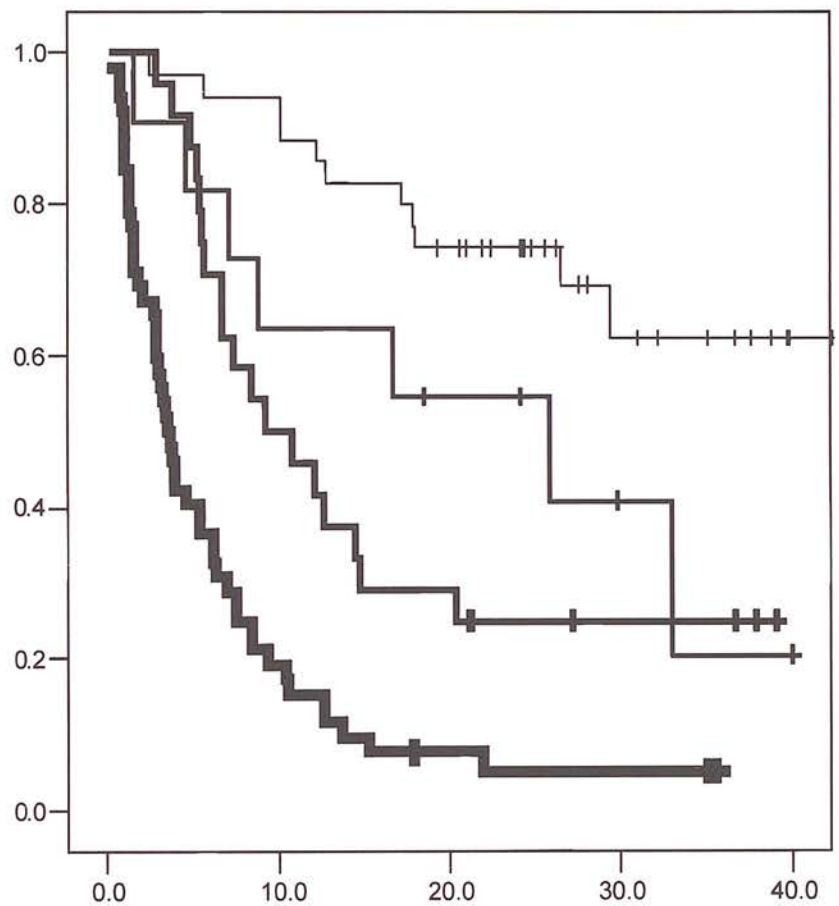
Serum CRP concentrations were able to discriminate between patients within the same treatment groups (Figure 9.4). Patients who underwent surgical resection and whose serum CRP concentrations were in the lowest tertile had a median survival >40 months versus those patients who underwent surgery and who had a CRP concentration in the highest tertile, median survival 25.9 months (p=0.043; log-rank test). Similarly, for patients who were palliated, those with a CRP concentration in the lowest tertile had a median survival of 9.2 months compared with those with a CRP concentration in the highest tertile, median survival 3.5 months (p=0.0007).

Serum CRP concentrations were also able to discriminate survival duration between patients within some of the same stage groupings (Table 9.3). Patients who were stage I or II and who had a CRP concentration within the lowest tertile had a median survival >40 months compared with those with a CRP concentration in the highest tertile who had a median survival of 16.7 months (p=0.002; log-rank test). Patients with stage IV disease and a low serum CRP concentration had a median survival of 7.3 months versus those with a high CRP concentration who had a median survival of 3.5 months (p=0.031). Serum CRP concentrations were not able to discriminate between patients with stage III disease overall (p=0.199), however, those patients with a CRP concentration in the lowest tertile had a median survival of 20.4 months compared with those whose CRP concentration was in the highest tertile and who had a median survival of 7.9 months, suggesting that CRP concentrations may predict early death rather than long term survival.

Table 9.3 Survival duration analysed by stage group and serum CRP tertile. *Log-rank test

	CRP Tertile	Median survival (months)	P value*
Stage I/II	Low CRP (n=31)	>40	0.002
	High CRP (n=11)	16.7	
Stage III	Low CRP (n=24)	20.4	0.199
	High CRP (n=22)	7.9	
Stage IV	Low CRP (n=18)	7.3	0.031
	High CRP (n=39)	3.5	

Figure 9.4 Kaplan-Meier survival curve analysing survival duration according to treatment intervention and CRP concentration. Light line = lowest CRP tertile and surgery (median survival >40 months), medium line = highest CRP tertile and surgery (median survival 25.9 months), heavy line = lowest CRP tertile and palliation (median survival 9.2 months), and heaviest line = highest CRP tertile and palliation (median survival 3.5 months) [p<0.001; log-rank test].



Number at risk:					
Surgery, low CRP	35	33	22	9	1
Surgery, high CRP	11	7	5	2	0
Palliation, low CRP	24	12	7	3	0
Palliation, high CRP	52	10	3	2	0

Longitudinal study of markers of systemic inflammation and survival duration

A second CRP and albumin measurement was performed in 77 (35%) patients after an average 95 days (range 41-151 days) from the time of the original measurement at diagnosis. Between the initial and follow-up measurements 48 patients had undergone surgical resection, 20 patients had received pre-operative chemotherapy but had not yet undergone surgery, and 9 patients had been palliated by non-surgical methods (for example, stenting). These measurements were performed a minimum of 92 days following surgery and a minimum 41 days following completion of pre-operative chemotherapy.

Changes in serum CRP and albumin concentrations between the first and second measurements are described in Chapter IV. An increase in serum CRP concentration between the initial measurement and those taken at the second or third time interval were not associated with reduced survival duration for the whole patient group (p=0.84; Mann-Whitney U test), or for each sub-group divided by treatment intervention; surgery (p=0.52), pre-operative chemotherapy (p=0.22), or palliative treatments (p=0.99). In addition, neither CRP nor albumin concentrations measured at the second time point were associated with prognosis (p=0.760 and p=0.130, respectively; Cox's univariate analysis).

3. Association between tumour tissue inflammation and prognosis

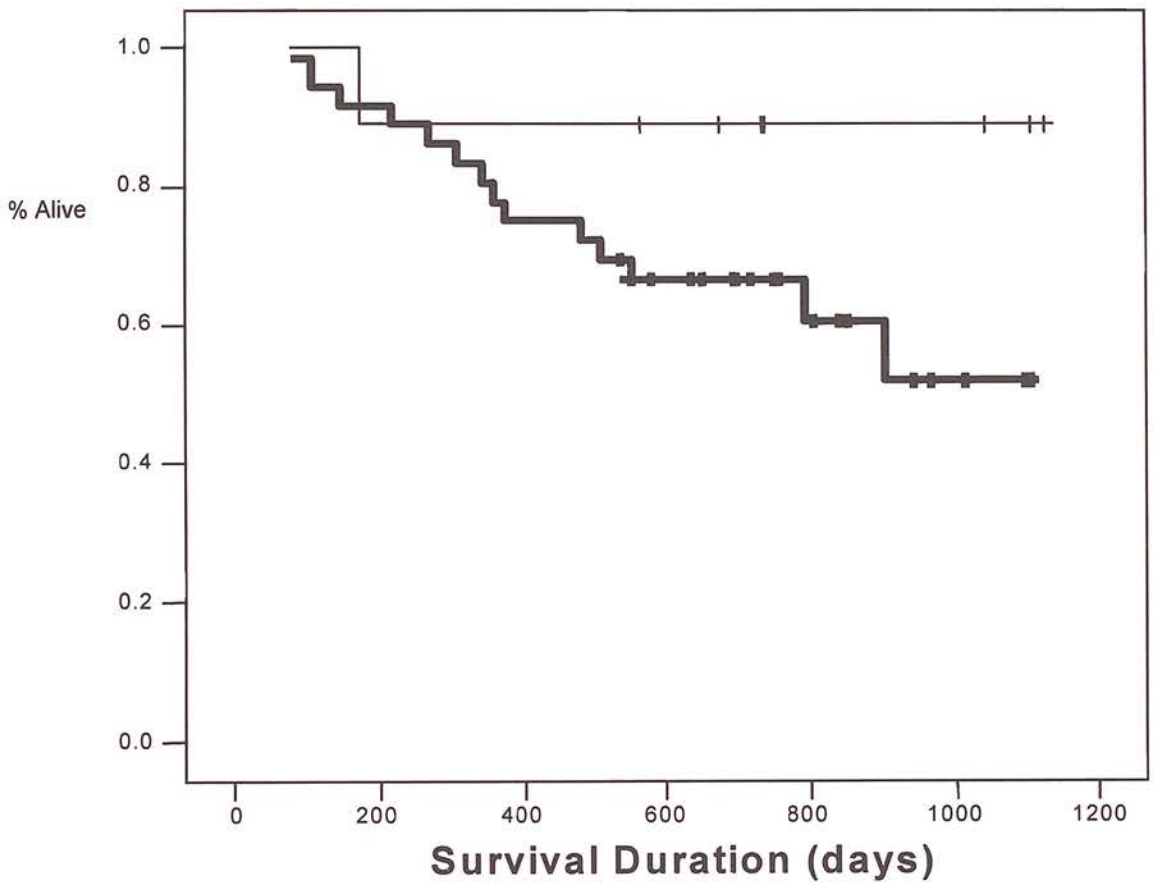
Relationship between tissue cytokine concentrations and survival duration

Tissue cytokine mRNA and protein concentrations were analysed by tertiles for patient survival. Neither mRNA nor tissue protein concentrations in benign or malignant tissue were associated with prognosis among these patients.

Relationship between the degree of chronic inflammatory cellular infiltrate and survival duration

A chronic inflammatory cell infiltrate was recorded in 75% of tumour tissues. The presence of an inflammatory response was associated with reduced prognosis (p=0.05, log rank test) (Figure 9.5).

Figure 9.5 Kaplan-Meier survival plot presented by the presence or absence of a chronic inflammatory cellular infiltrate within the tumour. The heavy line represents the presence of a chronic inflammatory infiltrate versus focal lymphoid aggregates alone, light line (p=0.05, log-rank test).



Number at risk:						
Inflammation	13	12	12	10	8	4
No inflammation	40	34	28	22	11	3

4. Multivariate survival analysis

Patient age at diagnosis, Karnofsky performance score, stage of disease, tumour grade and serum CRP concentrations were all identified as prognostic indicators on univariate analysis. Multivariate analysis was performed to identify which of these variables were independently associated with prognosis using Cox's proportional hazards model (Table 9.4). Advanced disease stage [hazard ratio 2.36 (95% CI 1.77-3.13), p<0.001], reduced performance scores [hazard ratio 0.97 (95% CI 0.95-0.98), p<0.001], and increased serum CRP concentrations [hazard ratio 1.21 (95% CI 1.04-1.41), p=0.012] were all identified as independent prognostic indicators for gastro-oesophageal cancer. Therefore, the likelihood of death increases by 21% for every logarithmic increase in serum CRP concentration. This means that an increase in serum CRP concentration from a value of 1 mg/l to 10 mg/l is associated with an almost 50% increase in likelihood of death.

Table 9.4 Multivariate survival analysis using Cox's proportional hazards model to identify independent prognostic indictors for gastro-oesophageal cancer (forward stepwise conditional method). Advanced disease stage (p<0.001), reduced performance scores (p<0.001), and increased serum CRP concentrations (p=0.012) were all identified as independent prognostic indicators for gastro-oesophageal cancer.

	Hazard Ratio	95% CI	P value
Age (Ln)	0.68	0.23-1.98	0.478
Karnofsky score	0.97	0.95-0.98	<0.001
Stage of disease	2.36	1.77-3.13	<0.001
Tumour grade	0.95	0.61-1.49	0.829
CRP conc (Ln)	1.21	1.04-1.41	0.012

Discussion

Concentrations of serum acute phase proteins and sTNF-R, but not concentrations of serum cytokines, are associated with survival duration. Serum CRP concentration was identified as the best individual prognosticator. Patient age, Karnofsky performance score, stage of disease, and tumour grade were also identified as prognostic indicators on univariate analysis. Multivariate analysis identified stage of disease, Karnofsky score and serum CRP concentrations as independent prognostic indicators for gastro-oesophageal cancer. The presence of a chronic inflammatory cell infiltrate into the tumour tissue was also associated with reduced survival duration.

Elevated concentrations of the positive acute phase proteins and reduced concentrations of the negative acute phase reactants were associated with reduced survival duration. Most acute phase proteins have been linked with survival duration but this study has identified CRP concentrations as the best predictor of prognosis. Previously serum CRP concentrations have been identified as an independent predictor of survival duration among patients with advanced pancreatic cancer and other groups have found CRP to be similarly discriminating (Rashid et al, 1982; Nozoe et al, 2001; Falconer et al, 1995; Alexandrakis et al, 2003). Repeat CRP measurements at the time of diagnosis in the same group of cancer patients have demonstrated good reproducibility (see Chapter IV) and CRP concentrations in different patient groups with similar disease processes have also demonstrated similar reproducibility. These findings suggest serum CRP concentrations are robust measure of systemic inflammation. In addition, CRP determination is a routinely performed laboratory investigation, familiar to most and is generally widely available without the need of specialist laboratory equipment. Some groups have combined CRP concentrations with albumin concentrations to develop a prognostic scoring system (Crumley et al, 2006). However, serum albumin concentrations may be influenced by malnutrition, hydration status, and trans-capillary escape, as well as the presence of an inflammatory response and therefore this study has investigated only single inflammatory variables (Fleck et al, 1985).

Serum IL-6 and IL-8 concentrations were not associated with prognosis and levels of IL-1β and IL-10 were generally not measurable in patients' serum (Chapter IV). Although

many researchers have found an association between serum cytokine concentrations and adverse prognosis, many have not, and the relationship between serum cytokine levels and measures of outcome in advanced cancer are unclear. The difficulties in reliably measuring circulating cytokine concentrations and their relevance have already been discussed elsewhere. In contrast, sTNF-R levels were negatively associated with survival duration and this probably reflects the increased stability of this molecule and its ability to behave like an acute phase protein (Spinas et al, 1992) (see Chapter IV).

The magnitude of the systemic inflammatory response was negatively associated with survival and this has been reported elsewhere in patients with colorectal, gastric, breast and lung malignancies (McMillan et al, 2001). Those patients with CRP concentrations in the highest tertile had significantly reduced survival duration compared with those with values in the lowest tertile. It is, therefore, not only the presence of systemic inflammation that has prognostic value, but also the magnitude of the response. This has clear implications when deciding on treatment strategies for individual patients and the CRP cut-off that is selected will depend on the survival end-point that is chosen (see Chapter XI).

Among irresectable pancreatic cancer patients serial CRP concentrations generally increase in individual patients as their disease progresses and the incidence of elevated inflammatory markers increases (Falconer et al, 1995; Barber et al, 1999b). In this study CRP concentrations increased in those patients who underwent surgical resection, decreased in those who received pre-operative chemotherapy, and remained the same for those who underwent palliative treatments alone (Chapter IV). We found no association between repeat CRP concentrations and survival duration, either for the whole group or when analysed by treatment sub-group. Given the high degree of reproducibility of CRP concentrations at the time of diagnosis, the change in CRP concentrations are likely to be influenced by treatment modality, and, therefore, may lose predictive value. The absence of any significant change in CRP concentrations among those patients who received palliative treatments alone may reflect the small number of subjects involved (n=9). An elevated post-operative CRP concentration has been linked with early disease recurrence in colorectal, pancreatic and gastric cancers (McMillan et al, 2003; Fujita et al, 1999; Jamieson et al, 2005). There was no association between an elevated post-operative CRP concentration and survival duration for

the 48 patients who had undergone surgery in this study. However, it must be acknowledged that this sub-analysis involved smaller numbers of patients with relatively few events and, therefore, the value of post-operative CRP measurements in predicting outcome in patients with gastro-oesophageal malignancy remains unclear.

Patient age at the time of diagnosis, Karnofsky performance score, stage of disease, and tumour grade were all identified as additional prognostic indicators on univariate analysis. All these variables are well established as prognostic indicators for malignant disease. Overall median survival by stage was: stage I/II >40 months, stage III 15.7 months and stage IV 6.3 months. These results are comparable with those previously published on equivalent patient populations (Dressner, 2000). There were no significant differences in survival duration by stage between oesophageal and gastric cancers. This supports the theory that cancer arising around the gastro-oesophageal junction have similar biological behaviour and may be justifiably analysed as a single group (Jankowski et al, 2000).

Within each treatment and stage grouping, serum CRP concentrations were able to discriminate between patients' survival duration. The median survival of patients who underwent surgical resection and had a CRP concentration in the lowest tertile was almost twice that of patients with a CRP concentration in the highest tertile. This was also true for patients who received palliative treatments. In addition, CRP concentrations were able to stratify patients in stages I, II and IV. Although differences in survival duration between CRP tertiles for stage III patients was not statistically significant, an elevated serum CRP was associated with a marked reduction in median survival (8 months versus 20 months). This clearly has clinical applications given that those patients who fail to survive longer than 12-18 months following surgery are better managed by alternative palliative methods (Blazeby et al, 2000). Measurement of pre-operative serum CRP concentrations may therefore aid the prospective identification of such patients.

Multivariate analysis identified stage of disease, Karnofsky score and serum CRP concentrations as independent prognostic indicators. Serum CRP concentrations were positively associated with disease stage suggesting an association between markers of systemic inflammation and tumour burden, however, multivariate analysis identified CRP as an independent prognostic variable making this explanation unlikely. Nozoe and Shimada and

co-workers have previously shown CRP as an independent prognostic variable in oesophageal cancer (Nozoe et al, 2001; Shimada et al, 2003). However, these studies from Japan included only patients suitable for surgical resection and patients generally had earlier stage disease and almost all tumours were of squamous cell subtype. It is recognised that Western populations differ significantly not only in histology and stage of disease at presentation, but also in outcomes when compared with their Eastern counterparts (Griffin, 2005). This study has shown that CRP has a prognostic value that is relevant to Western patients. In patients with gastric cancer elevated serum CRP and other acute phase protein concentrations have been linked with adverse outcomes (Rashid et al, 1982; Ogoshi et al, 1988) and early disease recurrence (Jamieson et al, 2005), but no study has determined whether markers of systemic inflammation are independently prognostic of stage and other pathological variables. Crumley *et al* devised an inflammatory scoring system to aid determination of prognosis among patients with gastro-oesophageal malignancy (Crumley et al, 2006). This system utilises serum CRP and albumin concentrations and these authors have shown these scores to be independently prognostic for patients with inoperable disease.

Conclusions

Serum acute phase protein concentrations are associated with survival duration in patients with gastro-oesophageal cancer. CRP concentrations are identified as the best individual marker of adverse outcome and serum CRP concentration is an independent prognostic indicator on multivariate survival analysis. It is not only the presence of systemic inflammation that has prognostic value, but also the magnitude of the response. An increase in serum CRP concentration from a value of 1 mg/l to 10 mg/l is associated with an almost 50% increase in likelihood of death. In contrast, neither serum cytokine concentrations nor tumour tissue cytokine concentrations are associated with prognosis. However, a chronic inflammatory cell infiltrate into the tumour tissue is linked with adverse survival.

The presence of a systemic inflammation is an ominous prognostic marker that reflects either a tumour phenotype with increased malignant potential or a host response that leaves the individual susceptible to metastatic spread. The next Chapter examines the role of cachexia as an aetiological factor in the link between systemic inflammation and adverse prognosis in patients with gastro-oesophageal malignancy.

CHAPTER X

CACHEXIA IS AN AETIOLOGICAL FACTOR IN THE LINK BETWEEN SYSTEMIC INFLAMMATION AND ADVERSE PROGNOSIS IN PATIENTS WITH GASTRO-OESOPHAGEAL CANCER

ABSTRACT

Cachexia is an important cause of morbidity and mortality among cancer patients. The syndrome is associated with reduced quality of life scores, lower response rates to chemotherapy, and overall poorer outcomes. The clinical sequelae of systemic inflammation may lead to the development of SIMS/cachexia and this association may account for the link between systemic inflammation and adverse prognosis in patients with gastro-oesophageal cancer. In this Chapter the changes in nutritional status in a cohort of patients with gastro-oesophageal cancer are described and the association between nutritional variables and markers of systemic inflammation and prognosis in these patients were examined.

Patients (n=220) newly diagnosed with gastro-oesophageal cancer were recruited at the time of diagnosis. Each patient underwent a nutritional assessment consisting of calculation of body mass index (BMI), measurement of total weight loss and rate of weight loss, anthropometry measurements, dysphagia scoring and estimation of dietary intake. Serum concentrations of acute phase proteins and cytokines were determined by ELISA. Clinical and pathological information was collected and survival duration was recorded.

At diagnosis, 83% of patients had lost weight with a median loss of 7% body weight (2.5% body weight loss per month) and reduced anthropometry measurements. At three months following diagnosis, 92% of patients had lost weight and patients had lost significantly more weight than measured at the time of diagnosis. Furthermore, weight loss occurred in these patients irrespective of treatment modality undertaken. Increased weight loss was associated with serum acute phase protein concentrations and sTNF-r and IL-8 concentrations, but not with serum IL-6 concentrations. The best predictor of the fastest rate of weight loss was CRP. Weight loss was also associated with poor performance status and advanced stage of disease stage. Multiple regression analysis identified level of dietary intake (estimate of size of effect 38%), serum CRP concentrations (estimate of size of effect 34%), and stage of disease (estimate of size of effect 28%) as independent variables in determining degree of weight loss. There was a highly significant negative association between the magnitude of weight loss and survival duration.

Nutritional depletion is common among patients with gastro-oesophageal cancer and processes other than reduced dietary intake or mechanical obstruction by the tumour are

involved in the nutritional decline. The clinical sequelae of systemic inflammation may contribute to the weight loss observed in these patients and weight loss may provide the mechanism by which systemic inflammation contributes to adverse prognosis.

INTRODUCTION

The previous Chapter has demonstrated an association between elevated serum markers of systemic inflammation and adverse outcome in patients with gastro-oesophageal cancer. This Chapter investigated the factors that might provide the link between systemic inflammation and adverse prognosis. The clinical sequelae of systemic inflammation include alterations in protein and fat metabolism, changes in energy expenditure and alterations in patterns of hormonal secretion (see General Introduction for a detailed description – Chapter II). This may lead to a collection of symptoms collectively known as 'systemic immune-metabolic syndrome' (SIMS) (Cerchetti et al, 2004). These metabolic changes are also associated with the syndrome of cachexia and the mechanisms underlying the metabolic changes accompanying SIMS and cachexia may follow common pathways, for example, through increased pro-inflammatory cytokine activity. Therefore, in patients with systemic inflammation, the clinical sequelae may lead to the development of SIMS/cachexia and this association may account for the link between systemic inflammation and adverse prognosis in patients with gastro-oesophageal cancer. In this Chapter, therefore, the changes in nutritional status in a cohort of patients with gastro-oesophageal cancer were described and the association between markers of systemic inflammation, nutritional status, and prognosis in these patients was examined.

Cachexia remains an important cause of morbidity and mortality among cancer patients, affecting up to 85% of patients with gastrointestinal malignancy at the time of diagnosis (DeWys et al, 1980). Cancer cachexia is associated with reduced quality of life scores, reduced performance status scores, lower response rates to chemotherapy, and overall

poorer outcomes (DeWys et al, 1980; Barber et al, 1999b). Around 20% of deaths from cancer may be directly related to cachexia (Studley, 1936; Inagaki et al, 1974). In a large study of patients with oesophageal cancer, weight loss greater than 10% premorbid weight was the only significant predictor of death in patients undergoing surgical resection (Kelson et al, 1998).

The metabolic similarities underlying SIMS and cachexia suggest that pro-inflammatory cytokines may also play a role in the aetiology of cachexia. Circulating concentrations of IL-1β, IL-6 and TNFα and other cytokines have been associated with anorexia and weight loss in rodent tumour models and in human subjects (Ghelin et al, 1991; Strassmann et al, 1993). More recently, IL-6 over-expression in the serum and in tumour specimens of pancreatic cancer patients have been linked with accelerated weight loss (Martignoni et al, 2005). Markers of systemic inflammation have also been linked with cachexia. Elevated serum CRP concentrations have been correlated positively with weight loss in human cancer patients (Falconer et al, 1995; Wigmore et al, 1997; O'Gorman et al, 1999). An APPR in pancreatic cancer patients is associated with elevated resting energy expenditure (REE) and with reduced energy intake (Falconer et al, 1994; Wigmore et al, 1997). In patients with cancer, therefore, an APPR has been associated with hypermetabolism, anorexia, increased weight loss, and adverse prognosis (Argiles et al, 1997).

MATERIALS AND METHODS

Study population

The same patient cohort was studied as described in Chapter IV. In summary, all patients diagnosed with gastric or oesophageal cancer within the Lothian and Borders regions between March 2002 and June 2004 were eligible for inclusion into the study. No patients were excluded and patients were recruited to the study within two weeks of diagnosis. Blood was collected from each patient at the time of recruitment for determination of serum cytokine

and acute phase protein concentrations. All patients underwent an assessment of their nutritional status (see below). Patients were staged and underwent various treatments following discussion at the multidisciplinary team meeting. Duration of survival, defined from time of histological diagnosis to death, was recorded for each patient.

Nutritional assessment

All patients underwent an assessment of their nutritional status at the time of diagnosis. An additional assessment was performed in a subgroup of patients later in the progression of their disease and following initiation of treatment. A description of the nutritional assessment undertaken and methods employed are detailed in the Materials and Methods section (Chapter III). Briefly, body mass index (BMI) was calculated following height and weight measurements. Pre-morbid patient weight was recalled by the patient and confirmed where possible from the medical notes. Total body weight loss was calculated and expressed as a percentage of weight lost from the pre-illness stable weight. Rates of weight loss were calculated by dividing the total weight lost by the number of months where symptoms were experienced. Anthropometry was undertaken on each patient and included measurement of mid-arm circumference (MAC) and triceps skin-fold thickness. Mid-arm muscle circumference (AMC) was calculated by means of Jelliffe's equation (Jelliffe, 1966). Measurements were corrected for age and sex and were normalised using standardised reference tables (Bishop et al, 1981). The severity of dysphagia was scored according to Knyrim *et al* (Knyrim et al, 1993). Dietary intake was estimated by all patients and scored as normal, reduced or poor/minimal. A small group of randomly selected patients provided detailed 3-day food diaries for comparison. Patients were instructed to record all food and drink consumed over a 3-day period, which included a weekend day and the data were analysed by a senior dietician who provided information on the level of intake of macronutrients using computer software (CompEat®, Nutrition Systems, Grantham, UK). Comparisons were made to dietary reference values issued by the Department of Health (Department of Health, 1991). Body composition analysis was also performed on this patient subgroup. Lean body mass was calculated from total body water assuming a hydration coefficient of 0.732 (Hannan et al, 1995).

Statistical analysis

Non-parametric analysis was performed in all instances. Correlations were investigated by Spearman's rank test. Independent variables were analysed by the Mann-Whitney U or the Kruskal-Wallis tests. Categorical data was analysed by the Chi-square test. The paired sample T-test was used to compare serial measurements for each patient. Receiver operator characteristics (ROC) curves were used to evaluate the ability of serum acute phase protein concentrations to identify patients with the fastest rate of weight loss. Multiple regression modelling was used to identify which variables were independently associated with weight loss and to calculate the estimates of effect size. Survival analysis was analysed by Log-rank test and Cox's multivariate model.

RESULTS

Study Patients

Group demographics are presented in Table 4.1 (Chapter IV). In summary, 220 patients were studied over a 28-month period. Ninety-five (43%) patients underwent surgical resection and 25 of these received pre-operative chemotherapy. The remaining patients (n=118; 54%) were not suitable for curative therapy and received palliative treatments. One-third (34%) of patients had metastatic disease (stage IV) at the time of presentation and most other patients (n=86; 39%) had locally advanced (stage III) disease at diagnosis. Approximately one-quarter (n=59; 27%) of patients had early stage disease (stage I/II).

Patients were followed-up for an average 32 months and minimum of 18 months (range 18-45 months). At the time of censoring the data 147 (67%) patients had died. Overall median survival was 13 months.

1. Description of nutritional status

Weight loss and anthropometry measurements

The nutritional variables for the patient group measured at the time of diagnosis are shown in Table 10.1. Patients had lost a median of 7.1% (inter-quartile range 1.2-14.2%) total body weight at the time of diagnosis compared with their pre-morbid stable body weight. This was equivalent to a median rate of weight loss of 2.5% total body weight per month of illness (inter-quartile range 0.3-6.5% per month) [defined from the onset of symptoms]. Only 38 (17%) patients remained weight steady at the time of diagnosis, while 85 (39%) patients had lost more than 10% body weight. Anthropometry values were reduced for MAC, median percentile group = 10 (inter-quartile range 1-25 percentile); triceps skinfold, median percentile group = 25 (inter-quartile range 10-50 percentile); and AMC, median percentile group = 10 (inter-quartile range 1-25 percentile). Lean body mass was calculated from total body water values obtained by electrical bioimpedence in a random subgroup of patients (n=17). All subjects were male. Median values were 43.0 kg (inter-quartile range 39.4-47.8 kg) [normal values for males = 43.9 kg].

Assessment of dietary intake

Eighty-five (39%) patients described their dietary intake as normal, 103 (47%) patients described their intake as reduced compared to normal, and 32 (14%) patients had a poor or minimal food intake at diagnosis. Detailed 3-day food diaries were collected from 22 (10%) patients. When normalised to the estimated average requirement (EAR), total energy intake was below recommendations; median 87% of EAR kcal (inter-quartile range 68-93%), however, levels of protein intake (g/day) were above the reference nutritional intake (RNI) value; median 142% (inter-quartile range 109-170%). Actual food intake was compared with perceived food intake for those patients who completed food diaries. Eleven (50%) patients described their intake as normal, 9 (41%) said their intake was reduced, and 2 (9%) patients had a poor or minimal intake. Patient perception of reduced food intake was associated with reduced total calorie intake; 'normal' intake median 90% EAR kcal (inter-quartile range 81-105) versus 'reduced/poor' intake median 76% EAR kcal (inter-quartile range 56-89)

Table 10.1 Nutritional variables for the patient group measured at the time of diagnosis.

Pre-illness BMI	26.4 (24.1-30.1)
BMI at diagnosis	24.6 (21.4-28.0)*
Total body weight loss (%)	7.1 (1.2-14.2)
Rate of weight loss (% per month)	2.5 (0.3-6.5)
Mid-arm circumference (percentile group)	10 (1-25)
Triceps skinfold thickness (percentile group)	25 (10-50)
Arm-muscle circumference (percentile group)	10 (1-25)
Lean body mass[#]	43.0 (39.4-47.8)
Dietary intake	
Normal	85 [39]
Reduced	103 [47]
Poor/minimal	32 [14]
Food diary intake[¶]	
Energy kcal (% of EAR)	87 (68-93)
Protein (% of RNI)	142 (109-170)
Dysphagia score	
0	89 [40]
1	52 [24]
2	43 [19]
3	32 [15]
4	4 [2]

[#]Calculated from a subgroup of 17 patients. [¶] Calculated from a subgroup of 22 patients. EAR = estimated average requirement. RNI = reference nutritional intake. Values are median (inter-quartile range). [%].

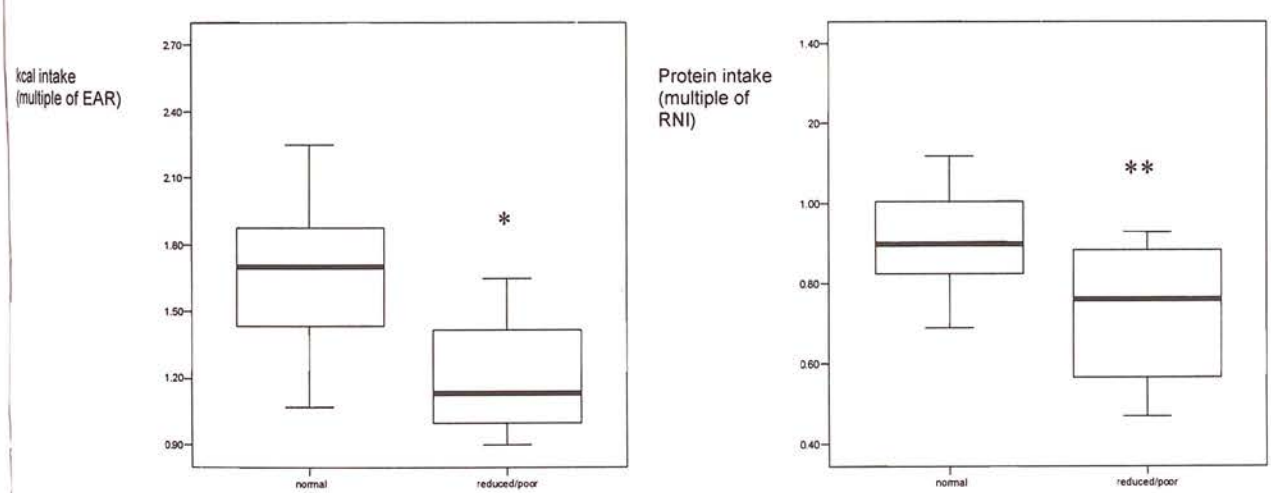
[p=0.040; Mann-Whitney U test], and reduced protein intake; 'normal' intake median 170% RNI g/day (inter-quartile range 133-197) versus 'reduced/poor' intake median 113% RNI g/day (inter-quartile range 99-148) [p=0.003] (Figure 10.1). However, there was no association between reduced calorie or protein intake and degree of weight loss, rate of weight loss, adverse anthropometry measurements, reduced lean body mass, or increased dysphagia scores for this subgroup of patients (data not shown).

Analysis of the whole patient group, however, identified reduced dietary intake as perceived by the patient to be associated with a lower BMI at diagnosis (p=0.007, Kruskal-Wallis test), increased percentage total body weight loss (p<0.001), increased rate of weight

loss (p<0.001), and reduced anthropometry measurements, although only triceps skin-fold thickness reached statistical significance (triceps p=0.025, MAC p=0.08, AMC p=0.11). In addition, reduced food intake was associated with reduced Karnofsky performance scores (p<0.001, Kruskal-Wallis test) and increased dysphagia scores (p<0.001, chi-square test).

Eighty-nine (40%) patients had no dysphagia at diagnosis, 95 (43%) were able to swallow solids or semi-solid foods, 32 (15%) were able to swallow liquids only, and 4 (2%) patients had total dysphagia. As a consequence of the association between dysphagia scores and reduced dietary intake, advancing dysphagia was also linked with adverse nutritional status.

Figure 10.1 Patient perception of reduced food intake was associated with reduced total calorie intake; 'normal' intake median 90% EAR kcal (inter-quartile range 81-105) versus 'reduced/poor' intake median 76% EAR kcal (inter-quartile range 56-89) [p=0.040; Mann-Whitney U test], and reduced protein intake; 'normal' intake median 170% RNI g/day (inter-quartile range 133-197) versus 'reduced/poor' intake median 113% RNI g/day (inter-quartile range 99-148) [p=0.003]. *p<0.05; **p<0.01



Longitudinal study of nutritional status

Seventy-seven (35%) patients underwent a second nutritional assessment after an average 95 days (range 41-151 days) from the time of the original measurement at diagnosis. Between the initial and follow-up measurements 48 patients had undergone surgical resection, 20 patients had received pre-operative chemotherapy but had not yet undergone surgery, and 9 patients had been palliated by non-surgical methods (for example, stenting). These measurements were performed a minimum of 92 days following surgery and a minimum 41 days following completion of pre-operative chemotherapy.

A comparison of nutritional variables measured at the time of diagnosis and those measured approximately 3 months later are shown in Table 10.2. Seventy-one (92%) patients had lost weight at three months following diagnosis compared with 83% at the time of diagnosis. Compared with the measurements taken at the time of diagnosis there was a significant decrease in BMI ($p<0.001$, Paired T test), increase in total body weight loss ($p<0.001$), increased rate of weight loss ($p<0.001$), and reduction in anthropometry measurements [MAC ($p<0.001$), triceps skin-fold thickness ($p<0.001$), and AMC ($p<0.001$)] at the follow-up assessment. There was a significant improvement in dysphagia scores between the two time points ($p<0.001$, chi-square test), but despite this food intake was significantly reduced ($p<0.001$, chi-squared test).

Effect of treatment on nutritional status

Sub-group analysis was performed to investigate any effect of treatment modality on nutritional status. There was no difference in total weight loss or rates of weight loss between patients who had undergone surgical resection, pre-operative chemotherapy, or palliative treatments alone (data not shown). Patients who had undergone surgery had significantly improved dysphagia scores compared with those who received pre-operative chemotherapy or who were palliated ($p<0.001$, chi-square test), but conversely the surgical patient group had significantly reduced dietary intake compared with the other two patient groups ($p=0.018$, chi-square test).

Table 10.2 Comparison of nutritional variables calculated at the time of diagnosis with those measured approximately 3 months later (n=77).

	1st assessment	2nd assessment	Paired T test		
			P value	Mean change	95% CI
BMI at diagnosis	25.8 (22.6-29.0)	24.0 (20.4-27.2)	<0.001	-1.8	-2.2 to –1.4
Total body weight loss (%)	3.2 (0-9.2)	10.3 (6.1-16.8)	<0.001	6.8	5.4 to 8.1
Rate of weight loss (% per month)	0.6 (0-3.1)	3.3 (2.0-5.4)	<0.001	1.9	1.3 to 2.5
Mid-arm circumference (percentile group)	10 (5-25)	5 (1-17.5)	<0.001	-8.3	-11.5 to –5.2
Triceps skinfold thickness (percentile group)	25 (21.3-50)	25 (10-50)	<0.001	-5.6	-8.3 to –2.9
Arm-muscle circumference (percentile group)	10 (1-25)	5 (1-10)	<0.001	-7.5	-10.6 to –4.5
Dysphagia score					
0	35 [45]	49 [64]	<0.001*	-	-
1	24 [31]	16 [21]			
2	10 [13]	9 [12]			
3	8 [10]	3 [4]			
4	0	0			
Dietary intake					
Normal	44 [57]	29 [38]	<0.001*	-	-
Reduced	26 [34]	36 [47]			
Poor/minimal	7 [9]	12 [16]			

Values are median (inter-quartile range). *Chi-squared test. [%].

2. Association between nutritional status and patient demographics

Clinical characteristics

Female sex was associated with reduced triceps skin-fold thickness ($p<0.001$, Mann-Whitney U test) and reduced dietary intake ($p<0.001$, chi-square test). In contrast male sex was associated with reduced MAC ($p<0.001$, Mann-Whitney U test) and AMC ($p<0.001$)

measurements. Despite these differences in anthropometry measurements and dietary intake there were no differences in amount of weight loss (p=0.68, Mann-Whitney U test) or BMI (p=0.28) between the sexes. Increasing patient age was associated with reduced triceps skin-fold thickness (p=0.03, Mann-Whitney U test), but age was not associated with any other nutritional variable. Reduced performance status was associated with reduced oral intake (p<0.001, chi-square test), increased dysphagia scores (p<0.001, chi-square test), increased weight loss (p<0.001, Kruskal-Wallis test), and reduced anthropometry values, although these did not quite reach statistical significance (MAC p=0.07, Triceps p=0.07, AMC p=0.08; Kruskal-Wallis test).

Pathological characteristics

Oesophageal tumours were associated with higher dysphagia scores (p<0.001, chi-square test), but not with reduced dietary intake (p=0.61) or increased weight loss (p=0.34, Kruskal-Wallis test). Total body weight loss was similar between the different histological subtypes (p=0.20). Advanced disease stage at the time of diagnosis was associated with greater percentage body weight loss (p<0.001), increased dysphagia scores (p=0.002), and reduced dietary intake (p=0.005), but with similar anthropometry measurements. Patients with stage III and stage IV disease had lost a median 9.5% of their total body weight by the time of diagnosis.

3. Relationship between markers of systemic inflammation and nutritional variables

a) Serum acute phase protein concentrations

Serum acute phase protein concentrations were associated with nutritional variables as shown in Table 10.3. Elevated concentrations of the positive acute phase proteins (CRP, α1-antichymotrypsin, and haptoglobin) were associated with increased total weight loss and increased rates of weight loss at the time of diagnosis, whereas the converse was true for the

negative acute phase reactants (albumin and transferrin) (Figure 10.2). Low serum albumin concentrations were also correlated with reduced anthropometry measurements; MAC ($p<0.001$, $r=0.25$) and AMC ($p<0.001$, $r=0.31$), but not triceps skin-fold thickness ($p=0.624$, $r=0.03$). Patients were grouped into tertiles according to rate of weight loss; lowest tertile median rate of weight loss = 0 % per month (inter-quartile range 0-0.3 % per month); middle tertile median rate of weight loss = 2.4 % per month (inter-quartile range 1.5-3.3 % per month); highest tertile median rate of weight loss = 7.6 % per month (inter-quartile range 6.4-14.5 % per month). Increasing tertile was associated with elevated serum concentrations of CRP ($p<0.001$; Kruskal-Wallis test), α1-antichymotrypsin ($p<0.001$), and sTNF-R ($p=0.002$) and with reduced concentrations of albumin ($p<0.001$) and transferrin ($p=0.003$) (Table 10.4). Elevated serum haptoglobin concentrations were associated with increased rates of weight loss but did not quite reach significance ($p=0.079$).

Table 10.3 Relationship between serum concentrations of acute phase proteins and cytokines with markers of nutritional status at the time of diagnosis.

	Wt loss		Rate of wt loss		BMI		MAC		Triceps		AMC	
	r	P	r	P	r	P	r	P	r	P	r	P
CRP	0.30	<0.001	0.36	<0.001	-0.04	0.546	-0.03	0.627	0.10	0.168	-0.13	0.065
ACT	0.23	0.001	0.27	<0.001	-0.05	0.486	-0.05	0.506	0.07	0.333	-0.14	0.058
Haptoglobin	0.15	0.038	0.16	0.020	0.01	0.915	0.02	0.734	0.05	0.452	-0.01	0.927
Albumin	-0.31	<0.001	-0.35	<0.001	0.24	<0.001	0.25	<0.001	0.03	0.624	0.31	<0.001
Transferrin	-0.30	<0.001	-0.26	<0.001	0.15	0.035	0.12	0.105	0.01	0.970	0.13	0.074
IL-6	0.08	0.294	0.08	0.260	-0.02	0.17	-0.12	0.099	-0.02	0.833	-0.12	0.108
IL-8	0.17	0.020	0.09	0.203	-0.15	0.040	-0.12	0.100	-0.02	0.773	-0.11	0.152
sTNF-R	0.10	0.155	0.15	0.041	0.09	0.188	0.08	0.293	0.09	0.190	-0.01	0.973

Spearman rank analysis; r = correlation coefficient

Figure 10.2 Scatter plot illustrating the positive correlation between elevated serum CRP concentrations at diagnosis and subsequent increased rate of weight loss ($p<0.001$, $r=0.36$; Spearman rank analysis).

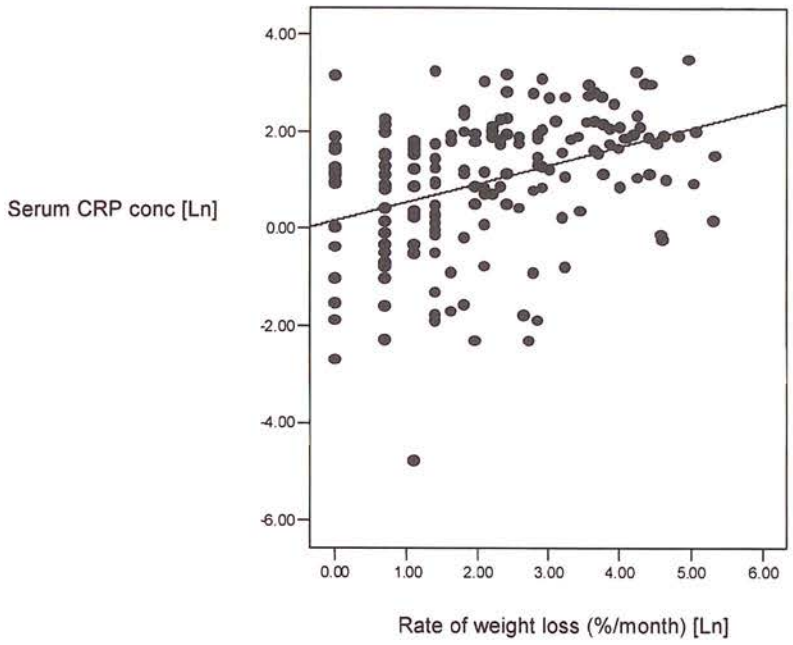


Table 10.4 Association between rate of body weight loss and serum acute phase protein and cytokine concentrations at diagnosis.

	Rate of weight loss (% body weight lost per month)			
	Lowest tertile	Mid tertile	Highest tertile	P value*
CRP	4 (2-14)	4 (2-18)	18 (7-47)	<0.001
ACT	424 (323-518)	412 (351-520)	572 (399-645)	<0.001
Haptoglobin	1965 (1224-2850)	2120 (1573-2853)	2460 (2020-3101)	0.079
Albumin	42 (38-44)	40 (37-42)	37 (33-41)	<0.001
Transferrin	2263 (1911-3353)	1852 (1594-2618)	1691 (1385-2913)	0.003
IL-6	0 (0-65.4)	17 (0-108.7)	11.3 (0-85.9)	0.223
IL-8	0 (0-0)	0 (0-148.8)	0 (0-240.1)	0.249
sTNF-R	3.4 (1.9-5.1)	2.4 (1.7-4.3)	4.4 (2.6-8.9)	0.002

Values are median (inter-quartile range). *Kruskal-Wallis test.

Serum CRP concentration was the best predictor of the fastest rate of weight loss, as identified by a receiver operating characteristics (ROC) curve [area under the curve = 0.72 ($p<0.001$; 95% confidence interval 0.65-0.79)] (Table 10.5).

Table 10.5 Areas under the receiver operating characteristics (ROC) curve for the ability of serum acute phase proteins and sTNF-R to predict the group of patients with the fastest rate of weight loss (AUC = area under the curve).

	AUC	95% CI	P value
CRP	0.72	0.65-0.79	<0.001
ACT	0.68	0.60-0.77	<0.001
Albumin	0.70	0.63-0.79	<0.001
Transferrin	0.61	0.52-0.70	0.015
sTNF-R	0.63	0.55-0.71	0.003

b) Serum cytokine concentrations

Elevated serum IL-8 concentrations were correlated with increased total weight loss ($p=0.020$, $r=0.17$; Spearman rank analysis), reduced BMI ($p=0.040$, $r= -0.15$), and were associated with reduced dietary intake ($p=0.071$) (Table 10.3). Serum sTNF-R concentrations were positively correlated with rate of weight loss ($p=0.041$, $r=0.15$). In contrast, serum IL-6 concentrations were not associated with any nutritional variable.

4. Multivariate nutritional analysis

Levels of dietary intake, dysphagia score, karnofsky performance score, stage of disease, and serum CRP concentrations were all associated with degree of weight loss among the patient group. Multiple regression analysis was performed to identify which variables were independently associated with weight loss. Dysphagia score and performance score are clearly confounding variables and are therefore excluded from the multivariate analysis. Dietary intake ($r = 0.28$, $p<0.001$), stage of disease ($r = 0.17$, $p=0.013$), and serum CRP concentrations ($r = 0.17$, $p=0.017$) all retained independent association in determining extent of weight loss (Table 10.6). A multivariate general linear model was then used to calculate the estimates of size of effect size on degree of weight loss for each variable (Table 10.6). These data suggested that 38% of weight loss was determined by level of dietary intake, 34% by serum CRP concentrations (systemic inflammation), and 28% was attributable to stage of disease.

Table 10.6 Multiple regression analysis of variables associated with increased weight loss. Dietary intake, stage of disease, and serum CRP concentrations all retained independent association in determining degree of weight loss. A multivariate general linear model was then used to calculate the estimates of size of effect on degree of weight loss for each variable.

	Regression coefficient	95% confidence interval	F-test	Estimates of effect size (%)	P value
Dietary intake	0.28	1.80 to 5.09	1.9	38	<0.001
Stage of disease	0.17	0.31 to 2.62	0.9	28	0.013
CRP conc (ln)	0.17	0.18 to 1.77	1.3	34	0.017

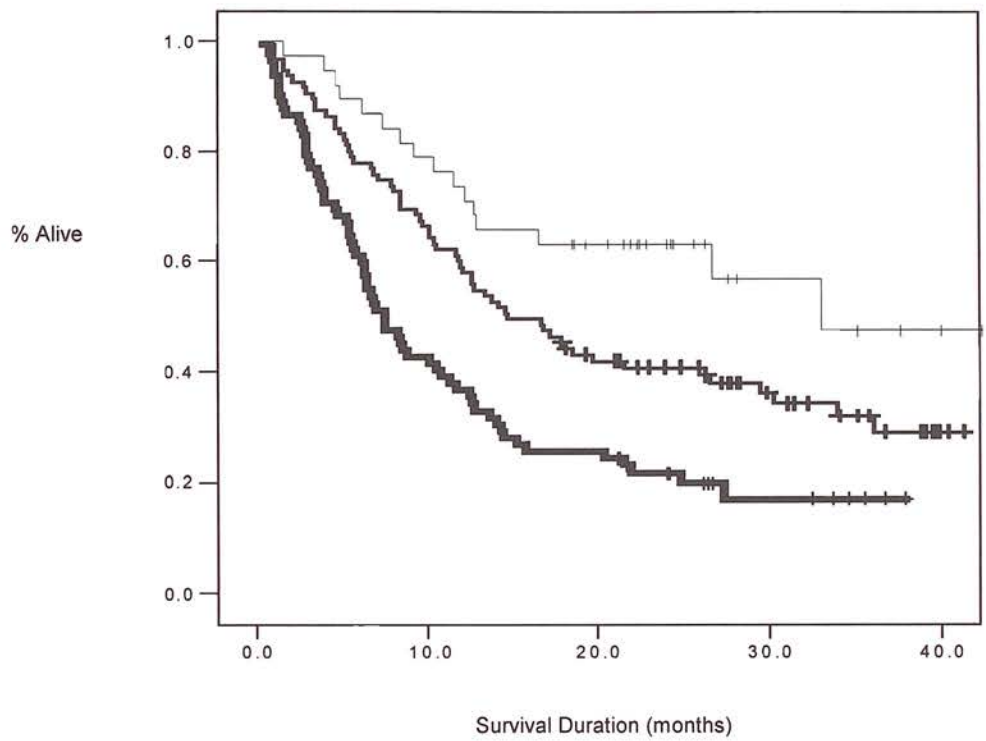
5. Relationship between nutritional status and prognosis

Increased total weight loss and increased rates of weight loss at diagnosis were associated with adverse prognosis [HR 1.44; p<0.001 and HR 1.42; p<0.001, respectively] (Table 10.7). Median survival duration was compared between patients who were weight stable (33.0 months; n=38, 17%), those patients who had lost up to 10% of their body weight (14.7 months; n=97, 44%), and those who had lost greater than 10% body weight (7.4 months; n=85, 39%) (Figure 10.3). Increasing magnitude of weight loss was associated with a corresponding reduction in survival duration (p<0.001; log-rank test). In addition, reduced dietary intake (p<0.001; log-rank test) and increasing dysphagia scores were also associated with reduced survival duration (p<0.001). In contrast, anthropometry measurements were not associated with prognosis and neither were lean body mass nor energy or protein intake as determined from food diaries, however, these groups involved smaller numbers (n=17 and n=22, respectively) and may have been subject to type II error.

Table 10.7 Relationship between nutritional variables and prognosis. Cox's hazard's model was used in univariate mode for the analysis of continuous variables and the log-rank test was used for categorical data.

		Cox' univariate analysis		
		Hazard ratio	**95% CI**	**P value**
BMI at diagnosis		0.95	0.92-99	0.005
Total body weight loss (%) [Ln]		1.44	1.20-1.73	<0.001
Rate of weight loss (% per month) [Ln]		1.42	1.23-1.63	<0.001
Mid-arm circumference (percentile group)		0.99	0.99-1.00	0.130
Triceps skinfold thickness (percentile group)		1.00	0.99-1.00	0.414
Arm-muscle circumference (percentile group)		1.00	0.99-1.01	0.532
Lean body mass		1.06	0.94-1.19	0.367
Energy kcal (% of EAR)		0.44	0.04-4.88	0.501
Protein (% of RNI)		1.16	0.42-3.15	0.778
		Log-rank test		
		Median survival (months)		**P value**
Dietary intake	Normal	26.7		
	Reduced	12.6		<0.001
	Poor/minimal	3.7		
Dysphagia score	0	16.7		
	1	26.7		
	2	9.8		<0.001
	3	7.4		
	4	1.0		

Figure 10.3 Increased weight loss at the time of diagnosis was associated with reduced survival duration; weight stable median survival 33.0 months (light line) versus 0-10% weight loss median survival 14.7 months (medium line) versus greater than 10% weight loss median survival 7.4 months (heavy line) ($p<0.001$; log-rank test).



Number at risk:

Weight stable	38	30	21	6	2
0-10% weight loss	97	64	38	19	2
>10% weight loss	85	37	22	6	0

DISCUSSION

This Chapter has described the changes in nutritional status in a cohort of patients with gastro-oesophageal cancer and has investigated the association between these nutritional variables and markers of systemic inflammation and outcome. At the time of diagnosis, patients with gastro-oesophageal cancer demonstrate a median loss of 7% body weight (2.5% body weight loss per month) and reduced anthropometry measurements. 83% of patients had lost weight at the time of diagnosis. At three months following diagnosis, 92% of patients had lost weight and patients had lost significantly more weight than measured at the time of diagnosis. Furthermore, weight loss occurred in these patients irrespective of treatment modality undertaken. Increased weight loss was associated with serum acute phase protein concentrations and sTNF-r and IL-8 concentrations, but not serum IL-6 concentrations. Weight loss was also associated with poor performance status and advanced stage of disease stage. Multiple regression analysis identified level of dietary intake (estimate of effect of size 38%), serum CRP concentrations (estimate of effect of size 34%), and stage of disease (estimate of effect of size 28%) as independent variables in determining degree of weight loss. There was a highly significant negative association between the magnitude of weight loss and survival duration.

Weight loss is common among cancer patients, particularly in patients with gastro-intestinal malignancy where up to 85% of patients may be affected during their illness (DeWys et al, 1980). One hundred and eighty-three (83%) patients in our study had lost weight at the time of diagnosis with almost half of these patients losing 10% or more of their pre-illness body weight. The median rate of weight loss was 2.5% body weight per month (approximately 1.9 kg/month) and compares with previous work relating to patients with pancreatic cancer where the mean rate of weight loss was 2.3 kg/month (Barber et al, 1999b; Fearon et al, 2003). Pancreatic cancer is notorious for its association with marked weight loss (DeWys, 1986; Galizia et al, 2002). In the present study we also found reduced anthropometry measurements: MAC and AMC median values in the10 th centile and triceps skin-fold thickness in the 25th centile. Values below the 15th centile are generally representative of some degree of nutritional depletion and these data may suggest a preferential loss of lean

body mass rather than fat mass as is characteristic of the cachexia syndrome (Bishop et al, 1981). However, we found no reduction in lean body mass as measured by bioimpedence analysis. The limitations of measuring lean body mass by electrical bioimpedence is documented elsewhere and better estimations could be made using either dual energy X-ray absorptiometry or magnetic resonance imaging.

One hundred and thirty-four (61%) patients described their level of dietary intake as reduced or poor compared with their normal levels of intake, and energy intake values were below national averages for those patients who completed food diaries (median 87% of estimated average requirement, approximately equal to 1920 kcal/day). Levels of protein intake exceeded minimum recommendations (142% reference nutritional intake); however, the average protein intake in the UK is 88g/day for men and 64g/day for females (Department of Health, 1991). Despite levels of protein intake in excess of reference recommendations the actual levels of protein intake were below the national average for both sexes (males 82g/day average; females 51g/day average). 1920 kcal/day may be insufficient to maintain constant weight even accounting for reductions in level of physical activity. Reduced dietary intake was associated with increased total weight loss, increased rate of weight loss, and reduced anthropometry measurements.

In this study we found that patients' perception of their level of dietary intake was associated with actual levels of energy and protein intake as determined from detailed food diaries. Diet diaries have been shown previously to reflect usual daily food intake (Bingham et al, 1982; Gibson, 1990) and it would appear that a patient's perception of their level of food intake may also reflect actual levels of dietary intake.

One hundred and thirty-two (60%) patients had symptomatic dysphagia at the time of diagnosis. Increasing dysphagia scores were associated with increased total weight loss, increased rate of weight loss, and reduced anthropometry measurements. Additionally, dysphagia was also associated with reduced levels of food intake. Patients with gastro-oesophageal malignancy are not only subjected to the systemic effects of the disease on their nutritional status (for example, anorexia, hypermetabolism, and altered protein metabolism), but also affected by the local effects of the tumour on the upper digestive tract. However, patients without dysphagia (and who had a 'normal' level of food intake) had still lost a

median 4.4% of their total body weight by the time of diagnosis (rate 1.5% per month) and therefore the systemic influences of the cancer on nutritional status remain important among these patients.

Patient performance status was strongly associated with reduced dietary intake and increased weight loss. In addition, reduced Karnofsky scores were linked with reduced anthropometry measurements, although these did not quite reach statistical significance. Performance score has previously been identified as an independent variable for weight loss in patients with advanced pancreatic cancer and similar findings have been reported elsewhere (Wigmore et al, 1997; O'Gorman et al, 1999). Clearly the functional status of the patient will influence appetite and desire and ability to eat. Likewise, nutritional depletion and cachexia will negatively influence performance status. It is therefore not surprising that performance status is adversely associated with nutritional variables.

A subgroup of 77 patients (35%) underwent a second nutritional assessment after a mean time interval of 95 days following diagnosis. These patients experienced on-going nutritional depletion and at an accelerated rate. Patients with unresectable pancreatic cancer demonstrate a similar inexorable progressive weight loss in an almost linear fashion as these patients approach death (Wigmore et al, 1997). In the present study, patients continued to lose weight irrespective of treatment intervention and despite improvements in the dysphagia scores of those patients who underwent surgery. In the case of those patients who underwent surgery the continued weight loss may reflect the catabolic response to the surgical insult and impaired muscle maintenance may continue for longer than 30 days following surgery (Petersson et al, 1990). A longer follow-up period would determine whether their nutritional status improves with recovery. However, certainly in the palliative treatment cohort, the general trend is for on-going nutritional decline among these patients.

Serum acute phase protein concentrations correlated with total weight loss and rate of weight loss and serum albumin concentrations also correlated with anthropometry measurements. Previously, a similar association between elevated serum CRP concentrations and increased weight loss in pancreatic cancer patients has been identified and other groups have found similar associations in other cancer types (Scott et al, 2003; O'Gorman et al, 1999; Barber et al, 1999b; Falconer et al, 1995). In addition, resting energy

expenditure (REE) in cancer patients is increased compared with controls and those patients with an APPR have greater REE compared with cancer patients without an APPR (Falconer et al, 1994). Cancer patients with an APPR have been shown to have elevated fibrinogen synthesis rates in both the fed and fasting states and it is this reprioritisation of protein metabolism that accompanies the APPR that may also contribute to the wasting observed in cancer cachexia (Preston et al, 1998; Barber et al, 2000). In this study CRP and albumin concentrations were identified as the best predictors of rate of weight loss by ROC curves. However, serum albumin concentrations may be influenced by malnutrition, hydration status, and trans-capillary escape, as well as the presence of an inflammatory response (Fleck et al, 1985) and therefore serum CRP concentrations were chosen for entry into the multivariate analysis.

Serum cytokine concentrations were generally not associated with any nutritional variable. IL-8 concentrations were correlated with increased total weight loss, but not rate of weight loss or any other nutritional variable and it is most likely that this result is due to multiple statistical testing. Although many other groups have demonstrated associations between serum cytokine concentrations and markers of nutritional depletion we have found no such association in this study or previously in a study of pancreatic cancer patients (Falconer et al, 1994). The difficulties in reliably measuring circulating cytokine concentrations has already been discussed in the Introduction (Chapter II), and the relevance of such measurements must also be considered. Local tissue cytokine production by inflammatory cells (or tumour cells) is likely to be a better indicator of cytokine activity in cancer cachexia (Falconer et al, 1994; Martignoni et al, 2005). However, circulating acute phase proteins (for example, CRP) remain robust indices of systemic pro-inflammatory status.

Increased total weight loss, increased rate of weight loss, reduced BMI, increased dysphagia scores, and reduced dietary intake were all associated with adverse prognosis. Moreover, the magnitude of weight loss was inversely associated with survival duration. The value of weight loss as a prognostic indicator will be investigated in a multivariate model in the following Chapter (Chapter XI).

Conclusions

Weight loss and reduced measures of nutritional status are common in patients with gastro-oesophageal malignancy. 83% of patients exhibit weight loss at the time of diagnosis and 92% of patients have lost weight three months later and at an accelerated rate. Weight loss appears to occur irrespective of treatment modality and independently of dysphagia score and dietary intake. These findings suggest that processes other than reduced intake or mechanical obstruction by the tumour are involved in the nutritional decline. Serum markers of systemic inflammation (acute phase protein concentrations and IL-8 and sTNF-R concentrations) are significantly associated with degree of weight loss and the presence of systemic inflammation (CRP concentrations) may account for 34% of this association. Therefore, the clinical sequelae of systemic inflammation may contribute to the weight loss observed in these patients. In addition, there is a highly significant negative association between degree of weight loss and survival duration. The association between systemic inflammation and adverse outcome in patients with gastro-oesophageal cancer has been investigated in the previous Chapter (Chapter IX). Accelerated weight loss may provide one of the mechanisms whereby systemic inflammation acts to reduce survival duration.

In Chapter IX the association between systemic inflammation and outcome in patients with gastro-oesophageal cancer was investigated. Concentrations of serum acute phase proteins were associated with survival duration and serum CRP concentration was identified as an independent prognostic indicator on multivariate survival analysis. In the present Chapter, a significant negative association between weight loss and survival duration was identified. The next Chapter will investigate the value of systemic inflammation and adverse nutritional status in improving prognostic accuracy for patients with gastro-oesophageal cancer. Various clinical and investigative factors, including markers of systemic inflammation and cachexia, predictive of death from gastro-oesophageal cancer will be determined with the aim of constructing a novel risk prediction model that may improve prognostic accuracy. This model may then be used to assist in the prospective prognostic evaluation of patients with gastro-oesophageal malignancy and assist in the clinical decision making for these patients.

CHAPTER XI

A CLINICAL PROGNOSTIC SCORING SYSTEM TO AID MANAGEMENT DECISION-MAKING FOR PATIENTS WITH GASTRO-OESOPHAGEAL CANCER

Deans DAC, Wigmore S, Ross JA, de Beaux A, Paterson-Brown S, Fearon KCH
A clinical prognostic scoring system to aid management decision-making for patients with gastro-oesophageal cancer.
British Journal of Surgery 2007; 94(12): 1501-1508

ABSTRACT

Despite advances in pre-operative staging accurate prognostication for patients with gastro-oesophageal malignancy remains challenging, particularly in the identification of patients who are likely to benefit from surgical resection. Markers of systemic inflammation and adverse nutritional status have been linked with adverse outcome in these patients (Chapters IX and X). This Chapter investigated the usefulness of systemic inflammation and cachexia as prognostic indicators. Various clinical and investigative factors, including markers of systemic inflammation and cachexia, predictive of death from gastro-oesophageal cancer were determined with the aim of constructing a novel risk prediction model. This model may then be used to assist in the prospective prognostic evaluation of patients with gastro-oesophageal malignancy and assist in the clinical decision making for these patients.

All patients newly diagnosed with gastro-oesophageal cancer over a 2-year period were studied prospectively. Patients were staged as per unit policy and underwent treatments following discussion at the multi-disciplinary meeting. Clinical and investigative variables were collected and included markers of systemic inflammation and a nutritional assessment. Death within 12 and 24 months following diagnosis were the primary end-points.

Two hundred and twenty patients were studied with an overall median survival of 12.6 months. Advanced clinical stage (cTNM) ($p<0.001$), reduced Karnofsky performance score ($p<0.001$), weight loss ≥ 6% body weight ($p=0.026$), and serum CRP concentration >5 mg/l ($p=0.031$) were identified as independent prognostic indicators on multivariate analysis. A prognostic risk score was constructed using these four variables to estimate the probability of death. Applying the model gave an area under the receiver operating characteristic curve of 0.84 ($p<0.001$) to predict death at 12 months and an area of 0.85 ($p<0.001$) to predict death at 24 months.

The addition of biological (systemic inflammation – serum CRP concentration) and host-related (weight loss and performance score) factors to conventional anatomical-based clinical staging modalities can improve prognostic accuracy for individual patients with gastro-oesophageal cancer. The risk score is able to accurately estimate the probability of death within 12 and 24 months of diagnosis and may be used prospectively to guide management decisions and provide quality assurance in the MDT decision-making process.

INTRODUCTION

This thesis has so far described some of the mediators involved in the generation and progression of systemic inflammation in patients with gastro-oesophageal cancer. The clinical and nutritional sequelae of systemic inflammation in these patients have also been described. In addition, markers of systemic inflammation and adverse nutritional status have been linked with adverse outcome in these patients (Chapters IX and X). This Chapter investigated the usefulness of systemic inflammation and cachexia as prognostic indicators. Various clinical and investigative factors, including markers of systemic inflammation and cachexia, predictive of death from gastro-oesophageal cancer were determined with the aim of constructing a novel risk prediction model. This model may then be used to assist in the prospective prognostic evaluation of patients with gastro-oesophageal malignancy and assist in the clinical decision making for these patients.

Despite advances in surgical care and improvements in staging accuracy overall survival for patients with gastro-oesophageal malignancy remains poor (Dressner and Griffin, 2000). Even for patients undergoing surgical resection 5-year survival rates are in the order of 30% and for patients presenting with locally advanced disease, who represent approximately two thirds of patients suitable for resection, the survival rates are only in the order of 10% (Dressner and Griffin, 2000). Around half of these patients are dead within 18 months and do not achieve any benefit from surgery in terms of quality of life improvements or survival gain (Blazeby et al, 2000). The problem lies in prospectively identifying such patients. If clinicians could identify which patients are unlikely to survive 18 months following surgery then these patients could be offered more appropriate palliative treatments and avoid the considerable morbidity that may be associated with surgical resection. In addition, improved understanding of the factors important in determining outcome in these patients, such as the presence of systemic inflammation and cachexia, may assist in the identification of novel therapeutic targets and help guide individualised patient directed therapy, for example, anti-cachexia treatments.

Several prognostic models have been proposed in an attempt to improve prognostic accuracy in patients with gastro-oesophageal cancer. Some of these models are designed to predict operative risk and mortality (Tekkis et al, 2004; Bartels et al, 1998) and most studies that have proposed prognostic scores have utilised pathological variables in their scoring systems (Kologlu et al, 2000; Novotny et al, 2006). Other models have utilised complex computer models, such as artificial neural networks, to predict long-term survivors following surgical resection (Sato et al, 2005; Mofidi et al, 2006). Although these models have demonstrated improved survival predictions when compared with TNM staging alone, these models rely on the input of many variables including pathological information only available after resection and therefore may not be used prospectively. Relatively few studies have attempted to prospectively identify patients who are 'curable' using clinical/pre-operative staging modalities alone. Crumley *et al* studied patients with inoperable oesophago-gastric cancer and devised an inflammation-based prognostic scoring system (Crumley et al, 2006). A score was accrued depending on the presence of a CRP concentration >10 mg/l and/or an albumin concentration <35 g/l. Survival duration was significantly reduced with an increasing score, independent of disease stage. A group of Japanese researchers have similarly identified clinical stage (cTNM), weight loss (more than 2% body weight), and serum CRP concentrations (greater than 0.5 mg/dl) to be independently associated with survival on multivariate analysis for patients with oesophageal malignancy (Ikeda et al, 2003). Patients scored one point according to the presence of stage III or IV disease, CRP concentration >0.5 mg/dl, and weight loss >2% and the total score (0 to 3) was superior to the conventional cTNM staging at determining prognosis.

This Chapter will determine the clinical and investigative factors, including markers of systemic inflammation and cachexia, predictive of death from gastro-oesophageal cancer with the aim of constructing a novel risk prediction model that may be applied to these patients to assist in clinical decision-making. This model will aim to determine the likelihood of death within 12 and 24 months from diagnosis.

MATERIALS AND METHODS

Study population

The same cohort of patients as those studied throughout this thesis was investigated (see Chapter IV for a detailed description). No patients were excluded and no patients refused consent. Data were prospectively collected. Patient performance status was assessed at the time of diagnosis using the scoring system devised by Karnofsky (Karnofsky and Burchenal, 1949). In all instances a final clinical stage (cTNM) was agreed at the unit multidisciplinary team meeting (MDT) and management strategies were decided. Patients without evidence of metastatic disease (stages I-III) and who were medically fit were offered surgical resection. Those patients staged as T3 N0 or T2/3 N1 were considered for pre-operative chemotherapy. The remaining patients received a variety of palliative treatments, either in combination or alone. Survival duration, defined as time from histological confirmation of disease until death, was recorded in each case.

Variables that could not be used prospectively, such as number of lymph nodes with metastatic involvement, degree of chronic inflammatory cell infiltrate into the tumour tissue and final histopathological stage, were excluded from prognostic modelling. Serum cPTHrP concentrations were also excluded form the analysis as longer follow-up duration has subsequently shown that these concentrations are no longer directly prognostic.

Measurement of markers of systemic inflammation

Blood was collected from each patient at the time of diagnosis and before initiation of treatment. Serum cytokine and serum acute phase protein concentrations were determined by ELISA as described in previous Chapters.

Nutritional assessment

All patients underwent an assessment of their nutritional status at the time of diagnosis as described in Chapter X. Briefly, BMI was calculated and total weight loss and rate of weight loss per month were recorded. Anthropometry measurements were corrected for age and sex and were normalised using standardised reference tables (Bishop et al, 1981). The severity of

dysphagia was scored according to Knyrim *et al* (Knyrim et al, 1993). Dietary intake was estimated by all patients and described as normal, reduced, or poor.

Statistical analysis

Cox's proportional hazards model was used to determine the effects of clinical and investigative variables on survival duration and probability of death within 12 and 24 months following diagnosis in both univariate and multivariate analysis. Stepwise regression (using an entry value $p \leq 0.050$ and a removal value $p \geq 0.100$) was used to determine the factors predictive of death. Models were developed separately for clinical and investigative variables and then for a combination of the two sets of variables. Receiver operating characteristic (ROC) curves were used to select cut-off values for continuous variables. Values with the best combination of sensitivity and specificity were chosen. The performance of the prognostic model was assessed by determining the area under the ROC curve. The estimates of probability of death at 12 and 24 months were calculated from survival tables.

Results

Study patients

Two hundred and twenty patients were recruited to the study between March 2002 and June 2004. Patient demographics are shown in Table 11.1. Ninety-five (43%) patients underwent surgical resection and 25 of these received pre-operative chemotherapy. Ninety (95%) patients had a potentially curative resection. Of the remaining five patients, three patients had unexpected metastatic disease identified at laparotomy and therefore did not proceed to resection and two patients had residual macroscopic disease (R2 resection). Seven (3%) patients, all of whom had squamous cell carcinoma of the oesophagus, received chemo-irradiation with curative intent. The remaining 118 patients (54%) were not suitable for curative therapy and received palliative chemo/radiotherapy or underwent alternative palliative treatments, such as insertion of a stent, endoscopic dilatation, or endoscopic laser therapy.

Seventy-five (34%) patients had metastatic disease (stage IV) at the time of presentation and 86 (39%) patients had locally advanced (stage III) disease. Approximately one-quarter (n=59; 27%) of patients had early stage disease (stage I/II).

Table 11.1 Study patient demographics (n=220)

		Number [%]
Age (years)[+]		71 (62-78)
Sex	Male	145 [66]
	Female	75 [34]
Karnofsky score	30	4 [2]*
	40	1 [1]
	50	5 [2]
	60	17 [8]
	70	25 [12]
	80	36 [17]
	90	50 [24]
	100	70 [34]
	Unknown	12
Tumour Site	Oesophageal	101 [46]
	Proximal third	2
	Middle third	13
	Distal third	86
	Oesophago-gastric junction	40 [18]
	Gastric	79 [36]
	Proximal	25
	Body	26
	Distal	28
Histology	Adenocarcinoma	185 [84]
	Squamous cell carcinoma	30 [14]
	Small cell	2 [1]
	Indeterminate	3 [1]
Grade	Well differentiated	3 [2]*
	Moderately differentiated	63 [34]
	Poorly differentiated	118 [64]
	Not commented	36
Treatment undertaken	Surgery alone	70 [32]
	Pre-operative chemotherapy + surgery	25 [11]
	Chemoradiotherapy with curative intent	7 [3]
	Palliative Chemotherapy	28 [13]
	Palliative Radiotherapy	6 [3]
	Stent/dilatation/laser/symptomatic	84 [38]
UICC Stage	1	25 [11]
	2	34 [16]
	3	86 [39]
	4	75 [34]
Status	Alive	73 [33]
	Dead	147 [67]

[+]Values are median (interquartile range). * Values are expressed as percentages of known results.

Survival duration

Patients were followed-up for an average 32 months and a minimum of 18 months (range 18-45 months). At the time of censoring the data 147 (67%) patients had died. Three patients died in the post-operative period and 1 patient died from injuries sustained following a fall. Information obtained from death certificates indicated that all other deaths were disease related. One hundred and eight (49%) patients had died within 12 months of diagnosis and 136 (136 of 194 patients followed-up for at least 24 months; 70%) patients had died within 24 months. Overall median survival was 12.6 months.

Performance status

Seventy (34%) patients had a normal performance status at the time of diagnosis (Karnofsky score 100). The remaining 150 patients had some degree of impaired performance status (Table 11.1). Ten (5%) patients required considerable assistance and frequent medical care or hospitalisation (Karnofsky 30-50).

Nutritional status

The nutritional variables for the patient group measured at the time of diagnosis are the same as those data previously presented in Chapter X and are shown again in Table 11.2. Patients had lost a median of 7.1% (inter-quartile range 1.2-14.2%) total body weight at the time of diagnosis, equivalent to a median rate of weight loss of 2.5% total body weight per month of illness. Ninety-seven (44%) patients had lost up to 10% of their total body weight and 85 (39%) patients had lost more than 10% body weight. Only 38 (17%) patients remained weight steady at the time of diagnosis. Anthropometry values were globally reduced compared with reference values. 135 (61%) patients had a reduced dietary intake and 131 (60%) patients had some degree of dysphagia ta the time of diagnosis.

Table 11.2 Nutritional variables for the patient group measured at the time of diagnosis.

Pre-illness BMI		26.4 (24.1-30.1)
BMI at diagnosis		24.6 (21.4-28.0)
Total body weight loss (%)		7.1 (1.2-14.2)
Rate of weight loss (% per month)		2.5 (0.3-6.5)
Mid-arm circumference (percentile group)		10 (1-25)
Triceps skinfold thickness (percentile group)		25 (10-50)
Arm-muscle circumference (percentile group)		10 (1-25)
Dietary intake	Normal	85 [39]
	Reduced	103 [47]
	Poor/minimal	32 [14]
Dysphagia score	0	89 [40]
	1	52 [24]
	2	43 [19]
	3	32 [15]
	4	4 [2]

Values are median (inter-quartile range) [%]

Concentrations of serum markers of systemic inflammation

Concentrations of serum acute phase proteins and sTNF-R, but not concentrations of serum cytokines, are associated with survival duration (Chapter IX). Serum CRP concentrations were identified as the best predictors of prognosis among all the acute phase proteins and cytokines measured, as determined by receiver operating characteristics (ROC) curves [area under the curve = 0.67, $p<0.001$] (see Table 9.2; Chapter IX). The median serum CRP concentration for the patient group was 7 mg/l (inter-quartile range 2-25 mg/l). One hundred and twenty-one (55%) patients had a CRP concentration greater than 5 mg/l at the time of diagnosis.

Identification of prognostic variables

Table 11.3 shows the risk of death form gastro-oesophageal cancer associated with baseline clinical characteristics and results of investigations. Patient age, body mass index at the time of diagnosis, total body weight loss at diagnosis, rate of weight loss, Karnofsky performance score, serum CRP concentration, dysphagia score, level of dietary intake, tumour grade, and clinical stage were all associated with outcome for the whole patient group. Variables that could not be used prospectively to aid management decision making, such as number of lymph nodes with metastatic involvement and the presence of a chronic inflammatory cellular infiltrate into the tumour, were excluded from the prognostic modelling.

Table 11.3 Hazard ratio of death associated with baseline clinical characteristics and results of investigations for the whole patient group with gastro-oesophageal cancer (Cox's univariate analysis).

Variable	Hazard ratio (95% CI)	P value
Age (years)	1.00-1.04	0.015
BMI at diagnosis	0.92-0.99	0.005
Total weight loss at diagnosis (%) [Ln]	1.20-1.73	<0.001
Rate of weight loss (% per month) [Ln]	1.20-1.71	<0.001
Dysphagia score	1.10-1.49	0.002
Dietary intake	1.38-2.31	<0.001
Karnofsky score	0.95-0.97	<0.001
CRP (mg/l) [Ln]	1.25-1.56	<0.001
Tumour grade	1.14-2.51	0.009
Stage of disease (cTNM)	2.01-3.29	<0.001

Determination of end-points

Twelve and 24 months were selected as end-points for prognostic analysis. Receiver operating characteristic (ROC) curves were used to identify cut-off values with the best discrimination power for each continuous variable. Values with the best combination of

sensitivity and specificity were chosen (Figure 11.1). The following discrimination points were selected: age greater or equal to 70 years, body mass index less than or equal to 25, weight loss greater or equal to 6%, rate of weight loss greater than 2.75% per month, Karnofsky score less than or equal to 80, and CRP concentration greater than 5 mg/l. The risk of death for the new cut-off points selected for each variable are shown in Table 11.4.

Figure 11.1 Line graph depicting the sensitivity and specificity of increasing serum CRP concentrations on predicting 12 month survival. A CRP cut-off value greater than 5 mg/l had the best combination of sensitivity and specificity. [Heavy line = specificity, dashed line = sensitivity.]

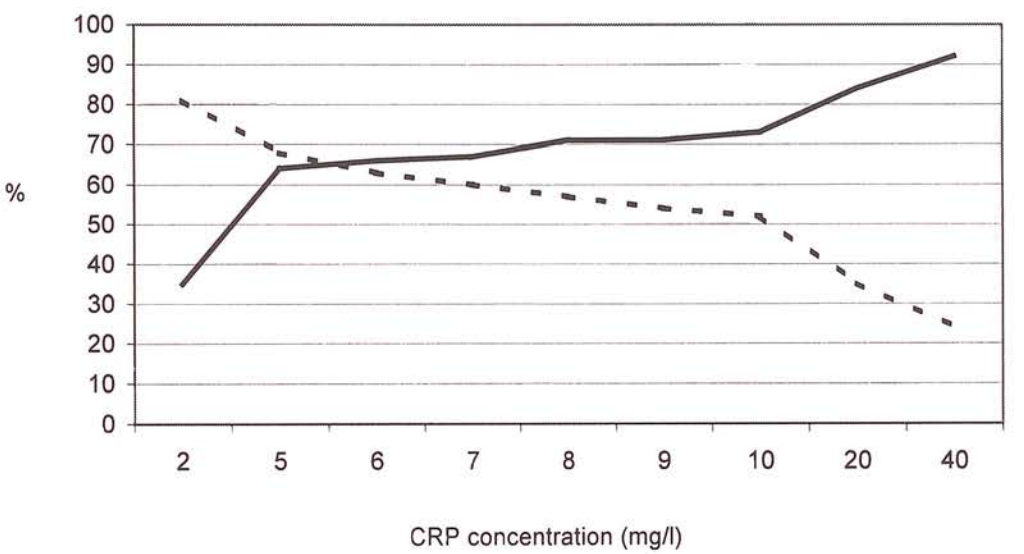


Table 11.4 Hazard ratio for risk of death for each new cut-off point chosen following discrimination analysis by ROC curves [Cox's univariate analysis].

Variable	Hazard ratio	95% CI	P value
Age (≥ 70 years)	1.6	1.15-2.24	0.005
BMI (≤ 25)	1.5	1.04-2.02	0.028
Weight loss (≥ 6%)	2.6	1.79-3.60	<0.001
Rate of weight loss (>2.75% per month)	2.5	1.86-3.75	<0.001
Dysphagia score			
1 versus no dysphagia	0.7	0.42-1.11	
2 versus no dysphagia	1.5	0.95-2.30	<0.001
3 versus no dysphagia	1.7	1.03-2.75	
4 versus no dysphagia	28.9	8.19-101.84	
Dietary intake			
Reduced versus normal	1.6	1.08-2.34	<0.001
Poor versus normal	3.6	2.21-6.00	
Karnofsky score			
80-90 versus normal	1.5	0.96-2.29	<0.001
60-70 versus normal	4.4	2.73-6.99	
≤ 50 versus normal	9.2	4.53-18.83	
CRP (>5 mg/l)	2.6	1.83-3.64	<0.001
Clinical stage of disease (cTNM)			
Stage II versus stage I	1.9	0.63-5.57	<0.001
Stage III versus stage I	3.0	1.11-8.35	
Stage IV versus stage I	11.2	4.02-30.96	
Tumour grade			
Poorly differentiated versus moderate	1.7	1.12-2.46	0.012

Multivariate survival analysis

Due to the relatively large number of prognostic variables multivariate analysis of clinical variables and investigative variables were initially analysed separately. Total weight loss and Karnofsky score were identified as independent prognostic variables from the analysis of the clinical variables. Serum CRP concentration and clinical stage were identified as independently prognostic from the analysis of investigative variables. These four variables were then analysed together and each retained independent prognostic value (Table 11.5).

Table 11.5 Clinical and investigative variables independently predictive of death, determined by using stepwise selection procedures in a general population of patients with gastro-oesophageal cancer. [Forward, conditional method of Cox's proportional hazards model.]

Variable	Hazard ratio	95% CI	P value
CRP (> 5mg/l)	1.6	1.04-2.34	0.031
Total weight loss (≥ 6%/month)	1.6	1.05-2.28	0.026
Karnofsky score			
80-90 versus normal	1.0	0.64-1.56	0.996
60-70 versus normal	2.1	1.30-3.54	0.003
≤ 50 versus normal	4.8	2.23-10.16	<0.001
Clinical stage of disease (cTNM)			
Stage II versus stage I	2.0	0.57-6.96	0.277
Stage III versus stage I	2.9	0.89-9.36	0.076
Stage IV versus stage I	8.8	2.68-28.60	<0.001

Development of a prognostic risk score for patients with gastro-oesophageal cancer

Serum CRP concentration greater than 5 mg/l, weight loss greater or equal to 6% body weight per month, reduced Karnofsky performance score, and increasing clinical stage were all identified as independent adverse prognostic variables. A prognostic scoring system was devised by logarithmically transforming the hazard ratio and multiplying by 100 (Daly et al, 2006). Therefore, for each patient a cumulative risk score may be calculated based upon the four prognostic variables (maximum score 202) (Table 11.6). The prognostic risk score could then be used to estimate the probability of death within 12 and 24 months (Table 11.7) (Figure 11.2). Applying the model developed on 75% of the patient population to the remaining 25% of the population gave an area under the receiver operating characteristic curve of 0.84 ($p<0.001$) to predict death at 12 months and an area of 0.85 ($p<0.001$) to predict death at 24 months (Figure 11.3).

Table 11.6 Risk score based on four prognostic variables.

Variable	Risk score*
CRP (mg/l)	
≤ 5	0
> 5	20
Weight loss (% body weight lost)	
< 6	0
≥ 6	20
Karnofsky score	
80-100	0
60-70	32
≤ 50	68
Clinical stage of disease (cTNM)	
Stage I	0
Stage II	30
Stage III	46
Stage IV	94

*Log [hazard ratio] multiplied by 100.

Table 11.7 Estimated probability of death from time of diagnosis to 12 months and 24 months stratified by prognostic risk score.

Risk score	Probability of death at 12 months (%)	Probability of death at 24 months (%)
0	0	0
20	0	0.5
30	0.5	2.7
40	1.0	3.3
46	2.7	9.1
50	4.5	11.6
66	11.5	21.3
70	13.8	24.1
72	14.6	24.8
78	16.1	26.2
82	16.8	26.9
86	23.0	35.4
94	27.5	40.7
98	28.4	43.0
102	29.3	44.5
114	42.7	55.1
118	51.6	63.8
134	66.3	77.5
138	67.5	78.3
146	73.5	82.5
154	74.8	84.1
166	90.7	94.2
182	92.0	95.0
202	100	100

Figure 11.2 Plot to assign estimated probability of death within 12 months (red) and 24 months (green) from diagnosis according to risk score (n=220).

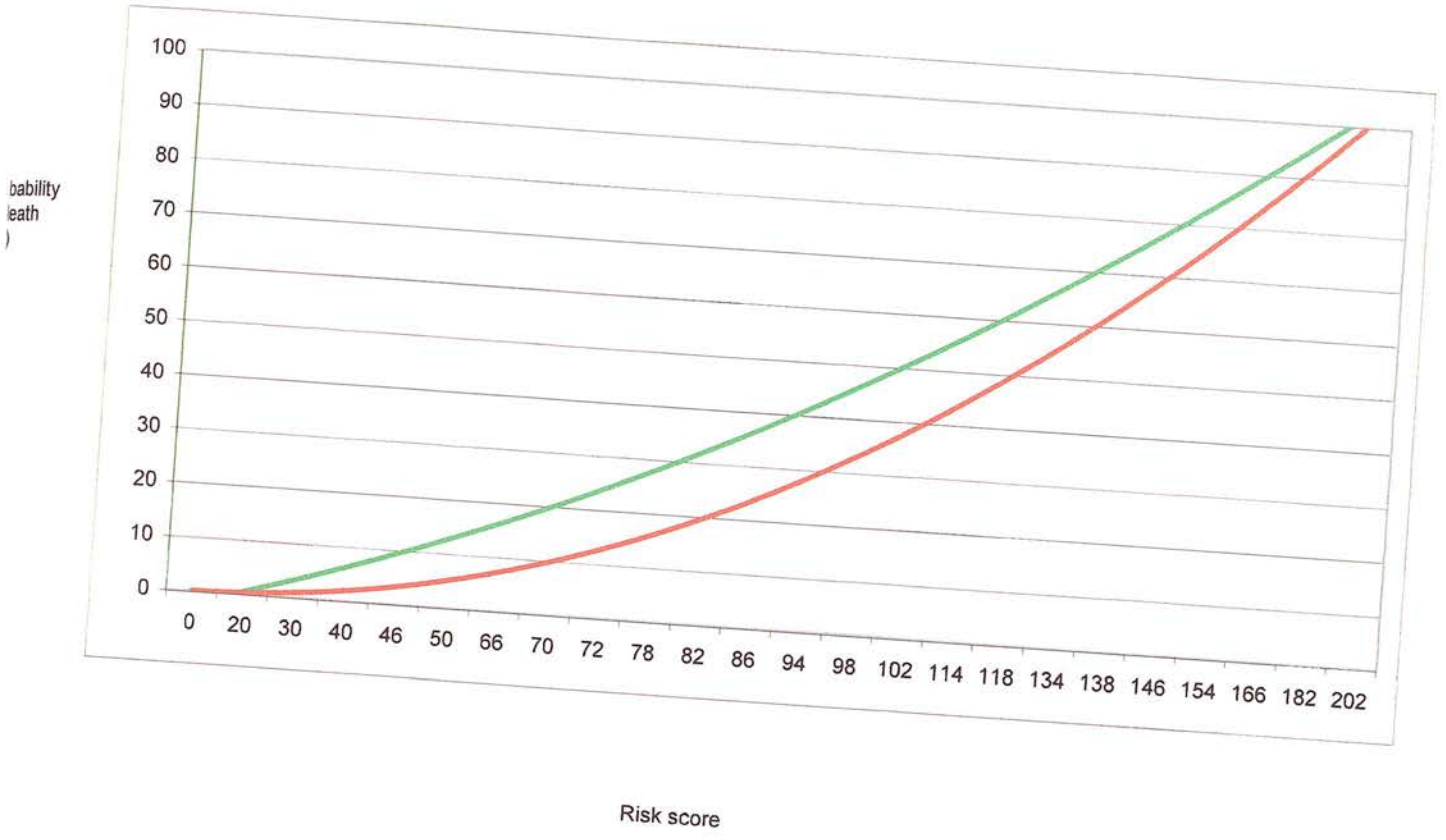
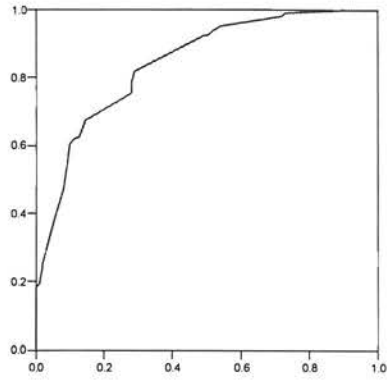
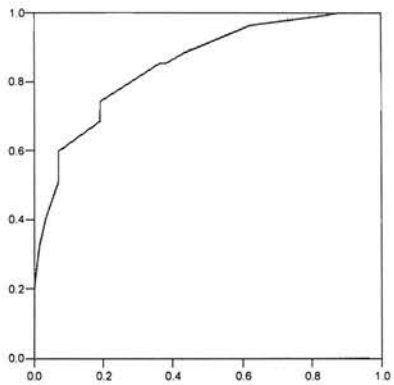


Figure 11.3 Receiver operating characteristic curves illustrating the predictive ability of the prognostic risk score to predict death at (A) 12 months [area under the curve = 0.84 ($p<0.001$)] and (B) 24 months [area under the curve = 0.85 ($p<0.001$)].

A



B



Subgroup analysis

Applying the risk score to the cancer subsites gave areas under the ROC curves for 12 and 24 months survival as: 0.84 and 0.89 for oesophageal cancer, 0.86 and 0.89 for gastric cancer, and 0.78 and 0.67 for tumours at the OGJ. The score was valid for adenocarcinoma at 12 and 24 months (area under ROC curve 0.86 and 0.87) and squamous cell carcinoma (0.78 and 0.68). Analysis by treatment type gave areas under the curve of 0.78 and 0.79 for 12 and 24 months survival in patients who underwent surgery with curative intent, and 0.77 and 0.79 for those treated by palliative methods alone.

DISCUSSION

This study has identified clinical disease stage, Karnofsky performance score, weight loss, and serum CRP concentrations as independent prognostic indicators on multivariate analysis for patients with gastro-oesophageal cancer. The development of a risk prediction score based on these four variables allows improved prognostic accuracy and risk prediction of death within 12 and 24 months from diagnosis.

Recent improvements in clinical staging accuracies have occurred due to advances in technology as well as increasing expertise in their use and the formation of special interest radiologists/gastroenterologists. Despite these advances such modalities are still failing to adequately stratify patients. Current staging strategies are directed at determining the anatomical spread of disease. Clearly, not all tumours within the same UICC anatomical stage group behave in the same way and, therefore, clinical staging and estimation of prognosis should additionally assess tumour biological behaviour.

Both the presence of systemic inflammation and nutritional depletion (cachexia) can be used as indirect measures of tumour biological activity. Systemic inflammation has been found in association with most epithelial malignancies and has been linked with adverse outcome (Rashid et al, 1982; Nozoe et al, 2001; Shimada et al, 2003; Crumley et al, 2006; Alexandrakis et al, 2003; Falconer et al, 1995; Cooper, 1988; Caspers et al, 1984; Forrest et al, 2003; McMillan et al, 2001; Masuda et al, 1998; Kodama et al, 1999; Elahi et al, 2005; McMillan et al, 2003; Fujita et al, 1999; Jamieson et al, 2005). In Chapter IX, an elevated serum CRP concentration was associated with reduced survival duration, independent of stage of disease, in our cohort of patients with gastro-oesophageal cancer. The present study has confirmed this finding, where a serum CRP concentration greater than 5mg/l was associated with adverse outcome on multivariate survival analysis. The source of the pro-inflammatory stimulus in advanced cancer remains unclear, but it is likely that both the host immune cells and the tumour cells contribute to the generation of the systemic inflammatory response. Both the presence of a host chronic inflammatory cell infiltrate and tumour tissue cytokine concentrations have been associated with markers of systemic inflammation in previous studies (Chapters V). In addition, other tumour-derived mediators, such as PTHrP

(Chapter VII) and PIF (Chapter VIII), are also linked with elevated serum markers of systemic inflammation. The presence of systemic inflammation in association with malignant disease may, therefore, indicate a tumour with increased growth/metastatic potential.

Weight loss is common among patients with gastro-oesophageal cancer. In Chapter X, 83% of patients experienced some degree of weight loss at the time of diagnosis and weight loss was negatively associated with survival duration. Weight loss was also associated with advanced stage of disease and with markers of systemic inflammation, however, the current study has shown that rate of weight loss retains prognostic significance even when systemic inflammation and disease stage are co-analysed in a multivariate model. Weight loss greater than 10% total body weight has been associated with adverse outcome on multivariate analysis among patients with oesophageal cancer who had undergone surgical resection and chemo-irradiation (Kelson et al, 1998). The present study has identified weight loss greater than 6% as a more discriminating cut-off value in our patient cohort. These data suggest that weight loss and nutritional depletion reflects adverse tumour behaviour by additional unknown mechanisms, but may be linked with the production of pro-inflammatory cytokines or other tumour derived mediators, for example PTHrP and PIF.

The relevance of performance status to prognosis is well documented and is an important assessment tool in the selection of patients for chemo/radiotherapy. Among patients with gastro-oesophageal malignancy, performance status was identified as an independent prognosticator on multivariate analysis of over 1000 patients, along with the presence of metastatic disease and an elevated serum alkaline phosphatase concentration (Chau et al, 2004). Increased fatigue and reduced physical function have been associated with increased probability of death in the first 6 months following surgical resection of gastro-oesophageal cancer (Blazeby et al, 2001; Blazeby et al, 2005). A multicentre study of 1080 patients with locally advanced or metastatic gastro-oesophageal cancer treated with chemotherapy identified performance status, serum alkaline phosphatase concentration, and the presence of liver or peritoneal metastases as independent prognostic indicators on multivariate analysis (Chau et al, 2004). However, clinical stage was not co-analysed in the multivariate analysis. A Swedish study of oesophageal cancer patients also treated by

chemoradiotherapy similarly identified performance status, in addition to clinical stage, as independent prognostic variables (Bergqvist et al, 2004).

A number of prognostic scoring systems have been proposed for patients with gastro-oesophageal cancer. Most of these studies include pathological information gained from tumour resection and these data cannot be used at the time of diagnosis to prospectively stratify patients. Other prognostic studies have used sophisticated novel molecular techniques, such as DNA microarray, to prognosticate patients (Inoue et al, 2002) or have involved the development of complex computer models, for example, artificial neural networks, where extensive clinical, investigative, and pathological information is required to estimate patients' prognosis (Mofidi et al, 2006). Crumley *et al* studied patients with inoperable oesophago-gastric cancer and devised an inflammation-based prognostic scoring system (Crumley et al, 2006). A score was accrued depending on the presence of a CRP concentration >10 mg/l and/or an albumin concentration <35 g/l. Survival duration was significantly reduced with an increasing score. A group of Japanese researchers have similarly identified clinical stage (cTNM), weight loss (more than 2% body weight), and serum CRP concentrations (greater than 0.5 mg/dl) to be independently associated with survival on multivariate analysis for patients with gastro-oesophageal malignancy (Ikeda et al, 2003). Patients scored one point according to the presence of stage III or IV disease, CRP concentration >0.5 mg/dl, and weight loss >2% and the total score (0 to 3) was associated with significantly reduced survival duration.

The scoring system developed in this study allows accurate estimation of the probability of death within 12 and 24 months for individual patients. Clearly, the score selected as the cut-off, will influence the probability of survival beyond 12 or 24 months. A higher score would have better specificity, but some patients would still be offered surgery without much prospect of longer survival. Selection of a lower score would perhaps deny the opportunity of surgery to some patients who may benefit from resection. We believe that it is better to select a higher score and accept that a few patients may have been overtreated. The final cut-off is likely to depend on future validation studies and individual preferences.

Conclusions

This study has shown that the addition of biological (systemic inflammation – serum CRP concentration) and host-related (weight loss and performance score) factors to conventional anatomical-based clinical staging modalities can improve prognostic accuracy for individual patients. The novel prognostic scoring system may be used for patients with oesophageal, junctional and gastric tumours with similar accuracy and different histological tumour types. The risk score is based on four measurements that are widely available, reproducible, inexpensive and easy to perform. The score may be used prospectively to guide management decisions at the time of diagnosis and provide more realistic prognostic information for patients and their families. In addition, the risk model can be used to provide quality assurance in the MDT decision-making process. The model requires prospective validation in other centres before widespread introduction into clinical practice.

CHAPTER XII

GENERAL DISCUSSION

The aetiology and significance of systemic inflammation in patients with cancer is complex and incompletely understood. Whereas much is known about the innate immune system in cancer biology in general, the factors that activate this system in human subjects with specific types of tumour are not known. The present thesis has examined patients with gastro-oesophageal cancer in detail.

Prevalence of systemic inflammation in patients with gastro-oesophageal cancer

In the present study 43% of patients at diagnosis had evidence of systemic inflammation and the prevalence of systemic inflammation was comparable with those quoted in previous studies relating to gastric and oesophageal malignancy (range 23-32%) [Chapter IV] (Crumley et al, 2006; Rashid et al, 1982; Nozoe et al, 2001; Shimada et al, 2003). In addition, CRP concentrations demonstrated excellent reproducibility for measurements undertaken at the time of diagnosis. However, follow-up CRP concentrations were influenced by intervening treatments. Surgical resection was associated with a significant rise in serum CRP concentrations in the post-operative period. This may be explained by an ongoing metabolic response to surgical trauma or may represent subclinical complications during the post-operative period. In contrast, pre-operative chemotherapy was linked with a reduction in serum CRP concentrations and may occur secondary to generalised immunosuppression. Such findings are important to consider if markers of systemic inflammation are to be used to estimate prognosis or influence treatment decisions.

In contrast, in the present thesis, serum cytokine concentrations determined at diagnosis were not significantly different to those measured in healthy controls and demonstrated poor reproducibility [Chapter IV]. The similarities in serum cytokine concentrations measured in patients and healthy controls are likely to be related to the difficulties in reliably measuring circulating cytokine concentrations owing to variations in plasma concentrations and short half-lives (May et al, 1992). In addition, the end-organ effects of these cytokines will depend upon binding to transport molecules, receptor expression, receptor affinity, and local cytokine concentrations within target tissues (Lantz et al, 1990).

The conclusions of these studies are that serum positive acute phase protein concentrations (especially CRP concentrations) are robust markers of systemic inflammation when measured at the time of diagnosis and before initiation of treatment, whereas serum cytokine concentrations are not representative of true levels of systemic inflammatory activity. It is more likely that local cytokine concentrations within target tissues are a more accurate determinant of systemic inflammatory activity.

The aetiology of systemic inflammation in patients with gastro-oesophageal cancer

One of the key hypotheses in this thesis was that tumour cells not only produce pro-inflammatory cytokines but other mediators (for example, PTHrP and PIF), which may induce systemic inflammation in patients with cancer. The close relationship between tumourigenesis and host immunology has been described in detail in the introduction and it is likely that the interaction between host and tumour cell populations is key to the generation of systemic inflammation. Tumour escape and subversion of host immunosurveillance are facilitated by the creation of a local environment within the tumour (Seliger et al, 2001; Garrido and Algarra, 2001). Such a milieu consists of a mix of mediators, including pro-inflammatory cytokines, enabling the complex interaction between the host cells and tumour cells (Almand et al, 2001). Postulated outcomes of this interaction include carcinogenesis and tumour growth and metastasis (Dalgleish and O'Byrne, 2002; O'Byrne and Dalgleish, 2001; Sharma et al, 2003; Bottazzi et al, 1990). The generation of systemic inflammation may occur secondary to these locally produced mediators (Raddatz et al, 2005).

In this thesis pro-inflammatory cytokine (mRNA and protein) concentrations were significantly elevated in tumour tissue from patients with gastro-oesophageal cancer when compared with concentrations measured in tissue samples from healthy controls [Chapter V]. Although all cytokine concentrations were elevated, tumour tissue IL-1β was particularly highly expressed and protein concentrations correlated with markers of systemic inflammation (CRP). Previously, IL-6 over-expression has been associated with a pancreatic cancer phenotype linked with inflammation and cachexia (Martignoni et al, 2005). IL-1β is an important pro-inflammatory cytokine and it is the conclusion of the work in this thesis that in

patients with gastro-oesophageal malignancy, IL-1β is an important candidate mediator involved in the aetiology of systemic inflammation.

In this thesis, the extent of host immune cell infiltrate into the tumour tissue was also assessed [Chapter V]. Tumour tissue is commonly infiltrated by immune cells and up to 60% of the tumour mass may be accounted for by macrophages and lymphocytes (van Ravenswaay et al, 1992). Tumour cells are thought to regulate immune cell numbers through production of chemotactic factors (Koide et al, 2004). It is postulated that tumour cells produce mediators such as IL-4 and IL-10, which lead to alternative macrophage activation favouring the tumour (O'Byrne and Dalgleish, 2001). These host immune cells may also contribute to the generation of systemic inflammation (O'Riordain et al, 1999). In this thesis a chronic inflammatory cell (lymphocyte) infiltrate (TIL's) was noted in 75% of tumours and was associated with markers of systemic inflammation (CRP), but not with elevated tissue cytokine concentrations. TIL's may become activated as they pass through the tumour tissue by locally produced mediators (cytokines). These lymphocytes may interact locally with macrophages within the tumour (TAM's). Macrophages are then free to circulate in the bloodstream and release cytokines locally within target tissues, thereby conveying the systemic effects of inflammation, without increasing local cytokine concentrations within the tumour tissue itself. Previously, circulating PBMC's from patients with pancreatic cancer and an APPR demonstrated greater rates of pro-inflammatory cytokine (IL-6 and TNFα) production when compared with those without a systemic inflammatory response (O'Riordain et al, 1999). These cells are then capable of stimulating increased CRP production by hepatocytes (O'Riordain et al, 1999). Other groups have also identified the ability of certain tumours to sensitise PBMC's and induce IL-6 expression from these cells (Martignoni et al, 2005). Rates of pro-inflammatory cytokine production by PBMC's from patients with gastro-oesophageal cancer were not measured in this thesis, but it is feasible that these cells would demonstrate increased cytokine activity. This may explain the association between TIL's and the presence of systemic inflammation in the present study.

The studies in this thesis were performed on homogenised tissue samples and total cytokine mRNA and protein concentrations within the tumour mass were measured. As stated previously, up to 60% of the tumour mass may be host immune cells and other cell types,

such as stromal cells and vascular endothelium (van Ravenswaay et al, 1992). Laser capture miscroscopy was used in an attempt to separate out these different cell populations in order to determine which of these cell types had undergone up-regulation of pro-inflammatory cytokines. Unfortunately, due to poor quality of isolated RNA this technique was not successful in this thesis. Alternative techniques to localise the main cell populations producing the pro-inflammatory cytokines would include insitu hybridisation or immunohistochemistry. These techniques may form the basis of future work. However, the studies described in this thesis show that pro-inflammatory cytokine protein concentrations (especially IL-1β) within the tumour mass are linked with the generation of systemic inflammation in the host (irrespective of the cell population that produced them).

The generation of a systemic inflammatory response in patients with gastro-oesophageal cancer is also influenced by host cytokine genotype. IL-6 and IL-10 genotypes were associated with markers of systemic inflammation and the IL-6 genotype was also associated with tumour tissue cytokine concentrations [Chapter VI]. These data suggest that cytokine polymorphisms are linked with differential levels of cytokine expression, which may then modify the systemic inflammatory response. These studies suggest that host factors are important determinants in the generation of systemic inflammation in patients with cancer, in addition to the tumour phenotype.

Mediators other than pro-inflammatory cytokines are involved in the generation of systemic inflammation in patients with gastro-oesophageal cancer

The role of two potential tumour-derived mediators, PTHrP and PIF, were investigated in this thesis. Elevated serum PTHrP concentrations were identified in 17% of patients with gastro-oesophageal cancer at the time of diagnosis without evidence of hypercalcaemia [Chapter VII]. Elevated concentrations were associated with markers of systemic inflammation. PTHrP expression has been linked previously with elevated serum cytokine and acute phase protein concentrations in cancer patients (Ogata, 2000; Takahashi et al, 2003; Funk et al, 1997). These data suggest a possible further enhancement of systemic inflammation in patients with cancer in association with elevated PTHrP.

Three months following diagnosis elevated serum PTHrP concentrations were measured in 49% of patients and elevated concentrations were associated with adverse nutritional status [Chapter VII]. This association occurred in the absence of hypercalcaemia and without apparent differences in dysphagia scores or levels of dietary intake. These findings support animal studies where elevated PTHrP concentrations in mice were associated with a significant reduction in body weight and tissue mass, an effect that was reversed by addition of an antibody to PTHrP (Iguchi et al, 2001). It is possible that the elevated serum PTHrP concentrations induce a 'sub-clinical' hypercalcaemia in these patients, which may promote anorexia and result in weight loss. Clinical trials involving administration of PTHrP antibodies to human subjects would provide additional valuable information on the role of PTHrP in cancer cachexia and may represent a valuable novel therapeutic agent for these patients.

In the present thesis, PIF protein was detected in the urine of approximately half the oesophagogastric cancer patients and was associated with adverse nutritional variables and demonstrated a trend towards elevated markers of systemic inflammation [Chapter VIII]. Urinary PIF has previously been linked with weight loss in patients with cancer (Todorov et al, 1996, Wigmore et al, 2000; Cariuk et al, 1997; Cabal-Manzano et al, 2001). This association may be mediated through increased skeletal muscle loss via activation of the ubiquitin-proteolytic pathway (Lorite et al, 1997; Lorite et al, 2001; Todorov et al, 1997). PIF has been shown to stimulate production of pro-inflammatory cytokines (IL-6 and IL-8) *in vitro* and in the present study a trend was observed between the presence of PIF in urine and elevated serum CRP concentrations and reduced serum albumin concentrations (Watchorn et al, 2001). PIF may, therefore, also contribute to the systemic inflammatory response in patients with cancer.

PIF mRNA expression was significantly elevated in tumour tissue when compared with levels measured in tissue from healthy controls, but patterns of expression did not correlate with markers of systemic inflammation or nutritional status or indeed with urinary PIF protein [Chapter VIII]. PIF is highly glycosylated and the carbohydrate component is essential for its proteolytic function (Todorov et al, 1996). Levels of core peptide mRNA, therefore, have little relevance to the concentration of functionally active glycosylated PIF. In addition, the relatively high prevalence of PIF mRNA detected in tissue from healthy controls (36%), albeit at low levels, was unexpected as previous studies have suggested that PIF mRNA is not

expressed in normal tissues. The findings in this study may reflect the high sensitivity of real-time PCR. Alternatively, the primers may have lacked sufficient specificity for the PIF sequence and what was detected may not have been the PIF gene. Sequencing the PCR product may have helped resolve this issue. In addition, questions remain over the issue of whether indeed there is a human homologue of PIF. The apparent lack of specificity of the only antibody available for the purification of PIF and the low abundance of PIF makes this a very difficult issue to resolve at present.

Therefore, in addition to pro-inflammatory cytokines, other tumour-derived mediators may play a role in the generation of systemic inflammation in patients with gastro-oesophageal cancer. These mediators may also have additional direct effects on the host, outwith the context of systemic inflammation, such as weight loss and cachexia. Further research is required to resolve these issues.

Is nutritional status (cachexia) a key factor linking systemic inflammation with adverse prognosis in patients with gastro-oesophageal cancer?

In the present thesis, elevated serum acute phase protein concentrations at the time of diagnosis of oesophagogastric cancer were associated with reduced overall survival duration [Chapter IX]. CRP concentrations were identified as the best marker of prognosis in these patients and the magnitude of serum CRP concentrations were negatively linked with survival duration. An increase in serum CRP concentration from a normal value of 1 mg/l to 10 mg/l was associated with an almost 50% increase in likelihood of death within two years of diagnosis. CRP concentrations were also able to discriminate survival duration between patients within the same stage group and within the same treatment group. However, there was no association between post-operative CRP concentrations and survival duration in patients who had undergone surgical resection. As already stated, the effect of treatment or even disease progression may influence CRP concentrations and this reduces the value of CRP as a prognostic indicator. The data from this thesis suggests that CRP measured at the time of diagnosis, and before commencement of treatment, is a robust and reliable marker of systemic inflammation and is an independent predictor of outcome for patients with gastro-oesophageal malignancy.

The mechanisms linking systemic inflammation with adverse prognosis remain obsure. However, the metabolic changes associated with systemic inflammation, such as increased rates of skeletal muscle proteolysis and elevated resting energy expenditure, may lead to nutritional depletion and the cachexia syndrome has been linked with adverse outcome in patients with cancer (Falconer et al, 1994; DeWys et al, 1980; Barber et al, 1999b; Studley, 1936). In the present study, 83% of patients had lost body weight at the time of diagnosis (median weight loss 7%) and by three months 92% of patients had lost weight (median weight loss 10%) [Chapter X]. Increasing weight loss was associated with reduced survival duration. Weight loss in patients with gastro-oesophageal cancer has commonly been associated with reduced food intake due to dysphagia/early satiety. In the present thesis, although dietary intake was a significant contributor to weight loss (size of effect 38%), on multivariate analysis systemic inflammation was also identified as a significant independent contributory factor (size of effect 34%). These data emphasises the potential inflammation driven metabolic contribution to weight loss and cachexia in these patients and may provide the link between systemic inflammation and adverse prognosis. Therefore, therapeutic strategies should address the systemic inflammatory component, in addition to the quantity of dietary intake, in cancer patients with weight loss.

Multivariate survival analysis identified increased weight loss, elevated serum CRP concentration at the time of diagnosis, reduced performance status, and advanced stage of disease as independent predictors of survival duration [Chapter XI]. A risk prediction model was constructed from these variables to devise a novel model with improved prognostic accuracy to aid clinical decision-making. It is anticipated that this model will be used to help inform treatment decisions for individual patients and provide quality assurance for the MDT process.

Conclusions and future directions

A diagrammatic overview of the main conclusions drawn from this thesis is shown in Figure 12.1. Systemic inflammation is common in patients with gastro-oesophageal cancer with 43% of patients demonstrating a systemic inflammatory response at the time of diagnosis. Serum acute phase protein concentrations (especially CRP), but not serum cytokine concentrations,

are robust measures of systemic inflammatory activity when measured at the time of diagnosis. Pro-inflammatory cytokines produced from the tumour tissue are important mediators involved in the generation of systemic inflammation in patients with cancer. IL-1β in particular is over-expressed in tumour tissue and may be a key determinant of systemic inflammation in patients with gastro-oesophageal malignancy. Cancer cells or host immune cells or a combination of the two cell types are likely to be the main source of these pro-inflammatory cytokines. Future studies employing techniques such as insitu hybridisation or immunohistochemistry may help determine which cell populations are the main cytokine producers driving the systemic inflammatory response. Transgenic murine models with inducible cell activation may also help resolve some of these issues.

Cytokine genotype also influences the genesis of systemic inflammation in patients with gastro-oesophageal cancer. Host genotype and tumour phenotype are both implicated in the generation of a systemic inflammatory response. Allelic association studies are, however, prone to a high false positive rate as a result of small sample size. The observations in the present thesis require to be confirmed in a separate population. Moreover, many other candidate genes need to be explored.

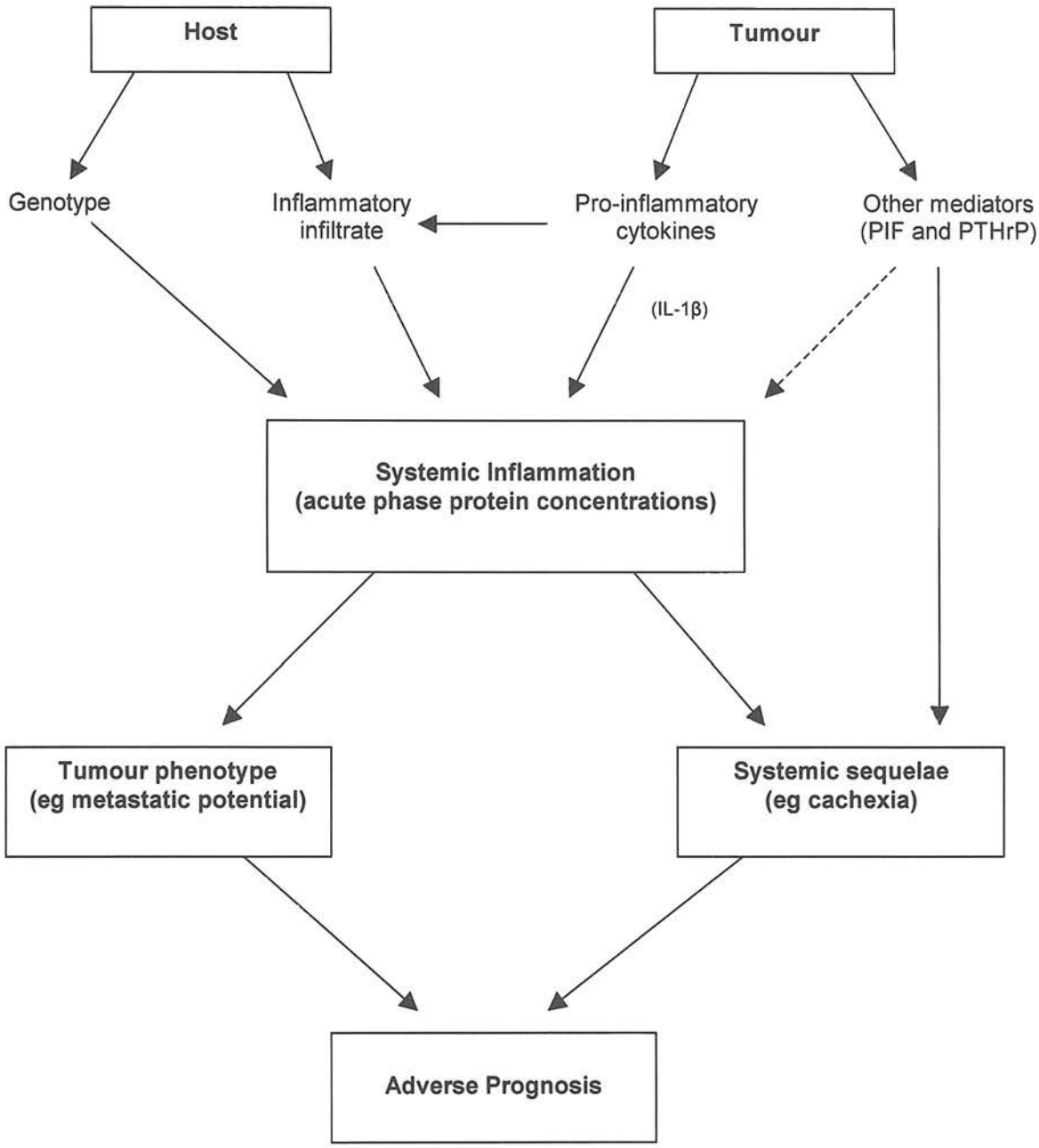
Additional factors, such as PTHrP and PIF, are potential tumour-derived mediators that may also play a role in the generation of systemic inflammation in patients with cancer. Both PTHrP and PIF are linked with markers of systemic inflammation and these factors may also have additional effects on the host, such as potentiating weight loss and cachexia. However, there are many questions querying the existence of PIF in human subjects and the outcome of ongoing work with mass spectroscopy is awaited.

Weight loss is common in patients with gastro-oesophageal cancer and is linked with the presence of systemic inflammation and adverse outcome. In addition to reduced levels of dietary intake, metabolic mechanisms, including systemic inflammation, are key processes involved in weight loss progression. Cachexia may be an aetiological factor involved in the link between systemic inflammation and adverse prognosis. Future clinical studies involving monoclonal antibodies against cytokines (such as anti-IL-1β) or PTHrP may provide valuable new information relating to tumour immunology and may represent much needed novel therapeutic strategies. Modification of the systemic inflammatory response in these patients

may reduce progression of rates of weight loss, improve performance status and function, and perhaps influence prognosis.

Systemic inflammation, weight loss, performance status, and stage of disease are the main determinants of outcome in patients with gastro-oesophageal cancer. These four prognostic factors were used to devise a model to improve prognostic accuracy for these patients. Currently this novel prognostic scoring system is undergoing prospective validation as part of the MDT process. It is hoped that the use of this model will improve treatment decisions for patients with gastro-oesophageal malignancy and thereby provide better care for individual patients.

Figure 12.1 A diagrammatic representation of the main conclusions drawn from this thesis.



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APPENDICES

Appendix A

Patient Information Sheet

THE CAUSES OF INFLAMMATION IN CANCER AND ITS LINK WITH OUTCOME IN OESOPHAGEAL/STOMACH CANCER

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please ask if there is anything that is not clear or if you would like more information.

In the treatment of patients with oesophageal (gullet) or stomach cancer there are a variety of treatments available. These depend on the extent of the tumour and also the general fitness of the patient. At the present time estimating a patient's response to these different treatment options is difficult and often inaccurate. Improving this process will help both doctors and patients in choosing the most appropriate treatment options. We think that detecting the presence of inflammation in the body may help to improve this process.

Over the next two years we plan to study around 250 patients who develop oesophageal or stomach cancer in our region. If you decide to take part in the study we will require a single blood test (about a tablespoon) and a single urine test. As these samples will be taken at the time of your other tests you will not require an additional needle prick. If you go on to have surgery we would use some of the cancer cells taken out during the operation to study in the laboratory. In addition, we would take a small sample of muscle from your tummy wall (about a pea size). This will not affect you in any way after the operation.

Taking part in the study will not affect the treatment you will receive. All patients, whether involved in the study or not, will receive the same follow up in the outpatient clinic.

It is up to you to decide whether or not to take part in the study. If you decide to take part you will be asked to sign a consent form and you should keep this information sheet. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

You may contact myself (the main researcher) directly by telephoning 0131 536 3821 for further information at any time. Alternatively, you may contact Mr Rowan Parks (Senior Lecturer in the Department of Surgery) who is acting as an independent advisor – contact 0131 536 3820.

Thank you for your time.

Mr Chris Deans
Department of Surgery
Royal Infirmary of Edinburgh

March 2002

Consent Form

THE AETIOLOGY OF SYSTEMIC INFLAMMATION AND ITS LINK WITH PROGNOSIS IN GASTRO-OESOPHAGEAL CANCER

- I agree to take part in the above titled study

- I confirm that I have read and understand the information sheet dated March 2002 for the above study and have had the opportunity to ask questions

- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason, without my medical care or legal rights being affected

RESEARCHER

Name Mr Chris Deans **Date**.....

Signature.....

PATIENT

Name..... **Hosp No**.....

Signature.....

Appendix B

Research Data Collection Sheet

Patient Details

Name/Addressograph	Date
	Sex M /F
	Age at diagnosis

Date of Diagnosis –

Duration of symptoms -

Co-morbidity/PMH -

Current Health (eg URTI) - Smoker

Current Medications
- NSAIDS
- Steroids
- Immunosuppressants
- Others

ASA

Pre-illness stable weight

Pre-illness BMI

Pathology Details

Tumour Site	**Oesophageal/Gastric**
Tumour Position	**Proximal/middle/distal/O-G Junction/etc**
Histology	**ACC/SCC/Other**
Differentiation	**Poorly/moderately/well**
Other Prognostic Features	**Inflammatory infiltrate**
	Signet cells
	Other

Staging Investigations -	CT	T	N	M
-	EUS	T	N	M
-	Lap/LUS	T	N	M
-	CXR			
-	Other (eg peritoneal washings)			

Final Stage:	**Clinical**	**T**	**N**	**M**
	Pathological	**T**	**N**	**M**

INVESTIGATIONS

	At Diagnosis	Clinic	Clinic	Clinic
DATE				
CRP				
Albumin				
Uric acid				
Alk Phos				
PIF				
Blood for storage				
Height				
Weight				
BMI				
Mid-arm Circumference				
Triceps skinfold thickness				
Arm muscle circumference				
Karnofsky Index				
Estimated oral intake				
Dysphagia score				

Tissue Samples

Source (eg theatre, UGIE) -

Ischaemic time - Time from stomach transected to freezing =

Time from oesophagus transected to freezing =

Skeletal muscle taken Y/N

Fat sample collected Y/N

Outcome

Treatment undertaken:

Surgery Yes/No

Operation –

Date –

Neoadjuvant chemotherapy – Y / N

Adjuvant Treatment –

Complications -

Palliation Chemotherapy

Radiotherapy

Stenting

Laser

Nothing

Date of Recurrence -

Date of death –

Cause of death -

KARNOFSKY INDEX

Normal, no complaints	100
Able to carry out normal activities, minor signs of disease	90
Normal activity but with effort	80
Self-caring but unable to carry out normal activity or work	70
Requires occasional assistance but able to care for most needs	60
Requires considerable assistance and frequent medical care	50
Disabled, requires special care and assistance	40
Severely disabled, hospitalisation indicated although death not imminent	30
Very sick, requires hospitalisation. Active supportive treatment necessary	20
Moribund	10
Dead	0

DYSPHAGIA SCORE

0	=	able to eat normal diet / no dysphagia.
1	=	able to swallow some solid foods
2	=	able to swallow only semi solid foods
3	=	able to swallow liquids only
4	=	unable to swallow anything / total dysphagia

Appendix C

Reagent recipes for ELISA's

Acute Phase Protein ELISA Reagents

Dilution Buffer: 0.01M Phosphate buffer, 0.5M NaCl, 0.1% Tween 20, pH 7.2

NaH_2PO_4	0.35g
Na_2HPO_4	1.34g
NaCl	29.22g
Tween 20	1ml
dH_2O	to 1000ml

Coating Buffer: 0.01M Phosphate buffer, 0.15M NaCl, pH 7.2

NaH_2PO_4	0.35g
Na_2HPO_4	1.34g
NaCl	8.47g
dH_2O	to 1000ml

Substrate Buffer:

OPD tablets	4
H_2O	12 ml
30% H_2O_2	5µl

Cytokine ELISA Reagents

Assay Buffer

Bovine serum albumin	5g
Tween 20	0.5 ml
NaCl	8g
KCl	0.2g
Na_2HPO_4	2.85g
KH_2PO_4	0.2g
dH_2O	to 1000ml

Appendix D

Western Blotting Reagents

Gel Preparation – for 2 gels:

4% Stacking gel	0.5M Tris-HCl (pH 6.8)	2.5ml
	10% SDS	100µl
	Acrylamide/bis (30%)	1.3ml
	dH_2O	6.1ml
	10% ammonium persulphate	50 µl
	TEMED (added last)	20 µl
12.5% Resolving gel	1.5M Tris-HCl (pH 8.8)	2.5ml
	10% SDS	100µl
	Acrylamide/bis (30%)	4.17ml
	dH_2O	3.12ml
	10% ammonium persulphate	100 µl
	TEMED (added last)	10 µl

British Journal of Cancer (2006) 95, 1568–1575

www.bjcancer.com

Elevated tumour interleukin-1β is associated with systemic inflammation: a marker of reduced survival in gastro-oesophageal cancer

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Systemic inflammation is associated with adverse prognosis cancer but its aetiology remains unclear. We investigated the expression of proinflammatory cytokines within normal mucosa from healthy controls and tumour tissue in cancer patients and related these levels with markers of systemic inflammation and with the presence of a tumour inflammatory infiltrate. Tissue was collected from 56 patients with gastro-oesophageal cancer and from 12 healthy controls. Tissue cytokine mRNA concentrations were measured by real-time PCR and tissue protein concentrations by cytometric bead array. The degree of chronic inflammatory cell infiltrate was recorded. Serum cytokine and acute phase protein concentrations (including C-reactive protein (CRP)) were measured by enzyme-linked immunosorbent assay. Proinflammatory cytokines were significantly overexpressed (interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α) both at mRNA and protein levels in the cancer specimens compared with mucosa from controls. Interleukin-1β was expressed in greatest (10–100-fold) concentration and protein levels correlated significantly with systemic inflammation (CRP) ($P=0.05$, $r=0.31$). A chronic inflammatory infiltrate was observed in 75% of the cancer specimens and was associated with systemic inflammation (CRP: $P=0.01$). However, the presence of chronic inflammation *per se* was not associated with altered cytokine expression within the tumour. Both a chronic inflammatory infiltrate and systemic inflammation (CRP) were associated with reduced survival ($P=0.05$ and $P=0.03$, respectively). Tumour chronic inflammatory infiltrate and tumour tissue IL-1β overexpression are potential independent factors influencing systemic inflammation in oesophagogastric cancer patients.
British Journal of Cancer (2006) **95,** 1568–1575. doi:10.1038/sj.bjc.6603446 www.bjcancer.com
Published online 7 November 2006

Keywords: inflammation; cytokines; real-time PCR

Systemic inflammation has been found in association with the majority of advanced solid epithelial malignancies and at the time of diagnosis up to 50% of patients may have an elevated acute phase protein response (APPR) (Falconer *et al*, 1995). The presence of an APPR has been associated with weight loss, the presence of hypermetabolism and anorexia, extent of disease, the development of recurrence in advanced cancer, and adverse prognosis (independent of stage of disease) (Rashid *et al*, 1982; Kodama *et al*, 1999; McMillan *et al*, 2001, 2003; Nozoe *et al*, 2001; Forrest *et al*, 2003). In patients with gastric cancer, the presence of systemic inflammation has been associated with a markedly reduced median survival (9 *vs* 53 weeks, $P<0.001$) (Rashid *et al*, 1982). Similarly, a study from Japan has identified a shortened survival in oesophageal cancer patients with an elevated serum C-reactive protein (CRP) at the time of diagnosis (Nozoe *et al*, 2001). More recently, a group from the UK has identified elevated serum CRP and reduced serum albumin concentrations as independent prognostic indicators among patients with inoperable gastro-oesophageal cancer (Crumley *et al*, 2006). The systemic inflammatory response is highly complex and is modulated, in part, by the interaction of pro- and anti-inflammatory cytokines. However, the precise origin of systemic inflammation among cancer patients remains obscure.

Human cancer cell lines have been shown to produce proinflammatory cytokines (Gelin *et al*, 1991; Strassmann *et al*, 1993a, b; Wigmore *et al*, 2002). However, such proinflammatory cytokines are not reliably detected in the circulation and probably act locally to promote inflammation and activate host inflammatory cells (e.g. peripheral blood mononuclear cells: PBMCs) passing through the tumour (Falconer *et al*, 1994; O'Riordain *et al*, 1999). Such cells can re-enter the circulation and release cytokines at distant target organs (e.g. the liver). More recently, Martignoni *et al* (2005) have suggested that interleukin (IL)-6 overexpression in pancreatic cancer patients is related to the ability of certain IL-6 producing tumours to sensitise PBMC and induce IL-6 expression in PBMCs. The main cytokines influencing the APPR in humans are thought to include IL-6, IL-1β, and tumour necrosis factor-α (TNF-α) (O'Riordain *et al*, 1999). Interleukin-6 is the main inducer of the APPR in human

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Received 6 June 2006; revised 15 September 2006; accepted 26 September 2006; published online 7 November 2006

hepatocytes and both IL-1β and TNF-α are capable of inducing IL-6 production from both tumour and host cells (Strassmann *et al*, 1993a, b). In cancer patients, the rates of production of IL-6 from isolated PBMCs can be linked to markers of systemic inflammation such as CRP (O'Riordain *et al*, 1999). The presence of such an acute phase reaction may then be used as an indirect marker of proinflammatory cytokine activity (IL-1β, IL-6, and TNF-α).

The source of the proinflammatory stimulus in advanced cancer remains unclear. It has been hypothesised that in patients with cancer, either the tumour cells or the host cells or a combination of the two are responsible for the production of the proinflammatory cytokines that induce the APPR. With a view to modulation of systemic inflammation in cancer, we hypothesise that dominant cytokines within tumour tissue drive the systemic inflammatory response and that these might be considered as targets for specific therapy. To investigate the role of tumour tissue in the genesis of systemic inflammation in cancer patients, we measured cytokine (IL-1β, IL-6, IL-8, and TNF-α) mRNA and protein concentrations in tumour tissue collected from patients with gastro-oesophageal cancer and tissue from healthy controls and related these measurements to systemic concentrations of cytokines and acute phase proteins (APPs). We also investigated the significance of a chronic inflammatory cellular infiltrate within these tissues and related these findings to tissue cytokine concentrations and to clinical outcome.

PATIENTS AND METHODS

Study patients

Patients diagnosed with gastric or oesophageal cancer within the Lothian and Borders regions between June 2002 and March 2004 were eligible for inclusion into the study. Patients were recruited at the time of diagnosis and all subjects provided written informed consent and the study received ethical permission from the Lothian Research Ethics Committee. All patients who had surgery were eligible and were studied. No patients were excluded or refused consent. Patients not suitable for surgical resection (advanced disease stage or comorbidity) were excluded from the study. Patients were staged according to the International Union Against Cancer (UICC), and final histopathological stage (pTNM) was used in all cases (Sobin and Wittekind, 2003). Tumours located around the oesophago-gastric junction were classified according to Siewert and those classified as types I and II were staged as oesophageal tumours and type III as gastric cancers (Siewert and Stein, 1998). All clinical and pathological information was collected prospectively, including documentation of the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and any other therapeutic agents that may influence the inflammatory response.

Determination of serum APP and cytokine concentrations

A random blood was collected from patients at the time of diagnosis and before any therapeutic intervention. All patients were free from infection at the time of blood collection. Samples were collected simultaneously from 22 healthy controls for comparison. Serum was obtained by collecting whole blood into lithium-heparinised tubes and centrifuging at 2000 r.p.m. for 10 min at 10°C (Mistral 3000i, Thermo Life Sciences, Basingstoke, UK). Aliquots were stored at −80°C until batch analysis.

C-reactive protein was determined using an immunoturbidimetric assay (Abbott TDX, Abbott Laboratories, Maidenhead, UK). A level above 10 mg l^{-1} defined the presence of an APPR. Serum albumin concentrations were measured by an automated bromocresol green dye-binding technique. The remaining APPs were determined by sandwich enzyme-linked immunosorbent assay as described previously (Wigmore *et al*, 2002). Briefly, 96-well plates were coated with 100 µl primary antibody (concentration 10 mg l^{-1}) and incubated overnight at 4°C (Dako, Ely, UK). The plates were washed with 0.1% Tween and diluted sera (100 µl) was added to the coated wells and incubated at room temperature for 2 h. Plates were washed as before and a secondary antibody conjugated with peroxidase was added to each well and incubated for 1 h (Dako, Ely, UK). The substrate used was OPD (Dako, Ely, UK) and the reaction was stopped with 0.5 M sulphuric acid. Plates were read at 490 nm using a Dynatech MR5000 automated plate reader. Standard curves were generated using standard APPs supplied by the manufacturer (Dako, Ely, UK).

Serum cytokines were analysed with module kits and performed according to the manufacturers instructions (Caltag, Bender MedSystems, Towcester, UK). The lower limit of sensitivity for each assay was; <1 pg ml^{-1} IL-1β, 1.4 pg ml^{-1} IL-6, 11 pg ml^{-1} IL-8, 0.8 pg ml^{-1} IL-10, and 5.8 pg ml^{-1} TNF soluble receptor (sTNF-R).

Tissue cytokine mRNA and protein measurement

Tissue collection Tissue was obtained from 56 patients at the time of surgical resection. A representative sample of tumour tissue was collected from each patient and tissues were snap frozen in liquid nitrogen before storage at −80°C until further analysis. An additional 12 patients were recruited as healthy controls. These patients underwent endoscopy as an elective procedure for investigation of dyspeptic-type symptoms. In all instances, the result of the procedure was normal, including both macroscopic and microscopic assessment. Mucosal tissue (seven oesophageal and five gastric) samples were collected from these patients with biopsy forceps at the time of endoscopy. All control subjects were considered healthy without established comorbidity or taking regular medications.

Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR)

RNA isolation and RT Total RNA was isolated from tissue samples using the RNeasy kit (Qiagen Inc., Crawley, UK). RNA quality and integrity was assessed using an Agilent 2100 bioanalyser (Agilent Technologies Ltd, Chesire, UK) in five randomly selected samples. For the remaining samples, purity and concentration were determined using spectrophotometry (Ultrospec 2000, Pharmacia Biotech, Bucks, UK). Reverse transcription was performed using 1 µg of total RNA following DNase treatment (Qiagen Inc., UK). All RNA samples were checked for genomic DNA contamination before RT using conventional RT-PCR. Two microlitres of total RNA was mixed with 1 µl MgCl (25 mM), 2.5 µl 10 × *Taq* DNA polymerase buffer with added MgCl, 2.5 µl dNTP (10 mM), 5 µl forward and reverse primers (10 µM), 11 µl DEPC-treated water and 1 µl *Taq* DNA polymerase (5 U µl^{-1}) (all reagents Promega, Southampton, UK). Primers for cytochrome *b* were used to detect DNA contamination. The forward primer sequence was GGTTCTGGAATAAGAATATAGG and the reverse primer sequence GACAACACAGTAAGAACCAGG, giving a product of 367 bp if contamination was present.

Reverse transcription was performed once DNA contamination had been excluded. The reaction mixture included the RNA (1 µg in 10 µl DEPC-treated water), 4 µl MgCl (25 mM), 2 µl 10 × reverse transcriptase buffer, 2 µl dNTPs (10 mM), 1 µl random hexamers (500 µg ml^{-1}), 1.5 µl AMV reverse transcriptase (10 U µl^{-1}), and 0.5 µl recombinant RNase inhibitor (40 U µl^{-1}) (all reagents Promega, Southampton, UK). Reverse transcription was performed at 42°C for 60 min followed by 95°C for 5 min.

Real-time PCR Quantitative PCR was performed using the ABI PRISM 770 real-time Sequence Detection System (Applied Biosystems, Warrington, UK). Reactions were performed in 50 µl

total volume, consisting of; 25 μl Taqman universal PCR mastermix (UNG $\times 2$), 14 μl primer/probe mix, 2.5 μl ribosomal 18S primer/probe mix (all reagents Applied Biosystems, UK), 3.5 μl DEPC-treated water, and 5 μl cDNA. Each sample was analysed in duplicate. The reaction conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15 s at 95°C and 1 min at 60°C. Genes studied included IL-1β, IL-6, IL-8, and TNF-α. The primers and probes were designed by Applied Biosystems, UK.

Quantification of gene expression was calculated using the comparative ($\Delta\Delta C_T$) method, where samples were compared with the positive control (Bustin, 2000). The level of gene expression within each sample was adjusted to an internal control (human ribosomal 18S) before expression was calculated as a percentage of the level of gene expression by the control sample. Samples that generated cycle numbers above 23 for the endogenous control (18S) were discarded and the samples were repeated.

Positive control Whole blood was collected from healthy donors and the white cells were isolated using histopaque (Sigma, Dorset, UK). The cells were cultured in lipo-polysaccharide (Sigma, Dorset, UK) for 48 h before isolation of the RNA. Total RNA was reverse transcribed as described above. Each real-time reaction used an aliquot from the stock solution of cDNA as a positive control.

Extraction of tissue protein Tissue lysates were prepared by homogenising 50 mg of tissue in 400 μl tissue homogenising buffer (0.4 ml 500 mM Tris, 0.2 ml 100 mM ATP, 1 ml 50 mM $MgCl_2$, 10 μl dithiothreitol, 1 $\times$ protease inhibitor, 8.4 ml water – Sigma, Dorset, UK). Samples were heated to 95°C for 5 min before centrifuging at 13 000 r.p.m. for 30 min. Protein concentration of the supernatants was determined by the Bradford method (Bio-Rad, Hemel Hempstead, UK) (Bradford, 1976). Samples were stored at −80°C until analysis.

Determination of tissue cytokine concentrations

Cytokine protein concentrations were determined using the Cytometric Bead Array System according to manufacturer's instructions (Human Inflammation Kit, BD Biosciences, Oxford, UK). This kit allows the measurement of cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α. Briefly, 50 μl of tissue extract was added to the reaction mix containing antibody-coated microbeads and incubated at room temperature for 3 h. Cytokine concentrations were determined by flow cytometry (BD FACScan, Oxford, UK). Results were calculated to take into account the total protein concentration of the tissue lysate and are expressed as pg mg^{-1} of total protein. Intra-assay variability ranged between 2 and 10% and interassay variability was 4–15%.

Histological analysis

Representative sections of tumour tissue were fixed with formalin and stained with haematoxylin and eosin. A single Consultant pathologist (HG) reviewed all the tissue sections and the extent of a chronic inflammatory cellular infiltrate was recorded. Sections were classified as either diffuse scanty (occasional) chronic inflammatory cells present, focal lymphoid aggregates only, diffuse chronic inflammatory cellular infiltrate present throughout the tissue, or patchy chronic inflammatory cells present (Figure 1). HG was blinded to the clinical data, serum APP/cytokine concentrations, and tissue cytokine concentrations relating to each patient.

Statistical analysis

Comparisons between groups of continuous variables were made by the Mann–Whitney *U*-test. Categorical variables were compared by Fisher's exact test. Correlations between continuous variables were assessed by Spearman's rank correlation coefficient. Survival between groups was analysed by the log-rank test and Cox's proportional hazards model. A *P*-value $\leqslant 0.05$ was considered statistically significant.

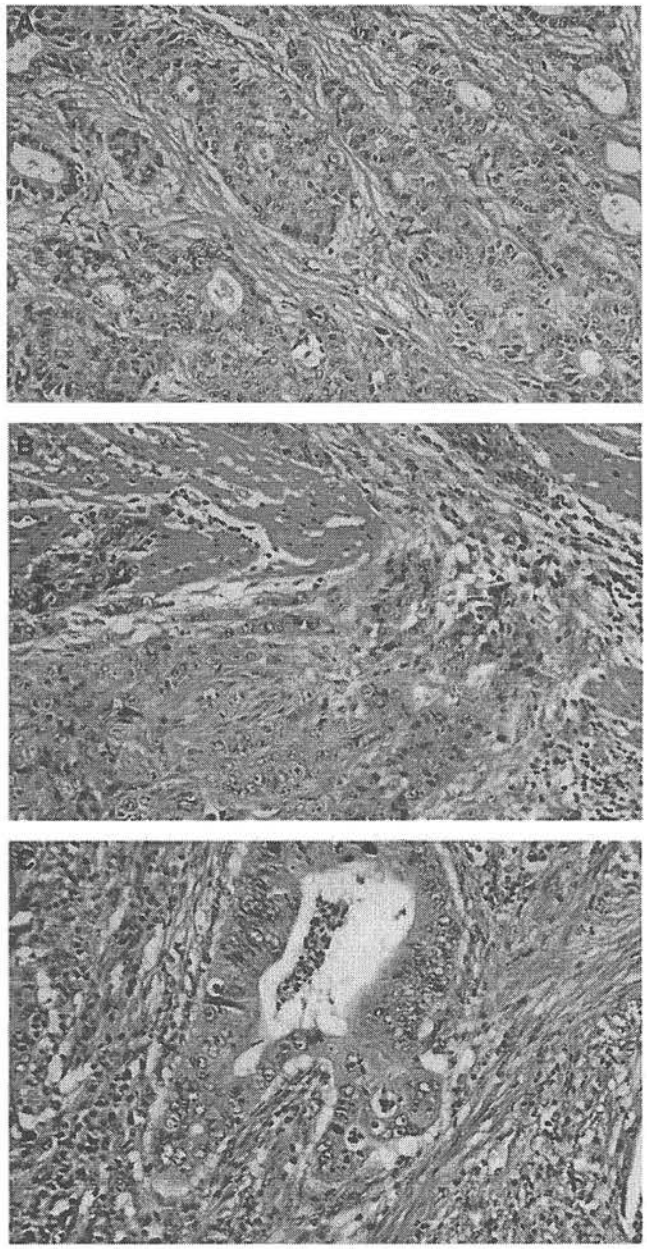

Figure 1 Representative photomicrographs taken from three patients with poorly differentiated adenocarcinoma of the oesophagus. Patient (**A**) demonstrates minimal/no inflammatory cell reaction. Patient (**B**) has a patchy chronic inflammatory cell infiltrate. Patient (**C**) shows a diffuse chronic inflammatory cellular infiltrate present throughout the tumour. Sections of tumour tissue were fixed with formalin and stained with haematoxylin and eosin (magnification $\times$ 100).

RESULTS

Study patients

Patient demographics are shown in Table 1. Subgroup analysis confirmed no significant differences in either tissue mRNA or

Table 1 Study patient demographics ($n = 56$)

	Number (%)
Age (years)[a]	66 (58–75)
Sex	
Male	40 (71)
Female	16 (29)
Tumour site	
Oesophageal	26 (46)
Oesophago–gastric junction	13 (23)
Gastric	17 (30)
Histology	
Adenocarcinoma	52 (93)
Squamous cell carcinoma	4 (7)
Grade	
Well differentiated	4 (7)
Moderately differentiated	24 (43)
Poorly differentiated	28 (50)
UICC stage	
1	17 (30)
2	13 (23)
3	21 (38)
4	5 (9)
Treatment undertaken	
Oesophagectomy	25 (45)
Gastrectomy	18 (32)
Preoperative chemotherapy followed by surgery	13 (23)
Status	
Alive	37 (66)
Dead	19 (34)

[a]Values given are median (interquartile range).

protein levels between patients who received preoperative chemotherapy and those who did not (data not shown). Similarly, there were no differences in tissue mRNA or protein levels or serum cytokine or APP levels among those patients taking NSAIDs or any other therapeutic agents that may modify the inflammatory response (data not shown). Therefore, all patients were included as a single group for analysis.

Serum cytokine and APP concentrations

Serum APP concentrations for the study patients and healthy controls are shown in Table 2. The patient group had significantly elevated concentrations of positive APPs compared with the control population; CRP ($P<0.001$, Mann–Whitney *U*-test), haptoglobin ($P<0.001$), and α1-antichymotrypsin ($P<0.001$). There was no difference in concentrations of the negative acute phase reactants; albumin ($P=0.242$) or transferrin ($P=0.346$). Ten (18%) patients had a serum CRP concentration $>10\,mg\,l^{-1}$, which was associated with reduced survival duration ($P=0.031$, log-rank test) (Figure 2). C-reactive protein concentration remained an independent prognostic indicator on multivariate analysis when analysed with stage, age, sex, and grade ($P=0.048$, hazard ratio 2.7 (1.1–7.3 95% CI); Cox's proportional hazards model).

Serum cytokine concentrations were similar between the healthy controls and cancer patients (Table 2). Serum cytokine concentrations did not correlate with serum APP concentrations (linear regression, data not shown) and patients with CRP levels greater than $10\,mg\,l^{-1}$ did not have significantly elevated serum cytokine concentrations.

Table 2 Serum concentrations of acute phase proteins and cytokines for the patient group ($n = 56$) and healthy controls ($n = 22$)

	Patient group ($n = 56$)	Control group ($n = 22$)	*P*-value[a]
CRP (mg/l)	4 (2–16)	1 (1–3)	<0.001
Haptoglobin (mg/l)	1869 (1421–2651)	821 (627–1157)	<0.001
ACT (mg/l)	409 (326–502)	245 (213–261)	<0.001
Albumin (g/l)	42 (39–44)	42 (39–45)	0.227
Transferrin (mg/l)	2076 (1565–2648)	2197 (1861–2451)	0.478
IL-1β (pg/ml)	0[b]	0	—
IL-6 (pg/ml)	0 (0–91)	11 (0–214)	0.412
IL-8 (pg/ml)	0 (0–57)	0 (0–118)	0.683
IL-10 (pg/ml)	0[c]	0	—
sTNF-R (ng/ml)	2.6 (1.3–4.1)	2.8 (1.3–3.6)	0.559

ACT = α1-antichymotrypsin; CRP = C-reactive protein; IL = interleukin; sTNF-R = soluble tumour necrosis factor receptor (p55). Positive acute phase protein concentrations were elevated in the patient group compared with the control group. There were no differences between concentrations of the negative acute phase reactants or serum cytokines. [a]Mann–Whitney *U*-test. [b]Only two patients had measurable serum IL-1β concentrations. [c]Only four patients had measurable IL-10 concentrations. Values are median (interquartile range).

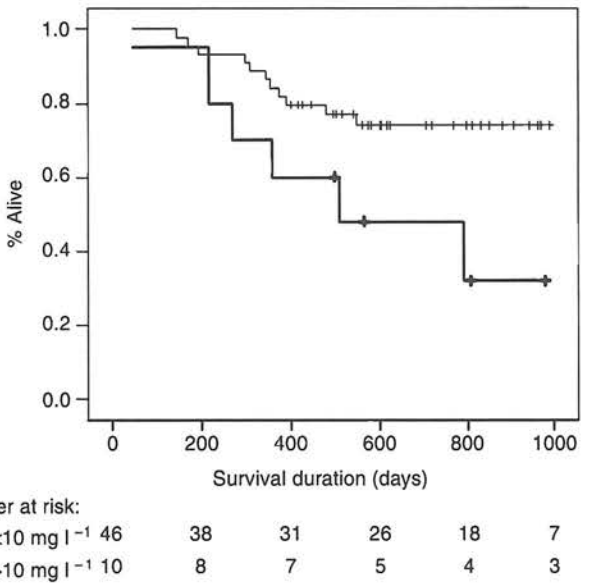

Figure 2 Kaplan–Meier survival plot presented by serum CRP concentration. Heavy line CRP $>10\,mg\,l^{-1}$ (median survival 509 days) *vs* light line CRP $<10\,mg\,l^{-1}$ (median survival >900 days); $P=0.031$, log-rank test.

Tissue cytokine mRNA and protein concentrations

Interleukin-6 and IL-8 mRNA were not measurable in any of the gastro-oesophageal mucosa samples collected from healthy controls and IL-1β and TNF-α were only detectable at very low concentrations (Figure 3A). In contrast, mRNA for IL-1β, IL-6, IL-8, and TNF-α were detected in tumour tissue at significantly elevated concentrations: IL-1β $P<0.001$; IL-6 $P<0.001$; IL-8 $P<0.001$; TNF-α $P=0.006$ (see Figure 3A).

Similarly, IL-6 protein was not detected in mucosal tissue samples from healthy controls and IL-1β, IL-8, and TNF-α were only measured at low concentrations (median concentrations; IL-1β $2.6\,pg\,mg^{-1}$ total protein, IL-8 $0.2\,pg\,mg^{-1}$ total protein, TNF-α $0.1\,pg\,mg^{-1}$ total protein). However, cytokine protein concentrations were significantly elevated in the tumour tissue: IL-1β

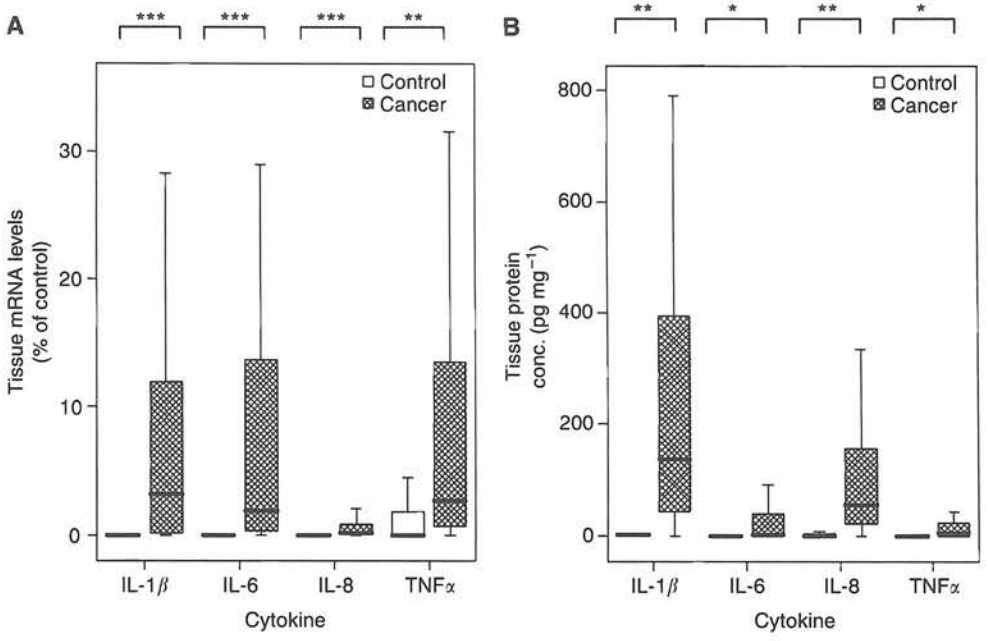

Figure 3 Comparison of cytokine levels of (**A**) mRNA and (**B**) protein between tissue from healthy controls and tumour tissue from patients with gastro-oesophageal cancer. The lines represent the median value, bars = interquartile range, error bars = extreme values. IL-1β = interleukin-1β, TNF-α = tumour necrosis factor-α. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Mann–Whitney U-test).

136 pg mg^{-1} of total protein (IQR 41–425), $P=0.007$; IL-6 3 pg mg^{-1} (IQR 0–46), $P<0.05$; IL-8 56 pg mg^{-1} (IQR 23–159), $P=0.007$; TNF-α 7 pg mg^{-1} (IQR 1–26), $P<0.05$ (Figure 3B). Of note, IL-1β concentrations were found at appreciably higher concentrations compared with the other cytokines (10–100-fold increase).

There was no correlation between tissue cytokine mRNA concentrations and cytokine tissue protein concentrations; IL-1β ($P=0.64$, $r=0.07$; Spearman's rank), IL-6 ($P=0.46$, $r=-0.1$), IL-8 ($P=0.55$, $r=0.09$), TNF-α ($P=0.90$, $r=0.02$). Increased mRNA concentrations were not associated with elevated tissue cytokine protein concentrations.

Tissue cytokine mRNA concentrations did not correlate with serum cytokine concentrations or serum APP concentrations (data not shown). However, tumour tissue IL-1β protein levels were positively correlated with serum CRP concentrations ($P=0.05$, $r=0.31$; linear regression) (Figure 4). Although TNF-α protein levels did not correlate with serum cytokine/APP concentrations there was a significant correlation between sTNF-R and serum CRP concentrations ($P=0.03$, $r=0.36$). There was no correlation between tumour tissue IL-6 and either circulating IL-6 or APP concentrations. There was also a trend towards a correlation between tumour IL-8 protein concentrations and serum sTNF-R concentrations, but this did not quite reach statistical significance ($P=0.06$, $r=0.32$).

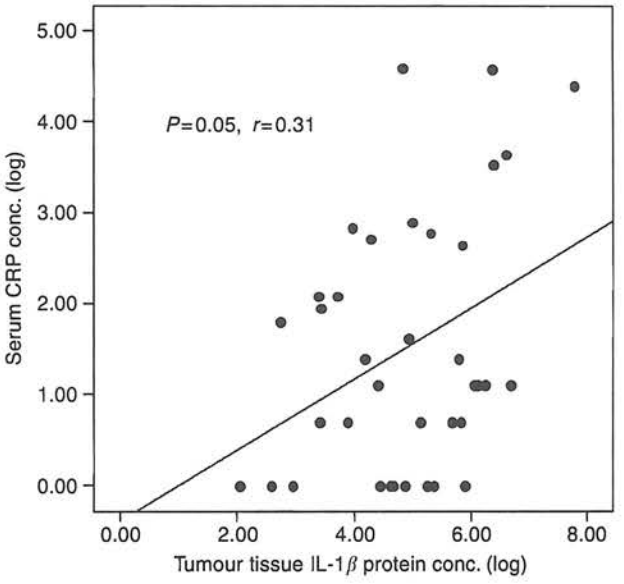

Figure 4 A scatter plot illustrating the relationship between serum CRP concentrations and tumour tissue IL-1β protein concentrations ($P=0.05$, $r=0.31$; linear regression).

Histological analysis

Histology from three patients recruited to the study could not be traced; therefore, 53 tumour sections were studied. Twenty-four (45%) tumour samples were classified as having scanty diffuse or patchy chronic inflammatory cells. Sixteen (30%) tumour samples had a diffuse chronic inflammatory cellular infiltrate visible throughout the whole tumour. The remaining 13 (25%) tumour sections had focal lymphoid aggregates only. When compared with tumour sections possessing lymphoid aggregates alone, tissues with a diffuse or patchy inflammatory cellular infiltrate were associated with elevated serum CRP and sTNF-R concentrations ($P=0.01$ and $P=0.007$, respectively, Mann–Whitney U-test) (Figure 5). In addition, a chronic inflammatory cellular response was associated with reduced prognosis ($P=0.05$, log-rank test) (Figure 6). A chronic inflammatory infiltrate remained an independent prognostic indicator on multivariate analysis when analysed with stage, age, sex, tumour grade, and serum CRP concentrations ($P=0.013$, hazard ratio 7.7 (1.5–38.0 95% CI); Cox's proportional hazards model).

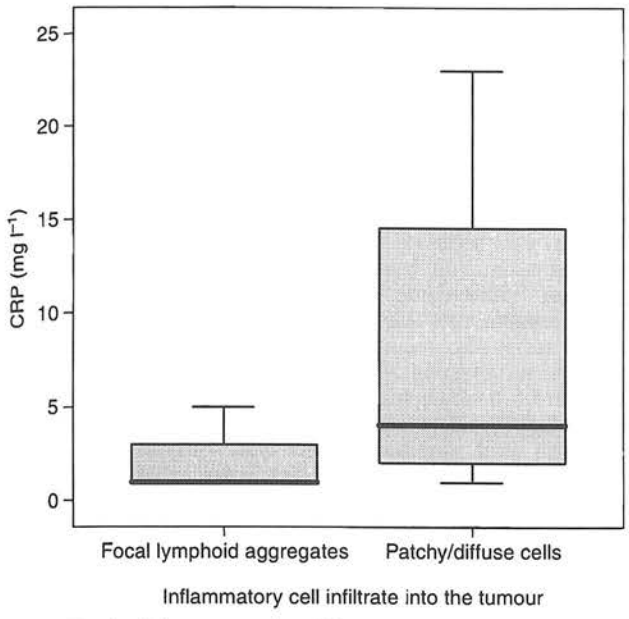

Figure 5 A diffuse or patchy inflammatory cellular infiltrate was associated with elevated serum CRP concentrations ($P = 0.01$, Mann–Whitney *U*-test). Thick bar represents median, the box represents quartiles, and lines represent extreme values.

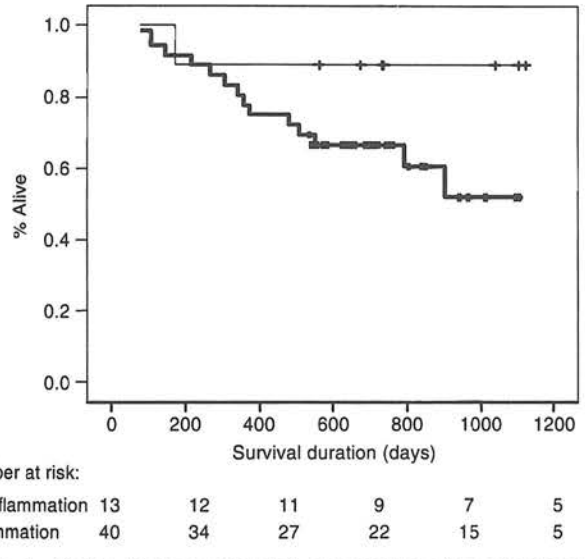

Figure 6 Kaplan–Meier survival plot presented by the presence or absence of a chronic inflammatory cellular infiltrate within the tumour. The heavy line represents the presence of a chronic inflammatory infiltrate *vs* focal lymphoid aggregates alone, light line ($P = 0.05$; log-rank test).

There was no correlation between the presence of a chronic inflammatory cell infiltrate and serum cytokine concentrations.

Six (11%) patients had histological evidence of *Helicobacter pylori* on the resected specimens. A chronic inflammatory cellular infiltrate was not associated with *H. pylori* infection ($P = 0.67$, Fisher's exact test).

Tumour necrosis was evident in 13 (25%) samples, including four samples collected from patients who received preoperative chemotherapy, but there was no association between treatment modality and the presence of tumour necrosis ($P = 0.92$, χ^2 test). The presence of tumour necrosis was associated with elevated serum haptoglobin but not CRP concentrations ($P = 0.045$ and $P = 0.07$, respectively, Mann–Whitney *U*-test). Tumour necrosis was not associated with differences in tissue cytokine concentrations or survival ($P = 0.62$, log-rank test).

Tissue cytokine (IL-1β, IL-6, IL-8, and TNF-α) mRNA and protein levels were found at similar concentrations within tumour tissues with a chronic inflammatory cell infiltrate and tumour samples with lymphoid aggregates alone.

DISCUSSION

In this study, we have shown that patients with gastro-oesophageal malignancy have elevated serum concentrations of APPs but similar serum proinflammatory cytokine concentrations compared with a control population. A range of proinflammatory cytokine concentrations (mRNA and protein) were significantly elevated in tumour tissue compared with tissue sampled from healthy controls. However, only IL-1β correlated with markers of systemic inflammation (CRP). In addition, a chronic inflammatory cellular infiltrate within the tumour was associated with elevated serum APP concentrations and reduced survival, but was not associated with elevated tissue cytokine mRNA and protein concentrations.

An APPR has been well documented among patients with cancer, including gastric and oesophageal malignancies, and an elevated serum CRP has been identified as an adverse prognostic indicator, independent of stage of disease, among these patients (Rashid *et al*, 1982; Falconer *et al*, 1995; Kodama *et al*, 1999; McMillan *et al*, 2001, 2003; Nozoe *et al*, 2001; Forrest *et al*, 2003). The present study has confirmed these findings. Patients with gastro-oesophageal cancer had significantly elevated serum concentrations of positive APPs compared with healthy controls. Moreover, the 10 (18%) patients with a CRP concentration above 10 mg l^{-1} at diagnosis had a reduced survival interval, which was independent of disease stage. Our study did not demonstrate any differences in serum cytokine concentrations between cancer patients and controls. Although some studies have shown an association between serum cytokines and APPs (Martignoni *et al*, 2005) several have failed to demonstrate such a link and determination of serum cytokines remains an unreliable measure of tissue cytokine activity (Falconer *et al*, 1994; Barber *et al*, 1999). Moreover, these findings suggest that circulating cytokines may not be the key mediators of the APPR.

Proinflammatory cytokine mRNA and protein concentrations were either not detectable or found at low levels in tissue collected from healthy controls. In contrast, mRNA and cytokine protein concentrations were measured at significantly higher concentrations in tumour tissue. In all instances, tissue cytokine concentrations were significantly elevated in tumour tissue compared with tissue from healthy controls. These findings are supported by Yuan *et al* (2000) who investigated IL-8 mRNA concentrations in tumour tissue and adjacent normal lung tissue among patients with non-small-cell lung cancer and also found increased cytokine expression within the tumour tissue. Other groups have similarly demonstrated increased tissue cytokine concentrations associated with progression along the metaplasia–dysplasia–carcinoma sequence in Barrett's oesophagus (Tselepis *et al*, 2002; Dvorakova *et al*, 2004).

In the present study, median IL-1β concentrations were 10–100-fold higher than IL-6 in the tumour tissue and there was a weak but significant correlation between tumour tissue IL-1β concentration and serum CRP. There was a similar trend with IL-8. Both IL-1β and IL-8 are recognised as important cytokines in the generation of the systemic inflammatory response and it is possible that high tissue concentrations of these cytokines stimulate PBMCs as they pass through the tumour mass, which in turn act on target organs,

such as the liver, to induce the synthesis of APPs that are associated with systemic inflammation. Previously, we have demonstrated that PBMC from weight-losing pancreatic cancer patients control the hepatic APPR by a primarily IL-6-dependent mechanism (O'Riordain *et al*, 1999). Moreover, Martignoni *et al* (2005) have suggested that IL-6 overexpression in cachectic pancreatic cancer patients is related to the ability of certain IL-6 producing tumours to sensitise PBMC and induce IL-6 expression in PBMCs. In the latter study, screening by DNA microassay analysis followed by quantitative PCR identified only IL-6 mRNA expression to be significantly increased in tumour samples of cachectic patients compared with noncachectic patients or pancreas samples from normal controls. Immunohistochemistry suggested the source of IL-6 to be tumour cells rather than host cells. The results of the present study, however, identify that at least in patients with gastro-oesophageal cancer IL-1β rather than IL-6 may be important as an initiator of the proinflammatory APPR. Interleukin-6 may form a common final pathway via activated PBMCs. Interestingly, in the colon-26 murine model of cancer cachexia associated with systemic inflammation there appears to be a complex intratumoural amplification loop between IL-1β and IL-6, which can be downregulated by IL-10 (Yasumoto *et al*, 1995; Fujiki *et al*, 1997).

In this study, we did not find any correlation between tissue cytokine mRNA concentrations and systemic cytokines or APP concentrations. Raddatz *et al* (2005) did identify an association between tissue cytokine mRNA levels and systemic CRP concentrations in Crohn's disease. These differing results may be partly explained by the lack of correlation between tissue mRNA concentrations and protein concentrations in this study. Although some groups have demonstrated a correlation between IL-1β and IL-6 mRNA and protein concentrations in an animal model of inflammatory joint disease, they also failed to show any correlation for TNF-α mRNA and protein concentrations (Rioja *et al*, 2004). The difficulties of relating mRNA concentrations to protein concentrations has been extensively documented elsewhere, but it is also important to consider that real-time PCR is an exquisitely sensitive technique and that what we are detecting in some patients, although elevated, may have little or no functional significance as it may not be translated into protein. Cytokine protein concentrations are, therefore, likely to be a more robust measure of tissue cytokine activity than mRNA levels.

A chronic inflammatory cellular infiltrate was noted in 40 (75%) tumour samples and was associated with elevated levels of serum CRP and sTNF-R. In addition, a chronic inflammatory infiltrate was associated with reduced survival. The presence of an inflammatory infiltrate within tumours and its relevance to prognosis has been investigated in a number of cancer types. Tumour-associated macrophages have been associated with reduced disease-free survival among lung, head and neck, and endometrial cancer (Marcus *et al*, 2004; Ohno *et al*, 2004; Chen *et al*, 2005). In contrast, increased numbers of tumour-associated macrophages, eosinophils, mast cells, and lymphocytes have been linked with improved survival in colorectal cancer (Svennevig *et al*, 1984; Jass, 1986; Nielsen *et al*, 1999). The prognostic significance of tumour-associated inflammatory cells is less clear in gastro-oesophageal cancer. An increased macrophage infiltrate was associated with more advanced stage of disease among patients with gastric cancer in one study, whereas other studies have suggested a more favourable prognosis associated with a more pronounced macrophage infiltration (Heidl *et al*, 1987; Tsujitani *et al*, 1987; Ohno *et al*, 2003). Similarly, increasing tumour-infiltrating lymphocyte count has been linked with decreased risk of death from gastric cancer in one study, but associated with an adverse prognosis in another (Setala *et al*, 1996; Grogg *et al*, 2003). Studies relating to oesophageal cancer are equally contradictory (Ma *et al*, 1999; Koide *et al*, 2004). In this study, there were no differences in tissue cytokine concentrations (mRNA or protein) between tumours with a chronic inflammatory infiltrate and those without, suggesting that differential tissue IL-1β expression is likely to be tumour-cell derived.

Laser capture microdissection (LCM) enables single cell types to be separated from multiple cell populations and would have been helpful in separating our tissue samples into pure tumour cell and inflammatory cell populations (Emmert-Buck *et al*, 1996). This technique was attempted initially but abandoned owing to inconsistent results, which were related to poor RNA quality as a consequence of this technique. In addition, our results have shown a lack of correlation between mRNA levels and functional protein concentrations, questioning the relevance of measuring mRNA concentrations. Determining cellular cytokine protein concentrations by the cytometric bead array system following LCM was not possible owing to the low protein concentrations that were retrieved.

Tumour necrosis was evident in 25% of tissue samples and was not associated with receipt of preoperative chemotherapy. The presence of tissue necrosis was weakly associated with elevated serum APP concentrations and may be explained by the necrotic tissue behaving like an abscess and inducing a predominantly acute inflammatory response. Tissue necrosis did not have any prognostic value in this study.

In conclusion, systemic inflammation is associated with adverse prognosis in gastro-oesophageal cancer. Tumour tissue cytokine concentrations are elevated compared with healthy controls and IL-1β concentrations are positively associated with some markers of systemic inflammation. In addition, the presence of a chronic inflammatory cell infiltrate into the tumour is also associated with markers of systemic inflammation and reduced survival, but is not associated with differential expression of tissue preinflammatory cytokine concentrations. This raises the possibility that the role of the chronic inflammatory infiltrate in the generation of systemic inflammation may be independent of differential expression of proinflammatory cytokines by these cells. Different mediators or cell–cell interactions may be more important for their effects.

ACKNOWLEDGEMENTS

We thank Mr Andrew de Beaux, Consultant Surgeon, Edinburgh Royal Infirmary, for his help in the recruitment of patients and provision of assistance with the clinical aspects of this work. We also thank Ms Kathryn Sangster for her excellent technical advice and assistance.

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DOI: 10.1245/s10434-006-9122-9

Host Cytokine Genotype is Related to Adverse Prognosis and Systemic Inflammation in Gastro-Oesophageal Cancer

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Background: Systemic inflammation has been linked with reduced survival in cancer, however, the role of the host cytokine genotype versus tumour phenotype in the generation of this response is not clearly established. This study examined the relationship between cytokine polymorphisms (IL-1β 511, IL-6 174, IL-10 1082, TNFα 308 and LTα +252) and serum cytokine concentrations, serum CRP concentration and survival duration in patients with gastro-oesophageal malignancy.

Methods: Two hundred and three newly diagnosed patients with gastric or oesophageal cancer had serum CRP and cytokine concentrations determined by ELISA. SNP genotyping was performed by Taqman allelic discrimination genotyping and compared with the genotype observed in 266 healthy volunteers. Clinico-pathological information was collected prospectively and survival duration was recorded.

Results: Distribution of the cytokine genotypes was similar between patients and controls. The IL-6 174 CC and IL-10 1082 GG genotypes were associated with elevated serum CRP (P = .03, P = .01, respectively; Mann–Whitney U test) and sTNF-R (P = .015, P = .02) concentrations. These genotypes were also associated with reduced survival duration (P = .01, P = .047; log-rank test). TNFα AA genotype was also associated with reduced survival duration on univariate (P = .032) and multivariate analysis (P = .006, multivariate model), but not with inflammatory markers. No other cytokine polymorphisms were associated with systemic inflammatory markers or prognosis.

Conclusions: There is a pro-inflammatory cytokine haplotype (IL-6 CC, IL-10 GG, TNFα AA) that is associated with adverse prognosis that may act, at least in part, through an inflammatory mediated mechanism. Determining patients' cytokine haplotype may improve prognostication and allow stratification for intervention studies.

Key Words: Cytokines—Polymorphisms—Cancer—Survival—Inflammation.

Malignant tumours arising around the oesophagogastric junction have the fastest increase in incidence of any solid tumour in Europe and North America.[1,2] Together gastric and oesophageal malignancy are the third leading cause of cancer-related death in the UK.[3] Despite advances in both staging techniques and treatments, 5-year-survival rarely exceeds 30%.[4]

Received March 16, 2006; accepted June 13, 2006; published online November 11, 2006.
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Systemic inflammation has been associated with a number of malignant diseases, including gastric and oesophageal cancer, and is associated with adverse outcome.[5–15] Among patients with gastric cancer the presence of systemic inflammation has been associated with a markedly reduced median survival (53 vs. 9 weeks, $P < .001$).[14] Similarly, a recent Japanese study has identified a shortened survival in oesophageal cancer patients with an elevated serum C-reactive protein (CRP) at the time of diagnosis.[15] The systemic inflammatory response is modulated by the complex interaction of many pro- and anti-inflammatory cytokines. Although the production of these mediators is partly responsive to environmental influences, the origin of systemic inflammation among cancer patients remains obscure and may be influenced by host cytokine genotype. Variations in genotype for a number of cytokines have been associated with changes in both serum cytokine and serum acute phase protein concentrations and prognosis among cancer patients.

Tumour necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 are key pro-inflammatory cytokines involved in the generation of the inflammatory response. Increased production of these cytokines has been shown to contribute to adverse outcome in patients with sepsis, infections and inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis.[16–19] Our group has previously investigated the influence of *IL-1β* polymorphisms on systemic inflammation and survival in patients with pancreatic cancer.[20] The possession of the less common allele 2 was associated with increased levels of serum CRP concentrations, increased production of IL-1β by peripheral blood mononuclear cells (PBMC), and reduced overall survival. In contrast, another group failed to identify an association between *IL-1β* polymorphisms and outcome for patients with ovarian cancer.[21]

A single nucleotide polymorphism (SNP) at the 308 position of the *TNFα* gene lies within the promoter region and the A allele has been associated with higher levels of TNFα production in vitro following stimulation with endotoxin.[22] Variation at the 308 locus has been associated with conflicting levels of TNFα production both in in vitro studies and among patients with sepsis.[23,24] Our own work, again on pancreatic cancer patients, found no link between genotype and serum soluble TNF receptor (sTNF-R) concentrations or serum CRP concentrations.[25] There was a trend for reduced survival duration associated with possession of the AA genotype, but this did not reach statistical significance ($P = .13$). Another group identified that possession of the A allele was associated with elevated serum sTNF-R concentrations in patients with non-Hodgkin's lymphoma and was associated with reduced overall survival.[26]

A SNP at the 252 locus of the lymphotoxin α (*LT*α) (tumour necrosis factor β) gene has also been shown to modify levels of TNF production. Septic surgical patients homozygous for the AA genotype had significantly higher mortality than those patients who were homozygous for GG.[27] Among cancer patients, homozygotes had a more favourable prognosis than heterozygotes in advanced lung cancer and patients possessing the type 1 allele were shown to have poorer survival in oesophageal cancer.[28,29]

A SNP at 174 in the *IL-6* promoter region has been associated with increased serum IL-6 concentrations and worse outcome among cardiac surgery patients.[30] Survival data for cancer patients is conflicting. The possession of the C allele has been associated with earlier stage disease and better outcome (independent of stage) in women with ovarian cancer and breast cancer.[31,32] However, the same genotype has also been associated with adverse survival in another group of patients with breast cancer.[33]

Interleukin-10 (IL-10) is generally regarded as an anti-inflammatory cytokine important in the attenuation of the inflammatory response. The *IL-10* gene polymorphism at position 1082 lies within the promoter region and the AA genotype is generally thought to be associated with reduced levels of IL-10 production, but this is contradicted by one study.[34,35] The high *IL-10* producer genotype (GG) has been linked with more advanced stage among patients with gastric cancer.[36]

With a view to understanding more clearly the role of host genotype in the genesis of tumour-related systemic inflammation this study examined the relationship between cytokine polymorphisms (*IL-1β* 511, *IL-6* 174, *IL-10* 1082, *TNFα* 308 and *LTα* +252) and serum cytokine concentrations, serum CRP concentrations and survival duration among newly diagnosed patients with gastro-oesophageal cancer.

MATERIALS AND METHODS

Study Patients and Controls

Patients diagnosed with gastric or oesophageal cancer between March 2002 and May 2004 within Lothian and Borders regions were invited to take part in the study. All patients provided written informed

consent and the study received ethical permission from the Lothian Research Ethics Committee. Histological confirmation of disease was established in all cases following endoscopic biopsy. Patients were staged with a combination of computerised tomography (CT), endoscopic ultrasound (EUS) and laparoscopy/laparoscopic ultrasound (LUS) according to the International Union Against Cancer (UICC).[37] Final histopathological stage (pTNM) was only available for those patients who underwent surgical resection. In all other cases the final clinical stage (cTNM), as agreed at the unit multidisciplinary team meeting (MDT), was recorded. Tumours around the oesophago-gastric junction were classified according to Siewert and those classified as type I and II were staged as oesophageal and type III were staged as gastric.[38] For the purposes of analysis the cTNM stage was used for all patients. Separate subgroup analysis of those patients with final histopathological (pTNM) stage gave identical results. Clinical and pathological information was recorded prospectively for each patient, including treatment modality, tumour grade and histological subtype. Duration of survival, defined as time from histological diagnosis to death, was recorded for all patients.

In addition to the patient group, blood samples were also collected from healthy controls for genotyping. The control group used in this study comprised 266 British Caucasian bone marrow and solid organ donors, collected via the Histocompatibility and Immunogenetics Laboratory, Southampton University Hospitals. The mean age of these controls (140 males, 126 females) was 39.2 years at the time of blood collection (age range 3–69). All cytokine genotyping results in this control group have been published previously.[39,40]

Serum Cytokine and CRP Measurement

Whole blood was collected from each patient at the time of diagnosis. Samples were collected from patients without evidence of infection and at least 2 weeks following any invasive investigation to avoid artificial induction of an acute phase systemic response. CRP was determined using an automated immunoturbidimetric assay (Abbott TDX, Abbott Laboratories, Maidenhead, UK). A level above 10 mg/l was considered evidence of an acute phase response.

Cytokine concentrations were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using module kits and performed according to the manufacturers instructions (Caltag, Bender MedSystems, Towcester, UK) as described previously.[41] The lower limit of sensitivity for each assay was: < 1 pg/ml IL-1β, 1.4 pg/ml IL-6, .8 pg/ml IL-10 and 5.8 pg/ml sTNF-R.

Cytokine Genotyping

Genomic DNA was extracted from samples of lithium-heparinised blood using the Wizard Genomic DNA purification kit (Promega, Southampton, UK). The following SNPs were selected for genotyping due to their documented but variable association with cytokine production: IL-1β 511, IL-6 174, IL-10 1082, TNFα 308 and LTα +252. Genotyping was carried out by TaqMan allelic discrimination genotyping on the 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK). Primers and TaqMan probes were designed using Primer Express version 2.0 software (sequences shown in Table 1) and synthesised and supplied by Applied Biosystems UK. Ten microlitre PCR reactions containing 20 ng of DNA, .9 and .2 μM probes (final concentrations) were performed in 384 well plates. Each genotyping plate contained no DNA Template (water) controls; SDS version 2.1 software was used to analyse real-time and end-point fluorescence. Around 50 samples (~25% of the sample group) were randomly selected and included as replicates for each genotype tested. All replicates agreed. Only between 3–8 samples failed to genotype for each SNP (96.1–98.5% completion rate).

Statistical Analysis

Comparisons between groups of continuous variables were made by the Mann–Whitney U test. Categorical variables were compared by the Chi-squared test. Correlations between continuous variables were assessed by the Spearman rank correlation coefficient. Survival between groups was analysed by the log-rank test and Cox's proportional hazard's model was used for multivariate analysis. A P value $\leq .05$ was considered statistically significant. Significant P values were corrected for multiple comparisons using Bonferroni correction.

RESULTS

Study Patients and Genotype Distributions

Patient demographics are outlined in Table 2. Two hundred and three patients were genotyped. The

TABLE 1. *Cytokine TaqMan genotyping primer and probe sequences*

SNP	Sequence
(A) TaqMan MGB probe assays (Ta = 60°C)	
TNFα 308	
Forward primer	5′-CCAAAAGAAATGGAGGCAATAGGTT-3′
Reverse primer	5′-GGACCCTGGAGGCTGAAC-3′
G allele specific probe	5′-CCCGTCCcCATGCC-3′ (VIC-labelled)
A allele specific probe	5′-CCCGTCCtCATGCC-3′ (FAM-labelled)
LTα + 252	
Forward primer	5′-CAGTCTCATTGTCTCTGTCACACATT-3′
Reverse primer	5′-ACAGAGAGAGACAGGAAGGGAACA-3′
G allele specific probe	5′-CCATGgTTCCTCTC-3′ (FAM)
A allele specific probe	5′-CTGCCATGaTTCC-3′ (VIC)
IL-6 174	
Forward primer	5′-GCTGCACTTTTCCCCCTAGTT-3′
Reverse primer	5′-GCTGATTGGAAACCTTATTAAGATTGT-3′
G allele specific probe	5′-CTTTAGCATcGCAAGAC-3′ (VIC)
C allele specific probe	5′-CTTTAGCATgGCAAGAC-3′ (FAM)
IL-1β 511	
Forward primer	5′-GGTCTCTACCTTGGGTGGTGTT-3′
Reverse primer	5′-TCCTCAGAGGCTCCTGCAAT-3′
C allele specific probe	5′-TGCCTCgGGAGCT-3′ (VIC)
T allele specific probe	5′-TCTGCCTCaGGAGC-3′ (FAM)
(B) TaqMan TAMRA probe assays (Ta = 62°C)	
IL-10 1082	
Forward primer	5′-ACA CAC AAA TCC AAG ACA ACA CTA CTA A-3′
Reverse primer	5′-GGA GGT CCC TTA CTT TCC TCT TAC C-3′
G allele specific probe	5′-ATC CCT ACT TCC CCc TCC CAA AGA A-3′ (VIC)
A allele specific probe	5′-CCC TAC TTC CCC TTC CCA AAG AAG C-3′ (FAM)

For each probe, the position of SNP is marked in lower case.

median age was 71 years (interquartile range 62–78) and 66% of patients were male. The primary tumour sites were oesophageal (n = 91, 45%), gastric (n = 75, 37%) and those arising from the gastro-oesophageal junction (OGJ) (n = 37, 18%). Histological confirmation of disease was obtained in all cases and the predominant histological subtype was adenocarcinoma (85%). 113 (56%) patients underwent surgical resection, 22 of these received pre-operative chemotherapy. Seven (3%) patients, all of whom had squamous cell carcinoma of the oesophagus, received chemo-irradiation with curative intent. The remaining patients (n = 90) were not suitable for curative therapy and received palliative chemo/radiotherapy or underwent alternative palliative treatments, such as insertion of a stent or endoscopic laser therapy.

The prevalence of cytokine genotypes are presented in Table 3. There was a significant difference between cases and controls with the *IL-1β* 511 cc [P = .01, OR = 1.664 (95% CI = 1.137–2.434)] and ct [P = .03, OR = .657 (95% CI = .453–.955)] genotypes, but it did not remain so when corrected for multiple comparisons, making this finding most likely occurring due to chance. There were no significant differences between cancer patients and healthy controls in the distribution of any of the other genotypes. All genotype frequencies in both the patient and control groups were distributed in accordance with the Hardy–Weinberg equilibrium except for the *IL-6* patient group (P = .05), the *TNFα* control group (P = .04) and the *LTα* control group (P = .002).

Serum Cytokine and CRP Concentrations

The median serum concentration of IL-6 was 9.1 pg/ml (interquartile range 0–88 pg/ml) and for sTNF-R the median concentration was 3.3 ng/ml (interquartile range 1.9–5.8 ng/ml). IL-1β and IL-10 were only detectable in 4 (2%) and 10 (5%) patient's serum, respectively. 82 (41%) patients had an elevated acute phase protein response (CRP > 10 mg/l).

Serum sTNF-R concentration correlated with serum CRP concentration (r = .38, P < .001; spearman rank test), but there was no significant relationship between the other serum cytokine concentrations and CRP level.

Link between Genotype and Mediators/Markers of Systemic Inflammation

The *IL-6* CC genotype was associated with elevated serum sTNF-R concentrations when compared with the GC genotype (P = .015; Mann–Whitney U

TABLE 2. *Study patient demographics (n = 203)*

	Number (%)
Age (years)[+]	71 (62–78)
Sex	
Male	134 (66)
Female	69 (34)
Tumour site	
Oesophageal	91 (45)
Oesophago-gastric junction	37 (18)
Gastric	75 (37)
Histology	
Adenocarcinoma	172 (85)
Squamous cell carcinoma	26 (13)
Indeterminate	5 (2)
Grade	
Well differentiated	5 (2)
Moderately differentiated	69 (34)
Poorly differentiated	129 (64)
UICC stage	
1	23 (12)
2	33 (16)
3	76 (37)
4	71 (35)
Treatment undertaken	
Surgery with curative intent	91 (45)
Pre-operative chemotherapy/surgery	22 (11)
Chemoradiotherapy with curative intent	7 (3)
Palliative chemotherapy	24 (12)
Palliative radiotherapy	6 (3)
Stent/dilatation/laser/symptomatic	53 (26)

[+] values are median (interquartile range).

test), but not when compared with the GG genotype (P = .14). Similarly, the *IL-10* GG genotype was also associated with elevated sTNF-R concentrations when compared with the AG genotype (P = .05), but not when compared with the AA allele (P = .22). There was no association between any other serum cytokine concentrations and *IL-6* or *IL-10* genotypes. In addition, there was no association between any *IL-1β*, TNFα or *LTα* genotypes and serum cytokine concentrations.

The *IL-6* CC homozygous status was associated with significantly elevated serum CRP concentrations when compared with GC and GG genotypes [median 13 mg/l (range 4–35 mg/l) versus median 6 mg/l (range 2–22 mg/l)] (P = .03, Mann–Whitney U test) the GG *IL-10* genotype was also associated with elevated serum CRP levels [median 16 mg/l (range 3–34 mg/l)] when compared with the AA genotype [median 6 mg/l (range 3–13 mg/l)] (P = .02, Mann–Whitney U test) and the AG genotype [median 5 mg/l (range 2–24 mg/l)] (P = .03). The GG homozygous genotype was associated with elevated serum CRP levels (P = .01). None of the other cytokine genotypes were associated with serum CRP concentrations.

Possession of one or more of the following genotypes, *IL-6 CC, IL-10 GG* or *TNFα* AA, was associated with increasing serum CRP concentrations (P = .013, Chi square test) (Fig. 1). Moreover, multivariate analysis demonstrated that this positive association was independent of both treatment modality undertaken and stage of disease [regression coefficient = .16 (95% CI .05–.64), P = .021; linear regression analysis].

Survival Analysis

Survival of patients by genotype is presented in Figs. 2 and 3. Possession of the CC genotype for *IL-6* was associated with reduced survival duration when compared with GG or GC genotype (median survival 256 vs. 431 days) (P = .010; log-rank test) (Fig. 2A). The homozygous genotype GG for *IL-10* was associated with reduced survival compared with AA/AG genotypes (median survival 310 vs. 389 days, P = .047) (Fig. 2B). Similarly, AA homozygosity for the *TNFα* genotype was associated with adverse prognosis (median survival 194 vs. 409 days, P = .032) (Fig. 2C). Furthermore, the possession of more than one of these genotypes was associated with reduced survival (no alleles, median survival 512 days; 1 allele, median survival 269 days; 2 or 3 alleles, median survival 258 days; P = .004) (Fig. 3). Genotypes for *IL-1β* and *LTα* were not associated with prognosis.

Multivariate testing identified the AA genotype for *TNFα* as an independent adverse prognostic indicator when tested with age, sex, stage of disease, grade of tumour and serum CRP concentration (P = .006, hazard ratio 2.5; Cox's proportional hazard's model) (Table 4). Increasing age, male sex, advanced disease stage, elevated serum CRP concentration, and the *TNFα* AA genotype were all independently associated with poor prognosis. Genotypes for *IL-6* and *IL-10* were not independent prognostic indicators (P = .48 and P = .18, respectively).

Eighty-two (41%) patients had an elevated acute phase protein response (CRP > 10 mg/l) at the time of diagnosis and this was associated with a significantly reduced survival duration; CRP < 10 mg/l median survival 550 days versus CRP > 10 mg/l median survival 217 days (P < .001; log-rank test) (Fig. 4). Moreover, multivariate testing also identified CRP as an independent prognostic indicator (P = .002, hazard ratio 1.1; Cox's proportional hazard's model) (Table 4).

DISCUSSION

In the present study the *IL-6* 174 CC and *IL-10* 1082 GG genotypes were associated with elevated

TABLE 3. *Allelic distribution of cytokine genotypes among the study population*

Cytokine	Allele	Study patients n (%)	Controls n (%)	*P* value*	OR† (95% CL)
IL-1β 511	CC	89 (45.4)	87 (33.3)	.01 (.06)	1.664 (1.137–2.434)
	CT	81 (41.3)	135 (51.7)	.03 (.15)	.657 (.453–.955)
	TT	26 (13.3)	39 (14.9)	NS	Ref§
	HWE†	.27	.25		
IL-6 174	GG	71 (36.0)	79 (35.3)	NS	1.034 (.694–1.541)
	GC	83 (42.1)	101 (45.1)	NS	.887 (.603–1.304)
	CC	43 (21.8)	44 (19.6)	NS	Ref
	HWE	.05	.26		
IL-10 1082	GG	54 (27.0)	57 (25.6)	NS	1.077 (.699–1.659)
	AG	93 (46.5)	120 (53.8)	NS	.746 (.509–1.093)
	AA	53 (26.5)	46 (20.6)	NS	Ref
	HWE	.32	.24		
TNFα 308	GG	124 (62.0)	146 (68.2)	NS	.760 (.507–1.139)
	AG	61 (30.5)	56 (26.2)	NS	1.238 (.808–1.898)
	AA	15 (7.5)	12 (5.6)	NS	Ref
	HWE	.06	.04		
LTα +252	AA	82 (42.1)	92 (41.8)	NS	1.010 (.684–1.491)
	AG	84 (43.1)	83 (37.7)	NS	1.249 (.844–1.850)
	GG	29 (14.9)	45 (20.5)	NS	Ref
	HWE	.33	.002		

* *P* values were considered statistically significant when $P \leq .05$. Significant *P* values were corrected for multiple comparisons using Bonferroni correction (values in parenthesises).
† Odds ratio.
† *P* value for Hardy–Weinberg equilibrium (HWE) (Chi-squared test).
§ Reference genotype.

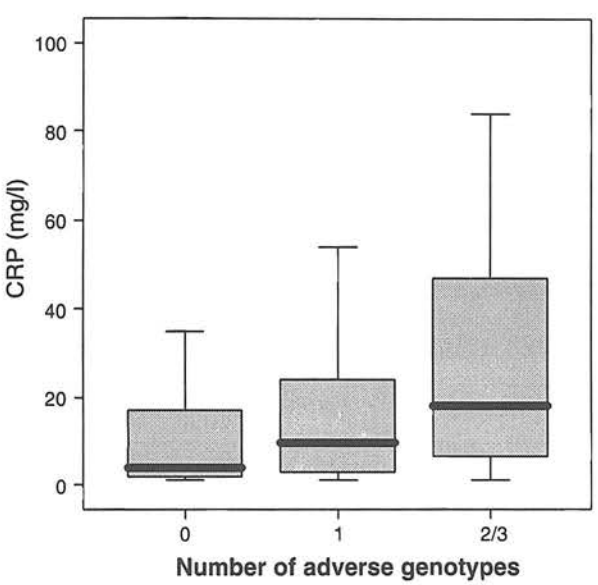


FIG. 1. Possession of one or more of the following genotypes, IL-6 CC, IL-10 GG or TNFα AA, was associated with increasing serum CRP concentrations ($P = .013$, Chi squared test).

markers of systemic inflammation (CRP and sTNF-R concentrations) and were associated with reduced survival duration. In addition, the *TNFα* 308 AA genotype was not associated with systemic inflammation, but was identified as an independent adverse prognostic marker. Therefore, there is a pro-inflammatory cytokine haplotype that is associated with adverse prognosis among patients with gastro-oesophageal cancer that may act in association with, but not entirely with, an inflammatory mediated mechanism.

IL-1β and IL-10 are not routinely measurable in the serum of healthy controls or cancer patients. In this study IL-1β and IL-10 were only detectable in 4 (2%) and 10 (5%) patient's serum, respectively. We, therefore, did not find an association between genotype and serum concentrations of these cytokines. We have, however, previously shown an association between *IL-1β* genotype and IL-1β production by PBMC in pancreatic cancer patients, but not with serum IL-1β concentrations.[20] PBMC production of IL-1β may better reflect tissue IL-1β levels rather than circulating serum concentrations and perhaps is a more accurate determinant of *IL-1β* activity.

TNFα is also rarely detected in the serum, however, soluble TNF receptors (sTNF-R) are shed in response to TNFα release and may be used as an indirect measure of TNFα concentration.[42] Serum sTNF-R concentrations were higher among those patients with elevated serum CRP concentrations. We found a significant correlation between serum sTNF-R and serum CRP concentrations ($r = .38$, $P < .001$) and we have shown a similar association among patients with pancreatic cancer.[43] There was no association between *TNF* genotype and serum sTNF-R concentrations either in this study or our previous work.[25] The data relating TNFα production

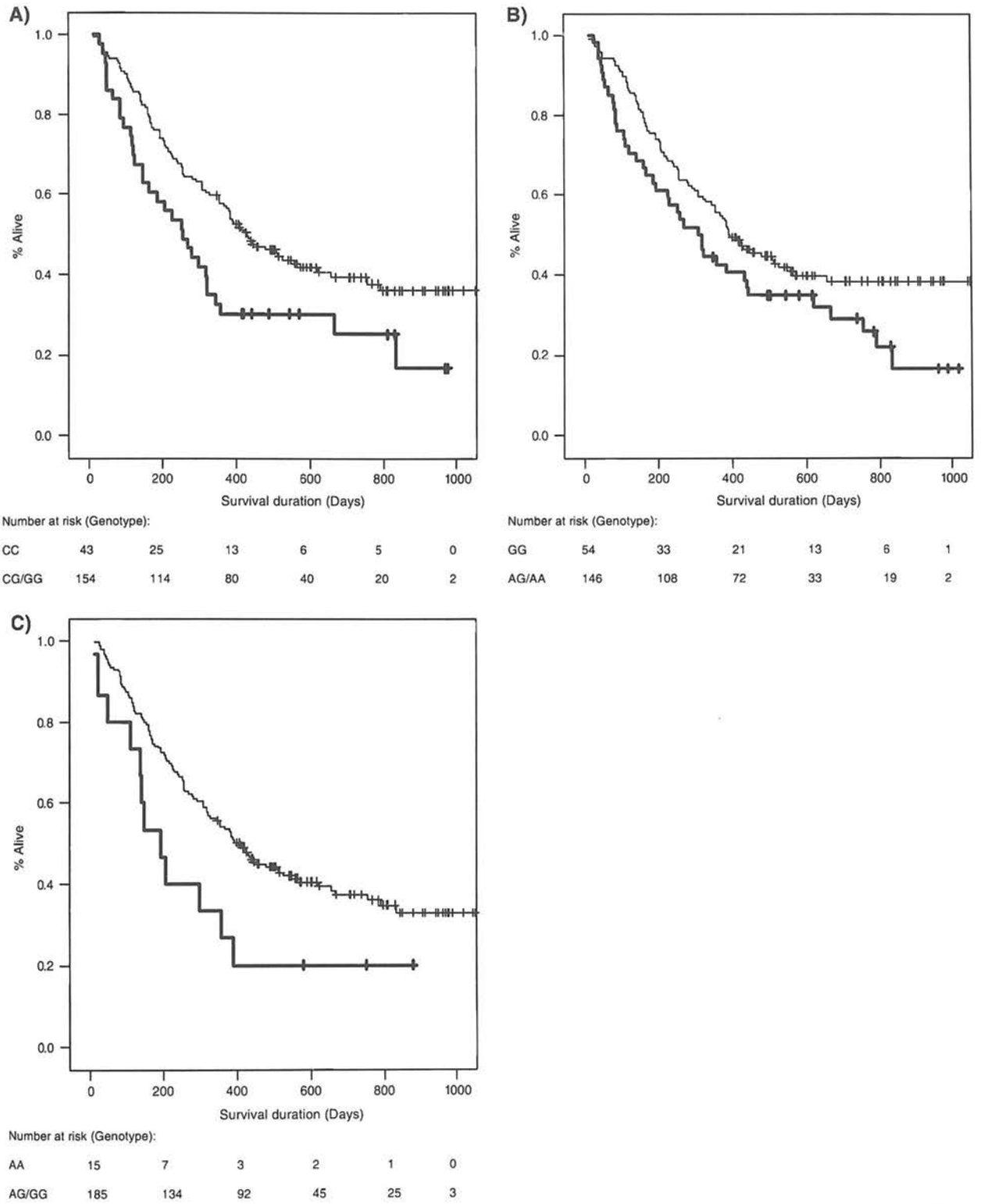
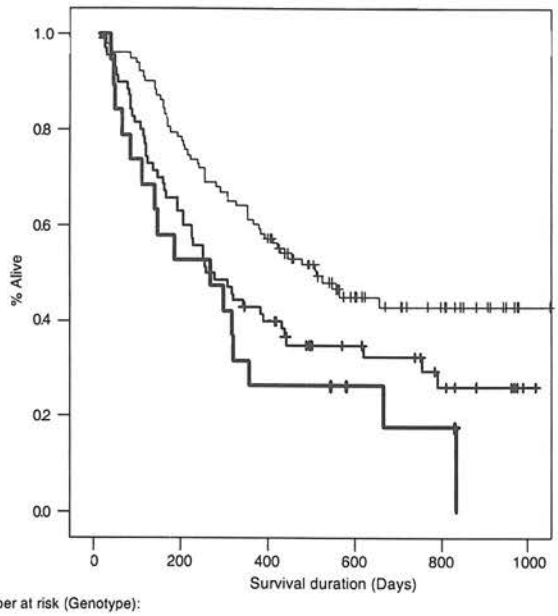


FIG. 2. Kaplan–Meier survival plots presented by **(A)** interleukin-6 (IL-6) genotype [CC median survival 256 days (heavy line), GC/GG median survival 431 days (light line)] (P = .010; Log-rank test); **(B)** interleukin-10 (IL-10) genotype [GG median survival 310 days (heavy line), AA/AG median survival 389 days (light line)] (P = .047) and **(C)** tumour necrosis factor α (TNFα) genotype [AA median survival 194 days (heavy line), AG/GG median survival 409 days (light line)] (P = .032).



Number at risk (Genotype):

0 Alleles	103	81	59	27	14	1
1 Allele	70	44	27	15	8	1
2/3 Alleles	19	10	5	3	2	0

FIG. 3. Kaplan–Meier survival plot stratified for possession of interleukin-6 (IL-6) CC genotype, interleukin-10 (IL-10) GG genotype and tumour necrosis factor α (TNFα) AA genotype. Possession of no alleles (light line), median survival 512 days; 1 allele (medium line), median survival 269 days; 2 or 3 alleles (heavy line), median survival 258 days (P = .004; Log-rank test).

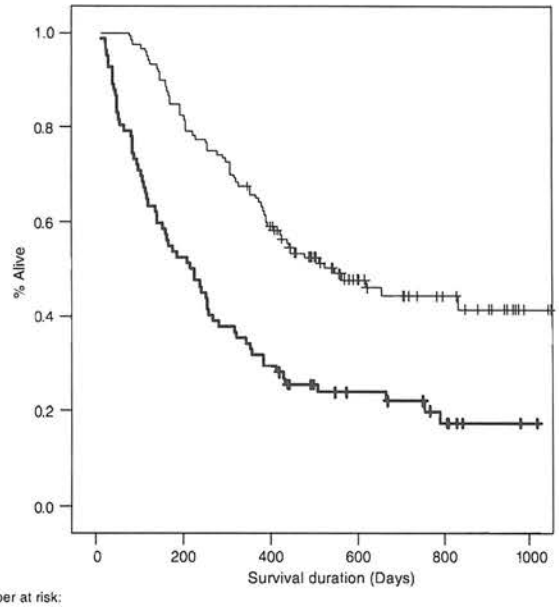
TABLE 4. *Multivariate survival analysis of 203 patients with gastro-oesophageal cancer using Cox's proportional hazard's model*

Variable	P value	Hazard ratio	95% CI
Age	.007	1.2	1.0–1.3
Sex	.04	1.8	1.0–3.0
Stage of disease	<.001	2.4	1.8–3.2
Grade of tumour	.88	1.0	.7–1.6
Serum CRP	.002	1.1	1.0–1.2
TNFα AA genotype	.006	2.5	1.3–4.9

A hazard ratio greater than 1 represents an increased risk of death. Increasing age, male sex, advanced disease stage, elevated serum CRP concentration, and the TNFα AA genotype were all independently associated with poor prognosis.

and genotype appears contradictory and at present there is no clear evidence relating *TNFα* genotype to circulating TNFα levels. However, tissue levels of TNFα may be a more relevant measure of TNF activity rather than systemic concentrations, which was not measured in the present study.

Forty-one percent of patients had an elevated acute phase response (CRP > 10 mg/l). Systemic inflammation has been found in association with the majority of solid epithelial malignancies and around



Number at risk:

CRP <10 mg/l	120	99	70	34	19	2
CRP >10 mg/l	82	43	24	13	7	1

FIG. 4. Kaplan–Meier survival plot according to the presence or absence of systemic inflammation. CRP < 10 mg/l median survival = 550 days (light line) versus CRP > 10 mg/l median survival 217 days (heavy line) (P < .001; Log-rank test).

50% of patients may have an acute phase response (APPR) at the time of diagnosis.[43] The presence of an elevated CRP has been associated with adverse prognosis in a number of types of cancer, independent of stage of disease.[5–15] We have similarly identified an elevated serum CRP concentration as an adverse prognostic indicator among patients with gastro-oesophageal cancer, also independent of stage of disease. The presence of systemic inflammation in malignant disease is an important marker of tumour behaviour and clearly has clinical relevance in assisting management decision making as well as emphasising the therapeutic potential of targeted anti-inflammatory strategies in advanced cancer.

IL-6 and *IL-10* genotypes were associated with serum CRP concentrations in this study. The *IL-6* CC genotype was associated with elevated serum concentrations of CRP and sTNF-R. A study of healthy volunteers identified the presence of the 174C allele to be associated with higher baseline CRP levels.[44] In cancer patients the rates of production of IL-6 can be linked to markers of systemic inflammation such as CRP. Although our study did not identify any association between *IL-6* genotype and serum IL-6 concentrations, it is possible that the CC genotype may be associated with elevated IL-6 production,

which acts at the tissue level to promote an acute phase response. The similar association between *IL-6* genotype and sTNF-R may reflect the more stable nature of the receptor molecule compared with the other cytokines, and in this regard sTNF-R may behave more as a marker of systemic inflammation.

We also found the *GG IL-10* genotype to be associated with elevated serum CRP and sTNF-R concentrations. The 1082 AA polymorphism is generally thought to be associated with reduced levels of IL-10 production.[34] One would therefore expect the GG genotype to be associated with increased levels of IL-10 production. An association between increased levels of an anti-inflammatory cytokine and elevated concentrations of acute phase proteins may initially appear contradictory, but may simply reflect the increased counter-regulatory activity of this important anti-inflammatory mediator in response to the presence of systemic inflammation.

We found no association between *IL-1β*, TNFα or *LTα* genotypes and CRP concentrations. In pancreatic cancer patients we previously demonstrated an association between allele 2 *IL-1β* genotype and elevated serum CRP levels, but in the present study there was no such association between the SNP at position 511 and CRP levels.[20] Similarly, there was no association between *TNFα* polymorphisms and CRP, a finding also in support of our previous work on pancreatic cancer patients.[25] Data relating to the 308 polymorphism and levels of TNFα production remain contradictory, however polymorphisms at this locus do not appear to influence serum CRP levels. Serum CRP concentrations were measured in a group of patients who had undergone cardiac surgery and this study did not find any differences between CRP levels and *TNFα* 308 genotypes.[45] Another group similarly failed to demonstrate differences in CRP concentrations by 308 genotype among smokers.[46]

The *IL-6* 174 CC and the *IL-10* 1082 GG genotypes were associated with reduced survival duration. The association between systemic inflammation and adverse prognosis among cancer patients has been well documented and in this study we have shown these two genotypes to be associated with elevated acute phase protein (CRP) concentrations. It is therefore possible that the adverse prognosis associated with these polymorphisms is related to the presence of systemic inflammation. This is supported by multivariate analysis where these genotypes lost their significance as prognostic indicators when CRP was co-analysed. The *CC IL-6* genotype has been linked with adverse prognosis among breast cancer patients, where possession of the CC polymorphism was associated with higher grade tumours and worse overall survival.[33] The GG genotype for *IL-10* 1082 has previously been associated with advanced stage in gastric cancer patients and associated reduced survival.[36]

We also found the *AA TNFα* 308 genotype to be related to adverse prognosis. In contrast to the *IL-6* and *IL-10* genotypes, we found no association between *TNFα* polymorphisms and systemic inflammation. It is therefore less likely that the reduced survival associated with this cytokine is related entirely to the generation of an inflammatory response. In addition, the AA genotype was an independent prognostic indicator on multivariate analysis, independent of CRP concentration, stage and other clinico-pathological characteristics that have previously been associated with adverse prognosis. We previously identified an association between the AA genotype and reduced survival in pancreatic cancer patients and another group similarly found the possession of the A allele to be linked with adverse outcome in patients with non-Hodgkin's lymphoma.[25,26] However, the AA genotype was only identified in 8% of patients, making it less useful in the clinical setting.

Distributions of the cytokine genotypes were similar between cancer patients and controls in this study and frequencies were similar to those previously published in studies of similar populations.[40,41] Although we did identify an association between gastro-oesophageal cancer and the *IL-1β* 511 CC genotype, this relationship lost its significance following correction for multiple comparisons. El-Omar et al. have proposed an association between *IL-1β* polymorphisms and an increased risk of gastric cancer among patients with helicobacter pylori infection.[47] We found no such association on sub-group analysis and other groups have similarly failed to demonstrate such an association.[48,49]

In summary, the possession of more than one of these genotypes (*IL-6 CC, IL-10 GG* and *TNFα* AA) resulted in a cumulative reduction in survival duration. This may relate to an increased magnitude of systemic inflammatory response or to some other unknown mechanism, but clearly there is a cytokine haplotype that is associated with adverse prognosis among these patients.

ACKNOWLEDGMENTS

We thank most sincerely Mr Simon Paterson-Brown and Mr Andrew de Beaux, Consultant Surgeons, Edinburgh Royal Infirmary, for help in the

recruitment of patients and provision of assistance with the clinical aspects of this work.

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Serum Parathyroid Hormone-Related Peptide Is Associated with Systemic Inflammation and Adverse Prognosis in Gastroesophageal Carcinoma

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Mr. A. de Beaux, Consultant Surgeon, Edinburgh Royal Infirmary, assisted with the recruitment of patients. The serum calcium measurements were performed by Mr. William Garden, F.I.B.M.S., Department of Clinical Biochemistry, Edinburgh Royal Infirmary. The c-terminal fragment of parathyroid hormone-related peptide radioimmunoassay kits were provided by Chugai Pharmaceutical Company, Ltd. of Japan, which also provided financial assistance for this work. Miss Amanda J. Lee, Medical Statistics Unit, University of Edinburgh, provided advice about suitable statistical methods for the analysis of the study data (but did not analyze the data or interpret the results).

Kevin Fearon received an honoraria from Chuga Pharma Company, Ltd.

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Received July 9, 2004; revision received November 29, 2004; accepted December 28, 2004.

BACKGROUND. Parathyroid hormone-related peptide (PTHrP) is a tumor-derived circulating factor that has been associated with hypercalcemia of malignancy. The role of PTHrP as a prognostic indicator remains unclear. Studies suggest that it may function as a growth factor; and, recently, the ability of PTHrP to induce cytokine expression has been described. PTHrP also has been proposed as a procachectic factor. In this study, the authors investigated the prognostic value of PTHrP in patients who had gastroesophageal carcinoma without hypercalcemia and determined whether PTHrP was associated with systemic inflammation and adverse nutritional status.

METHODS. Patients were recruited at the time of diagnosis. Serum was collected for determination of c-terminal fragment PTHrP (cPTHrP) levels (by radioimmunoassay) and calcium levels as well as levels of serum cytokines and acute-phase proteins (with an enzyme-linked immunosorbent assay). Nutritional assessment of patients was undertaken at the same time as serum collection. Patients underwent routine staging, and survival duration was recorded.

RESULTS. One hundred fifty-one patients with esophagogastric carcinoma were recruited. Six of 151 patients (4.0%) patients were hypercalcemic, and 26 patients (17.2%) had elevated serum cPTHrP levels. There was no association between the cPTHrP level and either serum calcium concentrations ($P = 0.72$) or adverse nutritional status. Elevated cPTHrP, however, was associated with significantly higher serum levels of soluble tumor necrosis factor receptor ($P = 0.008$) and with significantly lower levels of transferrin ($P = 0.009$) and albumin ($P = 0.02$). There was also a weak association with C-related protein levels ($P = 0.06$). Elevated cPTHrP levels also were associated with an adverse prognosis, as determined by reduced survival duration, on univariate analysis ($P = 0.038$), but not on multivariate analysis ($P = 0.15$).

CONCLUSIONS. Elevated serum cPTHrP levels were present in approximately 17% of patients with gastroesophageal carcinoma in the absence of hypercalcemia and was associated with markers of systemic inflammation and with an adverse prognosis. ***Cancer*** **2005;103:1810–8.** © *2005 American Cancer Society.*

KEYWORDS: parathyroid hormone-related peptide, hypercalcemia, cachexia, cytokines, prognosis.

Hypercalcemia is among the most frequent paraneoplastic syndromes, and it is estimated that hypercalcemia affects approximately 20% of patients during the advanced stages of malignant disease.[1] Parathyroid hormone-related peptide (PTHrP) is a tumor-derived circulating factor that has been associated with hypercalcemia of malignancy.[2] Elevated serum PTHrP has been reported in 50–90% of hypercalcemic patients with malignant disease.[3] PTHrP

DOI 10.1002/cncr.20972
Published online 30 March 2005 in Wiley InterScience (www.interscience.wiley.com).

mimics the actions of parathyroid hormone on the kidneys and bone to increase serum calcium concentration. More recently, PTHrP has been proposed as a growth factor with activity that may promote tumor growth, particularly aiding in the formation of osteolytic bone metastases.[4]

Recent interest in PTHrP has been directed toward its role in prognosis. In a study of 76 hypercalcemic patients with various types of advanced-stage malignancies, elevated serum PTHrP levels were found in 54% of patients.[3]. Increased PTHrP was an independent, adverse prognostic indicator for patients age < 65 years but had no prognostic role in older patients. Others investigators found no association between elevated serum PTHrP levels and reduced survival.[5] Differing conclusions also have been reached regarding the detection of PTHrP within tumors themselves and its role in prognosis. Patients with breast carcinoma who had tumors that produced PTHrP had a favorable prognosis in one study, but this observation was not corroborated by others.[6,7] The administration of a PTHrP antibody to mice bearing PTHrP-secreting tumors not only resulted in a reduction of serum calcium levels but also improved survival duration in the treated mice.[8] The role of PTHrP in determining prognosis remains unclear.

In addition, PTHrP has been proposed as a procachectic factor. PTHrP is produced by a human lung carcinoma model (HARA-B); and, when it is implanted into mice, results in a significant reduction in body weight and tissue mass, an effect that is reversed by the addition of an antibody to PTHrP.[9] This model was associated with elevated serum calcium levels, and it is not clear whether the observed cachexia was due to the systemic effects induced by hypercalcemia, because food intake in the weight-losing mice was lower than controls. The relation of PTHrP to adverse nutritional status needs further investigation.

PTHrP also may contribute to the proinflammatory cytokine cascade. Several studies identified elevated serum cytokines, in particular, interleukin-6 (IL-6) and tumor necrosis factor (TNF), in association with elevated serum PTHrP.[10,11] Another group demonstrated that PTHrP stimulates IL-6 production from osteoblasts in vivo.[12] Funk et al. investigated the effects of endotoxemia on PTHrP synthesis within hepatocytes: A near-lethal dose of endotoxin resulted in marked induction of PTHrP mRNA levels and PTHrP protein synthesis.[13] In addition, the administration of PTHrP to mice increased serum acute-phase protein levels. Those investigators also found that synovial cells from patients with rheumatoid arthritis produced increased levels of PTHrP, and these levels were enhanced further by the addition of IL-1β and TNF-α.[14]

Elevated serum PTHrP has been described in association with hypercalcemia in esophageal carcinoma, and PTHrP mRNA and protein also have been demonstrated within tumor cells.[15] In a large series from Japan that included 382 patients with esophageal carcinoma, hypercalcemia was identified in 1.3% of patients at the time of diagnosis.[16] The incidence of hypercalcemia increased to 38% in patients who were monitored within 2 months of death, and the authors reported observing a close association between hypercalcemia and PTHrP levels. Similarly, elevated PTHrP production has been reported in association with gastric carcinoma.[17,18] In one study, PTHrP expression was identified within tumor cells in 71 of 92 patients with gastric adenocarcinoma, none of whom had hypercalcemia.[19] Those authors also noted that PTHrP production was associated with poorly differentiated tumors.

The detection of PTHrP in tumors without associated hypercalcemia has raised the possibility that PTHrP may play a role in addition to calcium homeostasis, such as the regulation of cellular proliferation and differentiation. Epidermal growth factor and transforming growth factor β (TGF-β) have been implicated in the progression of gastric malignancies, and it has been found that these growth factors upregulate PTHrP gene expression in some cell lines.[20] Rats implanted with prostate carcinoma cells that overproduce PTHrP had significantly enhanced tumor growth and tumor size compared with control animals.[21] The gene encoding PTHrP is a downstream target for the protooncogenes K-*ras* and *Src* tyrosine kinase.[22,23] Whereas src expression is common in premalignant epithelium of Barrett esophagus,[24] c-K-*ras* expression is rare.[25] Whether the expression of *src* in gastroesophageal carcinoma correlates with increased expression of PTHrP and hypercalcemia is unknown but may provide a mechanism for the functional activation of tumor-derived PTHrP in this tumor type. Whether PTHrP expression alone is sufficient to cause hypercalcemia also is unclear, but a requirement for further modification may explain why PTHrP is produced without associated hypercalcemia.

For the current study, we investigated serum PTHrP production in patients with gastroesophageal carcinoma and determined its association with serum calcium levels in addition to serum acute-phase proteins and cytokines. The link between PTHrP levels and nutritional status was evaluated along with the role of PTHrP as a prognostic marker.

MATERIALS AND METHODS

Patients

Patients were recruited to the study within 2 weeks of diagnosis. All patients had histologic confirmation of

their disease after undergoing endoscopic biopsy. Blood samples were collected within 2 weeks of diagnosis and before patients started any treatment. All patients were free from systemic infection at the time of blood collection. No patients had symptoms related to possible hypercalcemia, and none had bone metastases identified after computerized tomography staging. No patients were receiving treatment either to supplement or to reduce calcium levels. No patients were taking calcium-containing "over the counter" medications. Renal function was assessed by measuring serum creatinine concentration, and values were within the normal range for all patients. Muscle wasting was not sufficiently severe to require correction for muscle bulk. Patients provided written informed consent, and the study received ethical permission from the Lothian Research Ethics Committee. Clinical information was collected prospectively. Patients were staged with a combination of computerized tomography, endoscopic ultrasound, and laparoscopy/laparoscopic ultrasound studies according to the International Union Against Cancer[26]; and final histopathologic stage (pTNM) was used when available (n = 59 patients). Final stage (pTNM) was available only for those patients who underwent surgical resection. In all other patients, the final clinical stage, as agreed at the unit multidisciplinary team meeting, was recorded. Tumors around the esophagogastric junction were classified according to Siewart: Type I and II tumors were classified as esophageal tumors, and Type III tumors were classified as gastric carcinomas.[27] Clinical and pathologic information was recorded for each patient, including treatment modality, tumor grade, and histologic subtype. Duration of survival, which was defined as the time from histologic diagnosis to death, was recorded for all patients. All deaths were disease related, except for three patients who died in the postoperative period from sepsis and multiorgan failure. All patients were included in the survival analysis.

Nutritional Assessment

Nutritional assessment included calculation of body mass index from the patients' weight and height and measurement of midarm circumference and triceps skinfold thickness, as described previously.[28] Midarm muscle circumference was calculated by means of Jelliffe's equation.[29] Premorbid patient weight was recalled by the patient and was confirmed from the medical notes when possible. Performance status was assessed using the Karnofsky index.[30]

Serum PTHrP and Calcium Concentrations

Whole blood was collected from patients at the time of diagnosis. Samples were centrifuged, and the serum was stored at − 70 °C until batch analysis. PTHrP has a very short half-life and is unstable unless it is stored in plasma with esterase inhibition. However, its breakdown products include a c-terminal region, which is stable when it is stored in serum and demonstrates direct correlation to circulating, intact serum PTHrP concentrations.[31] This is a direct breakdown product of PTHrP; therefore, its presence indicates PTHrP in plasma. In this study, the c-terminal fragment (cPTHrP) was measured by radioimmunoassay (Daiichi, Tokyo, Japan). This assay has no demonstrable cross-reactivity with parathyroid hormone. The sensitivity of this test is 10 pmol/L, the intraassay variability is 4.1%, and the interassay variability is 3.3%. Using this assay, serum PTHrP levels in healthy volunteers were (± 2 standard deviations from the mean) 13.8–55.3 pmol/L for males and 13.9–54.0 pmol/L for females.[32] No female patients had a cPTHrP serum concentration between 54.0 pmol/L and 55.3 pmol/L; therefore, for simplicity serum levels > 55.3 pmol/L were considered above the normal range for both genders.

Total serum calcium was measured by an automated analyzer (Clinical Biochemistry Department, Edinburgh Royal Infirmary, Edinburgh, UK). Calcium levels were adjusted for serum albumin concentration using the following formula, according to Truong et al., 2003[3]:

$$\frac{40 - \text{albumin concentration}}{4} \times 0.1 + \text{measured serum calcium concentration}$$

Note that the normal range for corrected calcium in our laboratory is 2.12–2.62 mmol/L.

Acute-Phase Proteins and Cytokines

C-reactive protein (CRP) levels were determined using an immunoturbidimetric assay (Abbott TDX; Abbott Laboratories, Maidenhead, UK). A CRP level > 10 mg/L represents the presence of an acute-phase response. Levels of the remaining acute-phase proteins and cytokines were determined by sandwich enzyme-linked immunoadsorbent assay, as described previously.[33] Serum cytokines were analyzed with module kits according to the manufacturer's instructions (Caltag; Bender MedSystems, Towcester, UK). The lower limit of sensitivity for each assay was < 1 pg/mL for IL-1β, 1.4 pg/mL for: IL-6, 11 pg/mL for IL-8, 0.8 pg/mL for IL-10, and 5.8 pg/mL for soluble TNF receptor (sTNF-R).

Acute-phase protein concentrations were determined using primary and secondary antibodies supplied by Dako (Ely, UK).[33] Briefly, 96-well plates were coated with 100 μL primary antibody (concentration, 10 mg/L) and incubated overnight at 4 °C. The plates were washed with 0.1% Tween, diluted serum (100 μL) was added to the coated wells, and the plates were incubated at room temperature for 2 hours. The plates were washed as described above, a secondary antibody conjugated with peroxidase was added to each well, and the plates were incubated for an additional hour. The substrate used was OPD (Dako), and the reaction was stopped with 0.5 M sulphuric acid. Plates were read at 490 nanometers using a Dynatech MR5000 automated plate reader (Dynatech, Billinghurst, UK). Standard curves were generated using standard acute-phase proteins supplied by the manufacturer (Dako).

Statistical Analysis

Correlations between serum cPTHrP and serum calcium concentrations were tested by linear regression analysis. Analysis of serum cPTHrP and continuous variables was tested by the Mann–Whitney U test. Analysis of serum cPTHrP and categoric data was undertaken with the chi-square and Fisher exact tests. Log-rank analysis was used to determine prognostic values for the univariate analysis, and a Cox proportional hazards model was used for the multivariate analysis. P values ≤ 0.05 were considered statistically significant.

RESULTS

Study Patients

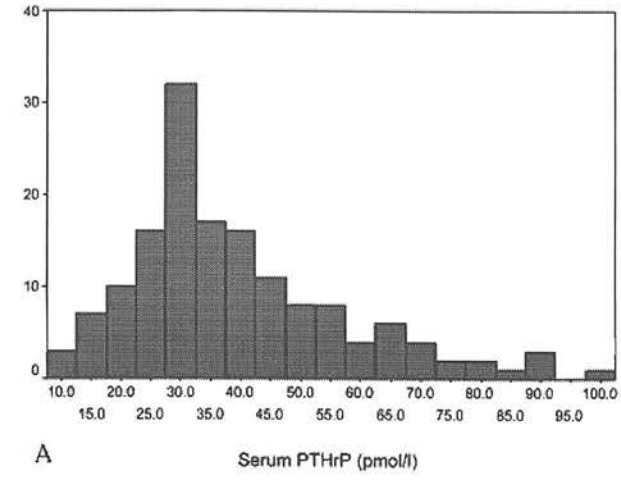
Patient demographics are outlined in Table 1. One hundred fifty-one patients were recruited to the study. Their median age was 72 years (range, 26–95 years), and 63% of patients were male. The primary tumor sites were esophageal (n = 64 patients; 42%), gastric (n = 59 patients; 39%), and tumors arising from the gastroesophageal junction (n = 28 patients; 19%). Histologic confirmation of disease was obtained in all tumors, and the predominant histologic subtype was adenocarcinoma (87%). There were no well differentiated tumors. Sixty patients (40%) underwent surgical resection, and 14 of those patients received preoperative chemotherapy. Three patients, all of whom had squamous cell carcinoma of the esophagus, received chemoirradiation with curative intent. Seventeen patients (11%) received palliative chemotherapy, and 4 patients (3%) received palliative radiotherapy. Sixty-seven patients (44%) were not suitable for curative therapy or palliative chemoradiotherapy and underwent alternative palliative treatments, such as insertion of a stent or endoscopic laser therapy, according to their symptoms.

TABLE 1
Patient Demographics

Characteristic	No. of patients (%)
Age (yrs)[a]	72 (63–78)
Gender	
Male	95 (62.9)
Female	56 (37.1)
Tumor site	
Esophageal	64 (42.4)
Esophagogastric junction	28 (18.5)
Gastric	59 (39.1)
Histology	
Adenocarcinoma	132 (87.4)
Squamous cell carcinoma	16 (10.6)
Indeterminate	1 (0.7)
Small cell carcinoma	2 (1.3)
Grade	
Well differentiated	0 (0.0)
Moderately differentiated	37 (24.5)
Poorly differentiated	114 (75.5)
UICC stage	
Stage I	13 (8.6)
Stage II	24 (15.9)
Stage III	54 (35.8)
Stage IV	60 (39.7)
Treatment undertaken	
Surgery with curative intent	46 (30.5)
Preoperative chemotherapy/surgery	14 (9.3)
Chemoradiotherapy with curative intent	3 (2.0)
Palliative chemotherapy	17 (11.3)
Palliative radiotherapy	4 (2.6)
Stent/dilatation/laser/symptomatic	67 (44.4)
Karnofsky score	
30	3 (2.0)
40	1 (0.7)
50	5 (3.3)
60	13 (8.6)
70	18 (11.9)
80	23 (15.2)
90	35 (23.2)
100	41 (27.2)
Not recorded	12 (7.9)
Survival	
Alive	100 (66.2)
Dead	51 (33.8)
Follow-up (days)[a]	379 (207–510)

UICC: International Union Against Cancer.
[a] Median (interquartile range).

Performance status was assessed using the Karnofsky index. The median score was 90 (interquartile range, 70–100), and the score was correlated strongly with disease stage ($P < 0.0001$) and mortality ($P < 0.0001$; data not shown). The median follow-up was 379 days (interquartile range, 207–510 days); and, at the time the data were censored, 51 patients (34%) had died.



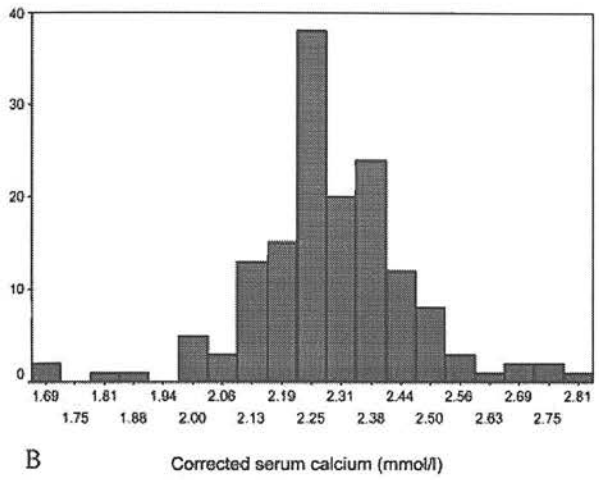


FIGURE 1. These graphs illustrate the distribution of serum levels of the (A) c-terminal fragment of parathyroid hormone-related (PTHrP) and (B) serum calcium within the study population.

Serum PTHrP and Calcium Concentrations

The mean ± standard deviation serum level of cPTHrP was 39.4 ± 17.9 pmol/L (range, 8.6–100.0 pmol/L) (Fig. 1A). Twenty-six patients (17%) had elevated serum cPTHrP levels. The mean ± standard deviation serum calcium concentration was 2.29 ± 0.17 mmol/L (range, 1.67–2.79 mmol/L) (Fig. 1B). Twelve patients (8%) were hypocalcemic, and 6 patients (4%) were hypercalcemic.

There was no correlation between serum cPTHrP levels and corrected calcium levels (linear regression; $r = 0.03$; $P = 0.72$) (Fig. 2). Patients with elevated serum cPTHrP levels did not have associated elevated serum calcium levels ($P = 0.63$; Mann–Whitney U test).

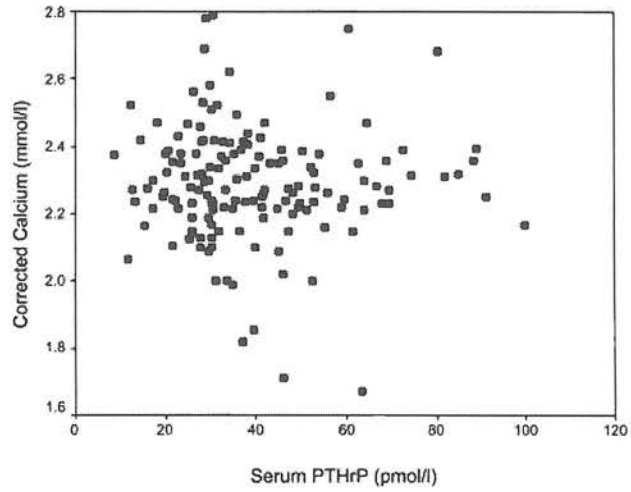


FIGURE 2. This graph illustrates the correlation between serum calcium and serum parathyroid hormone-related peptide (PTHrP) concentrations (*correlation coefficient* = 0.03; $P = 0.72$).

Serum PTHrP Concentrations Correlated with Patient Characteristics, Tumor Type, Disease Stage, and Tumor Grade

PTHrP levels and patient/tumor characteristics are outlined in Table 2. There was no association between serum cPTHrP concentration and diagnosis and pathologic data, including tumor location, histology, or stage. Although an association with cPTHrP and tumor grade did not reach significance ($P = 0.24$), poorly differentiated tumors were more likely to be associated with elevated cPTHrP levels. Serum calcium concentrations were significantly higher in patients who had squamous histology ($P = 0.005$; Mann–Whitney U test) and in females ($P = 0.01$; Mann–Whitney U test) (Fig. 3).

Serum PTHrP Concentration and Survival

Twelve of 26 patients (46%) who had elevated cPTHrP levels had died at the time the survival data were censored compared with 39 of 125 patients (31%) who had normal cPTHrP levels. Figure 4 shows a Kaplan–Meier survival curve that compares patients with normal serum PTHrP ($n = 125$ patients) and elevated serum PTHrP ($n = 26$ patients). Those patients who had elevated serum cPTHrP levels had a significantly reduced survival duration compared with patients who had normal serum levels ($P = 0.038$; log-rank analysis). However, when age, gender, disease stage, and tumor grade were included in the Cox proportional hazards model, in addition to serum cPTHrP, elevated cPTHrP was not an independent predictor of survival ($P = 0.45$).

TABLE 2
Serum Parathyroid Hormone-Related Peptide Levels and Patient Characteristics

Characteristic	Serum PTHrP (pmol/L) Normal (n = 125)	Elevated (n = 26)	P value
Age (yrs)			
Median	72	75	
Interquartile range	63–78	62–83	0.264[a]
Gender			
Male	80	15	
Female	45	11	0.702[b]
Tumor site			
Esophageal	52	12	
Esophagogastric junction	24	4	
Gastric	49	10	0.604[b]
Histology			
Adenocarcinoma	109	23	
Squamous cell carcinoma	13	3	
Indeterminate	1	0	
Small cell carcinoma	2	0	1.00[c]
Grade			
Well differentiated	0	0	
Moderately differentiated	33	2	
Poorly differentiated	92	22	0.243[b]
UICC stage			
Stage I	12	1	
Stage II	20	4	
Stage III	44	10	
Stage IV	49	11	0.949[c]
Karnofsky score			
30	1	2	
40	1	0	
50	4	1	
60	9	4	
70	15	3	
80	20	3	
90	33	2	
100	34	7	
Not recorded	8	4	0.194[a]

PTHrP: parathyroid hormone-related peptide; UICC: International Union Against Cancer.
[a] Mann-Whitney U test.
[b] Chi-square test.
[c] Fisher exact test.

Serum PTHrP Concentration and Nutritional Status

Nutritional status is shown in Table 3. There were no significant differences in the nutritional variables measured between patients with or without elevated serum cPTHrP concentrations, except for triceps skinfold measurements, which were higher in the group with elevated cPTHrP (P = 0.04). Patients with elevated serum cPTHrP had lower serum albumin levels compared with patients who had albumin levels within the normal range (P = 0.02).

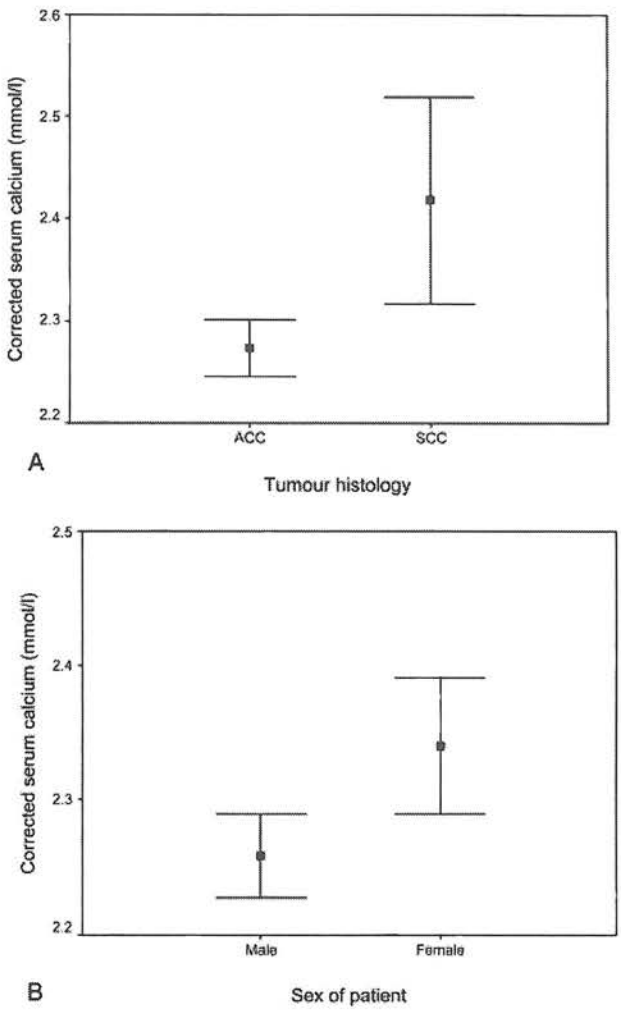


FIGURE 3. These graphs illustrate the mean serum calcium concentration stratified according to (A) tumor histology (P = 0.005; Mann–Whitney U test) and (B) gender (P = 0.01). Error bars indicate 95% confidence intervals. ACC: adenocarcinoma; SCC: squamous cell carcinoma.

Serum PTHrP Concentrations and Serum Acute-Phase Proteins and Cytokines

The correlation between cPTHrP and serum acute-phase proteins and cytokines is shown in Table 4. The median serum CRP and haptoglobin concentrations for patients who had elevated serum cPTHrP levels were above the normal ranges. Patients who had elevated serum cPTHrP levels had higher serum CRP levels compared with patients who had normal cPTHrP levels (P = 0.06). There was no difference in serum haptoglobin levels between patients with or without an elevated serum cPTHrP levels (P = 0.46). Patients who had elevated PTHrP levels had significantly lower serum transferrin levels compared with patients who had normal PTHrP levels (P = 0.009) (Fig. 5A). α-1-Antichymotrypsin levels were within the

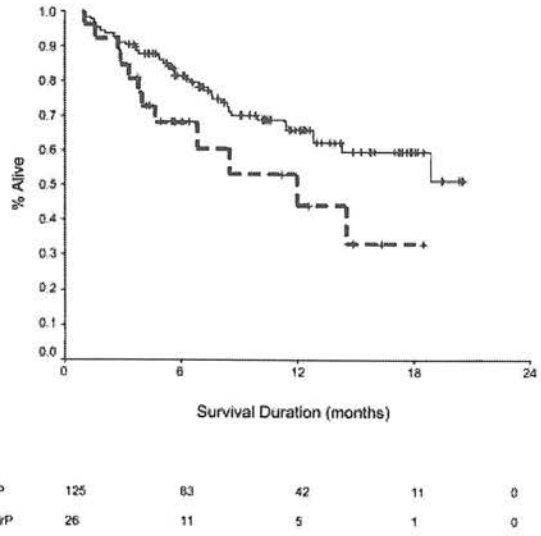


FIGURE 4. These Kaplan–Meier curves were constructed by comparing the survival of patients with normal levels of the c-terminal fragment of serum parathyroid hormone-related peptide (cPTHrP) (solid line) and patients with elevated serum cPTHrP (dashed line) (log-rank analysis; $P = 0.038$).

TABLE 3
Serum Parathyroid Hormone-Related Peptide Levels and Nutritional Variables

Nutritional parameter	Serum PTHrP (pmol/L)[a] Normal	Elevated	*P* value[b]
BMI at diagnosis (kg/m^2)	24.1 (21.2–28.0)	25.3 (22.3–28.8)	0.53
Weight loss (%)	7.6 (1.9–14.9)	6.7 (1.9–12.9)	0.73
Midarm circumference (cm)	27.0 (24.0–30.0)	28.0 (26.5–32.0)	0.14
Triceps skinfold (mm)	11.5 (8.0–15.0)	14.5 (9.0–18.0)	0.04
Arm muscle circumference (cm)	23.4 (20.8–25.2)	23.9 (21.7–26.2)	0.42
Albumin (g/L)	40.0 (36.0–43.0)	37.0 (34.0–40.5)	0.02

PTHrP: parathyroid hormone-related peptide; BMI: body mass index.
[a] Median (interquartile range).
[b] Mann-Whitney *U* test.

normal range for patients with or without elevated serum cPTHrP levels, and there was no difference between the 2 groups ($P = 0.47$).

IL-1β levels were not significantly different between the groups ($P = 0.34$) and were within the normal range, as were levels of IL-6 ($P = 0.46$), IL-8 ($P = 0.81$), and IL-10 ($P = 0.54$). However, patients who had elevated PTHrP levels had significantly elevated sTNF-R levels compared with patients who had serum cPTHrP levels within the normal range ($P = 0.008$) (Fig. 5B).

DISCUSSION

In the current study, only 6 patients (4%) were hypercalcemic, a proportion that is in agreement with the findings in a large Japanese study in which 1.3% of patients with esophageal carcinoma had elevated serum calcium levels at the time of diagnosis.[16] Patients with gastric or esophageal carcinomas experience hypercalcemia infrequently, and bone metastases are uncommon. Despite normal plasma calcium levels, 26 patients (17%) in the current study had elevated serum cPTHrP levels. Increased levels of cPTHrP were likely irrespective of tumor origin or histologic type. Similarly, there was no association between advanced disease stage and elevated cPTHrP levels, an observation that was reported previously in other studies. Although not statistically significant in the current series, the current data did suggest a trend toward a correlation between increased cPTHrP production and poor tumor differentiation. A study involving a much larger series of patients would be required to explore this potential association. A possible link between tumor grade and the overproduction of cPTHrP has been reported.[19]

Several tumor models have suggested that PTHrP is associated with adverse nutritional status.[9] No differences in nutritional status were observed between patients with or without an elevated cPTHrP, except for triceps skinfold thickness. Patients who had elevated cPTHrP levels had increased triceps measurements compared with patients who had normal serum levels of cPTHrP ($P = 0.04$). This finding likely represents sample error and may be a consequence of the higher proportion of females, who have a greater triceps skinfold thickness per kilogram of body weight compared with males, among the patients with elevated cPTHrP. The similarity in nutritional status between the two patient groups may be explained by the

TABLE 4
Serum Parathyroid Hormone-Related Peptide and the Acute Phase Proteins and Cytokines

Variable	Serum PTHrP (pmol/L)[a] Normal	Elevated	*P* value[b]
CRP (mg/L)	6.5 (2.3–23.8)	17 (4.5–60.8)	0.06
Transferrin (mg/L)	1.8 (1.4–2.2)	1.5 (1.1–1.8)	0.009
Haptoglobin (mg/L)	2094 (1521–2829)	2205 (1113–2639)	0.46
α1-Antichymotrypsin (mg/L)	402 (337–520)	451 (326–601)	0.47
IL-1β (pg/mL)	0	0	0.34
IL-6 (pg/mL)	8.9 (0.0–86.6)	0 (0.0–51.6)	0.46
IL-8 (pg/mL)	0 (0.0–147.0)	0 (0.0–126.2)	0.81
IL-10 (pg/mL)	0	0	0.54
sTNF-R (ng/mL)	3.2 (1.9–4.9)	5.3 (2.9–10.4)	0.008

PTHrP: parathyroid hormone-related peptide; CRP: C-reactive protein; IL-1β: interleukin 1β; sTNF-R: soluble tumor necrosis factor receptor.
[a] Median (interquartile range).
[b] Mann-Whitney *U* test.

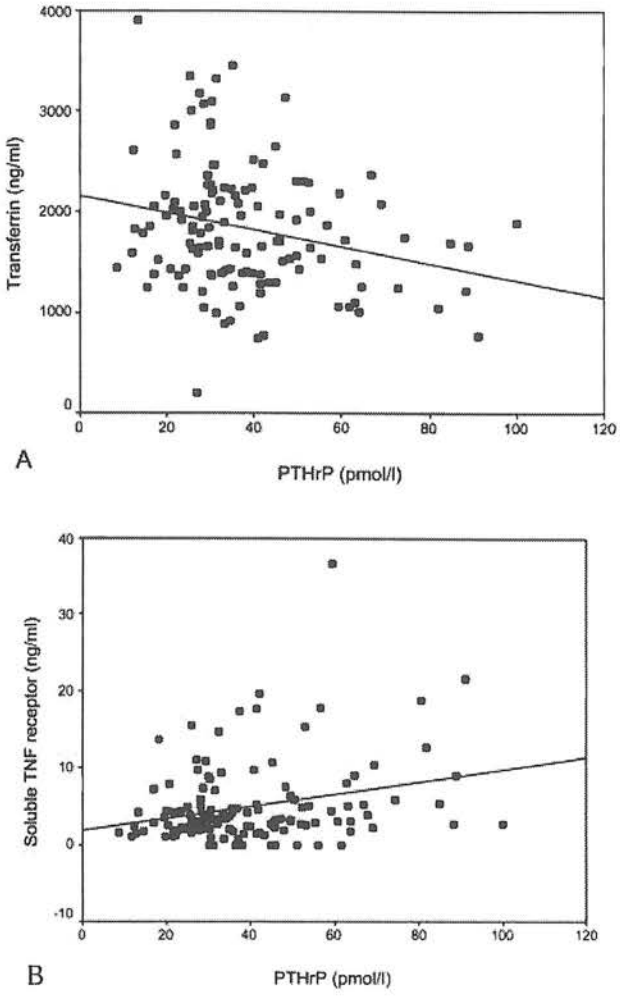


FIGURE 5. These graphs illustrate the correlations between (A) serum concentrations of the c-terminal fragment of parathyroid hormone-related peptide (PTHrP) with serum concentrations of transferrin ($P = 0.007$; $r = 0.24$; linear regression) and (B) with serum concentrations of soluble tumor necrosis factor (TNF) receptor ($P = 0.002$; $r = 0.26$).

fact that the sample population had normal serum calcium levels. It is possible that the correlation noted elsewhere between elevated PTHrP and weight loss is attributable to the systemic effects of hypercalcemia (e.g., anorexia). In patients with normal serum calcium levels, PTHrP may not have any independent effect on nutritional status.

Forty-four percent of the patients studied demonstrated an elevated, systemic, acute-phase protein response. The association between systemic inflammation and malignant disease has been documented well in the literature and is supported by the current data. Levels of CRP (which is a positive, acute-phase reactant) were higher, whereas levels of albumin and transferrin (both of which are negative, acute-phase reactants) were lower in patients who had elevated levels of PTHrP. These data suggest a possible further enhancement of systemic inflammation in association with elevated PTHrP. Plasma levels of the proinflammatory cytokines IL-1β, IL-6, and IL-8 did not differ significantly between the 2 groups. However, the correlations between serum cytokine levels and tissue inflammation or measures of outcome in patients with advanced carcinoma remain unclear. In contrast, sTNF-R levels were significantly higher in the group with elevated PTHrP levels. Again, this is consistent with a greater level of systemic inflammation. It is believed that plasma sTNF-R levels are a robust, indirect index of TNF-α production in tissue compartments.[34]

We found no correlation between serum calcium levels and cPTHrP levels. This raises the possibility of an additional role for cPTHrP as a growth factor that potentiates tumor progression. We found no clear association between elevated cPTHrP levels and disease stage. However, patients who had elevated serum levels of cPTHrP had a worse prognosis, which was significant on univariate analysis, but not on multivariate testing. The association between elevated serum cPTHrP and systemic inflammation may contribute to the adverse prognosis noted in this patient group.

Elevated serum cPTHrP levels were found to be associated with gastroesophageal carcinoma in the absence of hypercalcemia. Although serum proinflammatory cytokines (and, in particular, IL-6) were not higher in patients who had elevated levels of cPTHrP, overall, markers of systemic inflammation (as indicated by levels of acute-phase proteins and sTNF-R) were associated with elevated cPTHrP. Elevated serum cPTHrP also was associated with an adverse prognosis, but it did not predict outcome independent of other covariates. Circulating PTHrP levels were not associated with adverse nutritional status. However, given the strong correlation between systemic inflammation and cachexia/decreased survival, there is a possible indirect role for cPTHrP in these processes.

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British Journal of Cancer (2006) 94, 731 – 736

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Expression of the proteolysis-inducing factor core peptide mRNA is upregulated in both tumour and adjacent normal tissue in gastro-oesophageal malignancy

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Gastro-oesophageal cancer is associated with a high incidence of cachexia. Proteolysis-inducing factor (PIF) has been identified as a possible cachectic factor and studies suggest that PIF is produced exclusively by tumour cells. We investigated PIF core peptide (PIF-CP) mRNA expression in tumour and benign tissue from patients with gastro-oesophageal cancer and in gastro-oesophageal biopsies for healthy volunteers. Tumour tissue and adjacent benign tissue were collected from patients with gastric and oesophageal cancer ($n = 46$) and from benign tissue only in healthy controls ($n = 11$). Expression of PIF-CP mRNA was quantified by real-time PCR. Clinical and pathological information along with nutritional status was collected prospectively. In the cancer patients, PIF-CP mRNA was detected in 27 (59%) tumour samples and 31 (67%) adjacent benign tissue samples. Four (36%) gastro-oesophageal biopsies from healthy controls also expressed PIF-CP mRNA. Expression was higher in tumour tissue ($P = 0.031$) and benign tissue ($P = 0.022$) from cancer patients compared with healthy controls. In the cancer patients, tumour and adjacent benign tissue PIF-CP mRNA concentrations were correlated with each other ($P < 0.0001$, $r = 0.73$) but did not correlate with weight loss or prognosis. Although PIF-CP mRNA expression is upregulated in both tumour and adjacent normal tissue in gastro-oesophageal malignancy, expression does not relate to prognosis or cachexia. Post-translational modification of PIF may be a key step in determining the biological role of PIF in the patient with advanced cancer and cachexia.
British Journal of Cancer (2006) **94,** 731 – 736. doi:10.1038/sj.bjc.6602989 www.bjcancer.com
Published online 21 February 2006

Keywords: real-time PCR; cachexia; inflammation; prognosis

Although cachexia is a major cause of morbidity and mortality among cancer patients, the mechanisms remain unclear. Proteolysis-inducing factor (PIF) has been identified as a possible cachectic factor (Todorov *et al*, 1996). Initially isolated from a murine tumour model (MAC16), a human homologue of PIF was subsequently identified in human urine from weight losing cancer patients (Todorov *et al*, 1996; Cariuk *et al*, 1997; Wigmore *et al*, 2000). Proteolysis-inducing factor has been named as such because it has been shown to induce skeletal muscle proteolysis both *in vitro* and *in vivo* (Lorite *et al*, 1997, 2001; Watchorn *et al*, 2001). The molecule demonstrates a high degree of glycosylation, consisting of a peptide core of 4 kDa and carbohydrate residues contributing to the estimated total molecular size of 24 kDa (Todorov *et al*, 1997). The glycosylation appears essential for its proteolytic activities, as neither the peptide core alone nor de-glycosylated native PIF has any effect on weight loss in mice (Todorov *et al*, 1996).

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Received 14 October 2005; revised 12 January 2006; accepted 17 January 2006; published online 21 February 2006

In hepatocytes, glycosylated PIF has been shown to stimulate production of the cytokines interleukin-6 (IL-6) and IL-8 and the acute-phase protein C-reactive protein (CRP) via induction of transcription factors NF-κB and STAT3 (Watchorn *et al*, 2001). Therefore, PIF may contribute to the inflammatory state observed in conjunction with cancer cachexia in addition to its proteolytic function, and could conceivably also play a role in the generation of a proinflammatory state outwith the context of cancer.

Some gastrointestinal cancers and cancer cell lines have been shown to produce glycosylated PIF protein (Cabal-Manzano *et al*, 2001). Glycosylated PIF has been detected within tumour cells by immunohistochemistry and in the urine of the same subjects by Western blot. The presence of glycosylated PIF in tumour cells was also associated with increased weight loss among these patients. Proteolysis-inducing factor has also been identified from the human melanoma cell line (G361) (Todorov *et al*, 1999) and the pancreatic cell line (MIA PaCa2) (unpublished data).

Human-derived PIF has been designated human cachexia-associated protein (HCAP) by early identification of the human gene sequence (GenBank accession number AR053250) and patent (US 5834192) (Akerblom and Murry, 1998) and later by a group, which investigated its expression in prostate cancer patients (Wang *et al*, 2003). mRNA of HCAP was detected in 13 out of 15

radical prostatectomy specimens and seven out of nine bone metastases, but was not detectable in normal prostate or in any adjacent non-malignant prostate tissue from prostatectomy samples. *In situ* hybridisation confirmed that HCAP mRNA was only detectable in cancer cells and not in surrounding stromal cells or benign prostate tissue. Expression of HCAP mRNA was also detected from prostate cancer cell lines. When two of these cell lines (PC-3M and LuCaP35) were implanted into mice, there was a strong correlation between HCAP mRNA expression from the cancer cells and weight loss among the mice (Wang *et al*, 2003).

A BLAST search of the human genome using the PIF core peptide (PIF-CP) cDNA sequence (GenBank accession number AY590150) identified three products that arise from the single gene locus on 12q3.1; dermcidin (DCD), neuronal survival-promoting peptide, and a candidate breast cancer oncogene. Dermcidin is a novel peptide that was identified from human sweat and was shown to possess antimicrobial properties (Schittek *et al*, 2001). Unlike the PIF molecule, DCD appears to be unglycosylated. Dermcidin mRNA was detected in normal skin, benign naevi, and malignant melanoma cells by RT-PCR. Screening of adult and fetal tissue panels did not identify DCD mRNA in any other tissue. The authors therefore concluded that DCD was exclusive to skin and associated appendages.

Another PIF homologue has been identified following its isolation from the culture medium of neuronal cells grown under oxidative stress (Cunninghan *et al*, 1998). *In vivo* studies suggest that this protein confers survival benefits to hypoxic neuronal cells and the molecule has been termed neuronal diffusible survival-promoting peptide.

The third peptide, which has been mapped to the PIF gene locus, was identified using SAGE in breast cancer tissue (Porter *et al*, 2003). The gene was expressed only in a subset of invasive breast carcinomas and their lymph node metastases. *In situ* hybridisation studies located the mRNA to cancer cells only with no expression found within stromal cells. Northern blot analysis of 75 human adult and fetal tissues detected RNA expression only in the pons and paracentral gyrus of the brain. The protein was identified in 10% of invasive breast cancers, two out of 64 pancreatic cancers, and normal sweat glands. Receptors were found in the brain and on tumour cells that were producing the protein, suggesting an autocrine/paracrine mode of action. When the cDNA sequence was introduced into a breast cancer cell line (21NT), these cells had accelerated growth and were more resistant to oxidative stress and hypoglycaemia. The authors have suggested that this gene may function as an oncogene in breast cancer with survival-promoting properties.

More recently, a group of investigators probed a panel of normal human tissue cDNA for PIF-CP using real-time PCR and found absent or very low levels of expression (Monitto *et al*, 2004). In an analysis of unpaired breast tissue samples, minimal PIF expression in normal breast tissue was detected. However, significantly elevated PIF expression within breast tumours was observed. Mice implanted with tumours transfected with a PIF vector were found to produce increased levels of PIF mRNA and protein but the protein was not glycosylated and these mice did not develop wasting. However, the tumours from these mice were significantly larger than controls, supporting a previous hypothesis that PIF may confer cell survival properties (Cunningham *et al*, 1998).

PIF mRNA and protein are not expressed in most normal tissues and are overexpressed by certain tumours. We investigated the expression of the PIF-CP gene in paired tissue samples from patients with gastric or oesophageal tumours and compared levels of expression between tumour tissue and adjacent non-neoplastic tissue using the PIF mRNA sequence that our group had previously cloned (GenBank accession number AY590150). We also compared levels of gene expression with nutritional status and investigated the possible survival-promoting role of the PIF-CP gene on prognosis and survival among the patient cohort.

MATERIALS AND METHODS

Study patients

All patients diagnosed with gastric or oesophageal cancer between June 2002 and March 2004 within Lothian and Borders regions were invited to take part in the study. Patients who were not suitable for surgical resection were excluded. Patients provided written informed consent and the study received ethical permission from the Lothian Research Ethics Committee. Clinical information was collected prospectively. Patients were staged according to the International Union Against Cancer (UICC) (Sobin and Wittekind, 2003), and final histopathological stage (pTNM) was used in all cases. Tumours around the oesophagogastric junction were classified according to Siewert and those classified as type I and II were classified as oesophageal tumours, and type III as gastric cancers (Siewert and Stein, 1998). Full clinical and pathological information was recorded for each patient, including treatment modality. Patients provided urine samples at the time of recruitment and whole blood was collected at the same time. Duration of survival, defined as time from histological diagnosis to death, was recorded for all patients and all deaths were disease related.

Gastric or oesophageal biopsies were collected from 11 healthy volunteers at the time of endoscopic examination. These patients were undergoing endoscopic investigation of gastrointestinal symptoms. In all instances, both the macroscopic and microscopic assessments were considered normal. Those patients with abnormal findings were excluded as healthy controls. The tissue was sampled with biopsy forceps and the histological appearances were confirmed as normal by a consultant pathologist (HG).

Nutritional assessment

Nutritional assessment including calculation of body mass index from the patients' weight and height and measurement of mid-arm circumference (MAC) and triceps skinfold thickness (triceps) was performed as described previously (Wigmore *et al*, 2000). Mid-arm muscle circumference (AMC) was calculated by means of Jelliffe's (1966)equation. Premorbid patient weight was recalled by the patient and confirmed where possible from the medical notes. Anthropometry measurements were normalised using standardised reference tables (Bishop *et al*, 1981).

Tissue collection

Tissue was obtained from patients at the time of surgical resection. A section of tumour tissue and a sample of benign mucosa from the same organ were collected from each patient. A single consultant pathologist (HG) analysed tissue sections to confirm the presence of malignant cells in the tumour samples and the absence of malignant cells within the benign samples. Tissue samples were snap-frozen in liquid nitrogen before storage at −80°C until further analysis. The average time from tissue collection to freezing was 15 min (range 10–25 min). Tumour tissue and benign tissue were collected from the same patient in all cases. Tissue biopsies collected from healthy controls were snap-frozen within 1 min of collection.

Quantitative polymerase chain reaction (Q-RT-PCR)

RNA isolation and reverse transcription Total RNA was isolated from tissue samples using the RNAeasy kit (Qiagen Inc., Crawley, UK). RNA quality and integrity was assessed using an Agilent 2100 bioanalyser (Agilent Technologies Ltd, Chesire, UK) in five samples. For the remaining samples, purity and concentration were determined using spectrophotometry (Ultrospec 2000, Pharmacia Biotech, Bucks, UK). RNA samples were treated with

DNase (Qiagen Inc., UK) and all RNA samples were checked for genomic DNA contamination before reverse transcription using standard PCR for cytochrome *b*.

Reverse transcription was performed once DNA contamination had been excluded. The reaction mixture included RNA (1 μg in 10 μl diethyl-pyrocarbonate (DEPC)-treated water), 4 μl $MgCl_2$ (25 mM), 2 μl 10 × reverse transcriptase buffer, 2 μl dNTPs (10 mM), 1 μl random hexamers (500 $\mu g\,ml^{-1}$), 1.5 μl AMV reverse transcriptase (10 $U\,\mu l^{-1}$), and 0.5 μl recombinant RNase inhibitor (40 $U\,\mu l^{-1}$) (all reagents Promega, Southampton, UK). Reverse transcription was performed at 42°C for 60 min followed by 95°C for 5 min.

Real-time PCR Quantitative PCR was performed using the ABI PRISM 770 real-time Sequence Detection System (Applied Biosystems, Warrington, UK). Reactions were performed in 50 μl total volume, consisting of 25 μl Taqman universal PCR master-mix (UNG × 2), 14 μl primer/probe mix, 2.5 μl ribosomal 18S primer/probe mix (all reagents Applied Biosystems, Warrington, UK), 3.5 μl DEPC-treated water, and 5 μl cDNA. The reaction conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15 s at 95°C and 1 min at 60°C. The primers and probes were designed using Primer Express® software based on our previously registered sequence for PIF (GenBank accession number AY590150) and the sequences were checked for compatibility by Applied Biosystems (Warrington, UK). The primers were 5′-CAAAAGGAAAATGCAGGTGAAGA, 5′-TGGAAAAAGGCCTAGA CGGAG, and the probe 5′FAM-ACAGGCACCAAAGCCAAGGAAG CA-TAMRA.

Quantification of gene expression was calculated using the comparative ($\Delta\Delta C_T$) method, where samples were compared with the positive control (Bustin, 2000). Human ribosomal 18S was used as the internal control for all PCR reactions. The values generated for the gene of interest (PIF-CP) were normalised to the internal control as follows: the average threshold cycle numbers for PIF (C_T FAM) and for 18S (C_T VIC) were calculated for each sample and the mean VIC C_T values were subtracted from the mean FAM C_T values (FAM C_T − VIC $C_T = \Delta C_T$). Samples that generated cycle numbers greater than 23 for the internal control (18S; VIC) were considered too dilute for accurate analysis and these samples were repeated.

The level of gene expression within each sample was then expressed as a percentage of the total level of gene expression by the positive control (MIA PaCa2). Expression of PIF-CP by the positive control was also initially normalised to the internal control. Then, $\Delta\Delta C_T$ was calculated by subtracting the ΔC_T value of the positive control sample from the ΔC_T value for each sample (i.e. ΔC_T sample − ΔC_T control = $\Delta\Delta C_T$). The relative level of expression, normalised to the endogenous control and relative to the positive control, was then calculated by the formula $2^{-\Delta\Delta C_T}$. The level of gene expression of the positive control was assigned an arbitrary expression value of 1 and levels of gene expression in the samples were expressed as percentages of the positive control level.

Each sample was analysed in duplicate. The variability between duplicates was between 0 and 4.8%. To investigate inter-plate variability, between five and 10 randomly selected samples were repeated on different test plates and serial dilutions of the positive control cDNA were also performed. Inter-plate reproducibility was between 3.7 and 15.5%.

Positive control Previous work undertaken by our group has identified the pancreatic cancer cell line MIA PaCa2 to consistently produce PIF-CP mRNA. This cell line was, therefore, used as positive control for real-time PCR analysis. Total RNA was collected from cultured cells by the Trizol extraction method (Invitrogen, Renfrew, UK) before undergoing reverse transcription as described above.

Acute-phase proteins

C-reactive protein was determined using an immunoturbidimetric assay (Abbott TDX, Abbott Laboratories, Maidenhead, UK). A level above 10 $mg\,l^{-1}$ represents the presence of an acute-phase response. The remaining acute-phase proteins (transferrin, haptoglobin, and α1-antichymotrypsin) were determined by enzyme-linked immunosorbent assay as described previously (Wigmore *et al*, 2002).

Statistical analysis

Differences in levels of PIF-CP mRNA expression between different tissue types and PIF urinary expression were analysed by the Mann–Whitney *U*-test. Survival differences were tested by the log-rank test. Comparisons between tumour tissue PIF-CP gene expression and benign tissue expression were tested by linear regression analysis following natural logarithmic transformation of the data. Stage was analysed by the χ^2 test. Probabilities $\leqslant 0.05$ were considered significant.

RESULTS

Study patients

Patient demographics are shown in Table 1. Forty-six patients were recruited to the study, the median age was 65 years (inter-quartile range 58–75) and 32 (70%) of the patients were men. The primary tumour sites were oesophageal ($n = 22$, 48%), gastric ($n = 15$, 33%), and those arising from the gastro-oesophageal junction

Table 1 Patient demographics ($n = 46$)

	Number (%)
Age (years)	65 (58–75)
Sex	
Male	32 (70)
Female	14 (30)
Tumour site	
Oesophageal	22 (48)
Oesophago-gastric junction	9 (20)
Gastric	15 (33)
Histology	
Adenocarcinoma	43 (94)
Squamous cell carcinoma	3 (6)
Grade	
Well differentiated	4 (9)
Moderately differentiated	20 (44)
Poorly differentiated	22 (48)
UICC stage	
1	14 (30)
2	12 (26)
3	15 (33)
4	5 (11)
Treatment undertaken	
Oesophagectomy alone	20 (43)
Gastrectomy alone	15 (33)
Preoperative chemotherapy followed by surgery	11 (24)
Status	
Alive	35 (76)
Dead	11 (24)

Values are median (inter-quartile range). UICC = International Union Against Cancer.

($n=9$, 20%). Histological confirmation of disease was obtained in all cases and the predominant histological subtype was adenocarcinoma (94%). All patients underwent surgical resection and 11 (24%) of these received preoperative chemotherapy. At the end of the study, 11 (24%) patients had died.

All the control patients were considered healthy and all were weight stable. These patients underwent endoscopy as an elective investigation and in all instances the result of the procedure was normal, including both macroscopic and microscopic assessment. Helicobacter pylori was not detected in any of these tissue samples.

Expression of PIF-CP mRNA in tissues

Healthy controls mRNA of PIF-CP was detected in gastro-oesophageal biopsy tissue from four (36%) healthy controls. Of these positive tissue samples, three were gastric biopsies, and one was oesophageal tissue. Although PIF-CP mRNA levels were detectable, the levels of expression were significantly lower when compared with levels of expression in tumour tissue ($P=0.031$, Mann–Whitney *U*-test) and adjacent benign tissue ($P=0.022$) taken from cancer patients (Figure 1).

Study patients PIF-CP mRNA was detected in 27 (59%) tumour samples and 31 (67%) adjacent benign tissue samples. In 24 (52%) patients, PIF mRNA was detected in both the tumour tissue and adjacent benign tissue collected from the same patient (Table 2). There was a strong correlation between paired mRNA concentrations in tumour tissue and mRNA concentrations in adjacent benign tissue ($P<0.0001$, $r=0.73$; linear regression) (Figure 2). However, there was no difference in the level of gene expression between tumour tissue and adjacent benign tissue from the cancer patients ($P=0.51$, Mann–Whitney *U*-test) (Figure 1). In 12 (26%) patients, PIF-CP mRNA was not detected in either the tumour or adjacent benign tissue. For the remaining 10 patients, PIF-CP mRNA was detected in either tumour tissue only ($n=3$) or adjacent benign tissue only ($n=7$). There was no difference in the level of gene expression between patients who received preoperative chemotherapy and those who did not (tumour tissue, $P=0.58$; adjacent benign tissue, $P=0.72$: Mann–Whitney *U*-test).

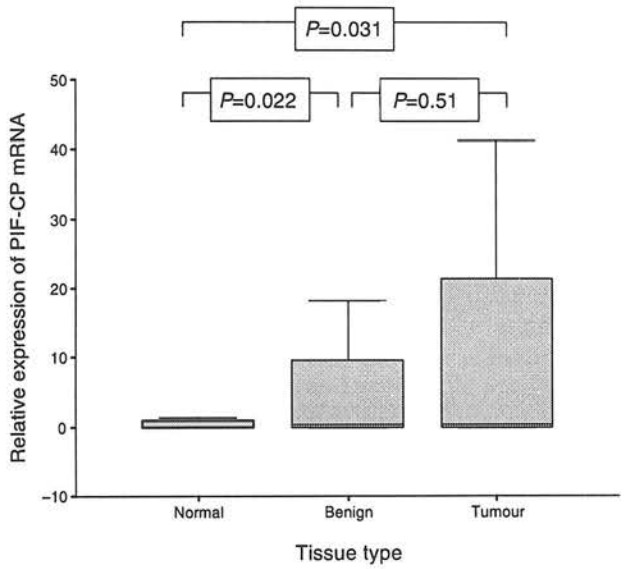

Figure 1 A comparison of relative expression of PIF-CP mRNA in tissue from healthy controls (normal), tumour tissue, and benign tissue collected from cancer patients (benign). The lines represent the median value, bars = inter-quartile range, error bars = extreme values (Mann–Whitney *U*-test).

Table 2 Tissue expression of PIF-CP mRNA in paired tumour tissue and benign tissue

Paired tumour tissue and benign tissue expression	Benign tissue expression only
24 (52%)	7 (15%)
Tumour tissue expression only	Neither tumour tissue nor benign tissue expression
3 (7%)	12 (26%)

PIF-CP = proteolysis-inducing factor core peptide.

Tissue PIF-CP mRNA expression and nutritional status

Tumour tissue PIF-CP mRNA did not correlate with weight loss ($P=0.37$; linear regression), MAC ($P=0.10$), triceps skinfold thickness ($P=0.37$), or AMC ($P=0.14$). Similarly, benign tissue mRNA concentrations did not correlate with weight loss ($P=0.84$) or any anthropometric measurements. Patients in whom PIF-CP mRNA was measurable in both tumour and adjacent benign tissues did not have adverse nutritional status when compared with patients without detectable PIF-CP mRNA in either tissue type (Table 3). There remained no correlation when the data were analysed according to dysphagia scores (data not shown).

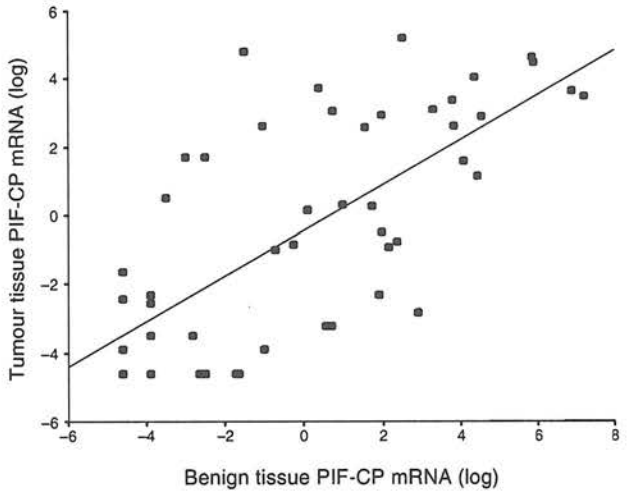

Figure 2 Correlation between paired tumour tissue PIF-CP mRNA concentrations and benign tissue PIF-CP mRNA concentrations ($P<0.0001$, $r=0.73$; linear regression). All values underwent natural logarithmic transformation.

Table 3 Tissue PIF-CP mRNA expression and nutritional variables

	PIF-CP mRNA detected in both tumour tissue and benign tissue ($n=24$)	PIF-CP mRNA not detectable in either tumour tissue or benign tissue ($n=12$)	*P* value
Weight loss (%)	3.3 (0.4–9.5)	5.3 (0–12.2)	0.93
MAC (percentile group)	10–25 (5–50)	10–25 (5–50)	0.96
Triceps (percentile group)	25–50 (10–50)	25–50 (25–50)	0.37
AMC (percentile group)	10–25 (5–50)	25–50 (5–50)	0.96

Nutritional variables were similar between patients in whom PIF-CP mRNA was measurable in both tumour tissue and benign tissue and patients who had no detectable mRNA (Mann–Whitney *U*-test). Absolute values were normalised into percentile groups before analysis. Values are median (inter-quartile range). PIF-CP = proteolysis-inducing factor core peptide, MAC = mid-arm circumference, triceps = triceps skinfold thickness, AMC = arm muscle circumference.

Tissue PIF-CP mRNA expression and systemic inflammation

Nine (20%) patients had an elevated serum CRP ($>10\,mg\,l^{-1}$), which may be used as a surrogate marker of systemic inflammation. There was no association between levels of PIF-CP mRNA in either tumour tissue ($P=0.89$) or adjacent benign tissue ($P=0.81$) and elevated serum acute-phase protein concentrations (Mann–Whitney *U*-test).

Tissue PIF-CP mRNA expression and prognosis

PIF-CP mRNA in either tumour tissue ($P=0.64$) or benign tissue ($P=0.51$) was not associated with adverse prognosis (Mann–Whitney *U*-test). Similarly, those patients with detectable mRNA in both tumour and benign tissue did not have adverse survival compared with patients with no detectable PIF-CP gene in either tissue type ($P=0.79$; χ^2 analysis).

We found no association between mRNA levels and age, sex, tumour position, histology, degree of differentiation, or stage (data not shown).

DISCUSSION

Previous studies have suggested that PIF-CP mRNA is absent or expressed at only minimal levels in normal human tissues and found at significantly elevated levels in some tumours and various cancer cell lines. However, we have detected PIF-CP mRNA in benign tissue in the majority of patients with gastro-oesophageal cancer, and these levels are equivalent to levels detected within adjacent tumour tissues. In addition, we detected PIF-CP mRNA in biopsy tissue from healthy controls, although at significantly lower levels of expression. In a previous study, PIF-CP (HCAP) mRNA appeared to be limited to cancer cells and their metastases (Wang *et al*, 2003). Our data suggest that PIF-CP gene expression is not limited to neoplastic tissue and that the PIF-CP gene may also be active in normal tissues. Furthermore, the level of gene expression appears to be similar in tumour tissue and adjacent benign tissue. Therefore, in some individuals with cancer, there may be upregulation of the PIF-CP gene not only in tumour tissue but also within the whole organ or even in distant tissues.

The relevance of PIF-CP gene upregulation remains obscure. Although PIF-CP has been identified as a putative cell survival factor we found no association between gene expression and prognosis, stage, or grade of tumour. Therefore, the present study is unable to confirm a tumour survival-promoting role for this gene in our study. Mice implanted with a tumour carrying the PIF-CP gene demonstrated increased rate of tumour growth compared with controls (Monitto *et al*, 2004). The present study did not evaluate tumour volume, but we did not identify any differences in tumour stage or grade between patients with elevated mRNA levels and those with undetectable mRNA levels.

Tissue PIF-CP mRNA expression was not associated with weight loss or any adverse nutritional variable. Previously, we have found an association between weight loss and urinary PIF (Todorov *et al*, 1996; Cariuk *et al*, 1997; Wigmore *et al*, 2000). In those studies, we detected the glycosylated PIF protein, whereas in this study, we have measured mRNA for the core peptide and so this finding is perhaps unsurprising. Only a small fraction of the total peptide core would be expected to be glycosylated, and as PIF is only present at 1 part in 10^8 of the total protein, expression of the peptide core would not be expected to be rate limiting for PIF formation, rather the expression of the glycosidases, their rate of catalysis, and substrate availability (Todorov *et al*, 1996). Whether variation in glycosylation of PIF occurs and whether this influences biological activity is unknown. Antibodies raised against the core peptide do not reliably detect urinary PIF.

Systemic inflammation was identified in 20% of patients and was not associated with PIF core tissue mRNA concentrations. *In vitro* work has demonstrated that hepatocytes produce increased levels of IL-6, IL-8, and CRP in response to PIF stimulation (Watchorn *et al*, 2001). This is thought to be mediated through transcription factors NF-κB and STAT3. Proteolysis-inducing factor may play only a minor role in the complex interaction between proinflammatory mediators and a direct relationship between PIF and acute-phase proteins may not be identified outwith the *in vitro* cell model.

It has been suggested that PIF is an important protein during embryological development and becomes quiescent during adult life (Watchorn *et al*, 2001). Neoplastic transformation would then allow the re-expression of the gene in adulthood (similar to carcinoembryonic antigen). We have found PIF-CP mRNA expression in normal healthy tissue albeit at reduced levels compared with tumour tissue and benign tissue from cancer patients and so this explanation is perhaps implausible for PIF-CP but may be important in re-expression of the glycosyltransferases. It is also important to consider that real-time PCR is an exquisitely sensitive technique and that what we are detecting in some patients, although elevated, may have little or no functional significance as it may not be translated into protein.

It is possible that the gene for the PIF-CP may confer a survival advantage to a tumour, promoting tumour growth and spread, although this study was unable to confirm this. Alternatively, the PIF-CP gene may be expressed to some degree in all tissue types and its diverse functions are due to post-transcriptional and post-translational modifications. The high degree of glycosylation of the PIF molecule may be due to aberrant glycosylation by tumour cells and this glycosylation may confer the proteolytic activity associated with PIF (Todorov *et al*, 1997). This aberrantly processed molecule could not be distinguished from any conventional versions by determination of the mRNA expression.

PIF-CP mRNA is expressed in healthy tissue and is found at significantly elevated levels within tumour tissue and adjacent benign tissue. However, measuring mRNA expression is unreliable as an indicator of glycosylated PIF protein expression and therefore has limited value both as a diagnostic and prognostic test. Attention needs to be directed at developing robust and reproducible quantitative assessments of glycosylated PIF protein. Such tests need to be able to identify PIF in complex matrices such as plasma or urine and must be able to identify glycosylation variants of the moiety. Similarly, such tests need to distinguish between different proteins carrying identical glycosylation motifs.

ACKNOWLEDGEMENTS

Mr Simon Paterson-Brown and Mr Andrew de Beaux, Consultant Surgeons, Edinburgh Royal Infirmary, were essential for the recruitment of patients and provided the tissue used in this study.

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Original article

Clinical prognostic scoring system to aid decision-making in gastro-oesophageal cancer

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Background: Accurate prediction of prognosis in gastro-oesophageal cancer remains challenging. The aim of this study was to develop a robust model for outcome prediction.

Methods: The study included 220 patients with gastric or oesophageal cancer newly diagnosed over a 2-year period. Patients were staged and underwent treatment following discussion at a multidisciplinary team (MDT) meeting. Clinical and investigative variables were collected, including performance and nutritional status, and serum C-reactive protein (CRP) level. Primary endpoints were death within 12 and 24 months.

Results: Overall median survival was 13 months. Advanced clinical stage ($P < 0{\cdot}001$), reduced performance score ($P < 0{\cdot}001$), weight loss exceeding 2·75 per cent per month ($P = 0{\cdot}026$) and serum CRP concentration above 5 mg/l ($P = 0{\cdot}031$) were identified as independent prognostic indicators in multivariable analysis. A prognostic score was constructed using these four variables to estimate a probability of death. Applying the model gave an area under the receiver–operator characteristic curve of 0·84 and 0·85 for prediction of death at 12 and 24 months respectively (both $P < 0{\cdot}001$).

Conclusion: This model accurately estimated the probability of death within 12 and 24 months. This may aid the MDT decision-making process.

Paper accepted 30 July 2007
Published online 17 August 2007 in Wiley InterScience (www.bjs.co.uk). **DOI:** 10.1002/bjs.5849

Introduction

The incidence of tumours arising around the oesophagogastric junction (OGJ) continues to rise, and gastric and oesophageal cancers together are the third leading cause of cancer-related death in the UK[1–3]. Overall survival figures remain disappointing, with many patients in the UK presenting with locally advanced tumours and very few with early-stage disease. The only real prospect of cure lies in surgical resection. This still carries significant risks in terms of morbidity and mortality with or without preoperative chemo/radiotherapy.

Despite advances in staging and treatment, the 5-year survival rate following resection rarely exceeds 30 per cent and is less than 10 per cent in patients with locally advanced disease (stages IIb and III)[4,5]. Indeed in this group, which represents approximately two-thirds of all patients suitable for curative resection, around 50 per cent do not survive more than 18 months. One study has shown that patients who fail to live for 2 years after surgery do not regain preoperative quality of life levels. Only those who survive beyond this time enjoy a return to their former state of well-being[6].

The problem lies in identifying such patients prospectively. Improvements in preoperative staging techniques, including spiral computed tomography (CT), endoscopic ultrasonography (EUS) and laparoscopy, are likely to help, but additional factors that can be used to refine prognosis are required. If clinicians could identify those unlikely to live more than 12–18 months following radical resection, such patients could be offered non-surgical palliative therapies.

Management decisions are currently undertaken as part of a multidisciplinary team (MDT) process. An objective measure of prognosis would therefore not only improve stratification of patients, but also provide a measure of quality assurance for the MDT. The aims of this study were to determine the clinical and investigative factors predictive of death from gastro-oesophageal cancer within 12 and 24 months of diagnosis in order to construct a risk

prediction model to assist in the prospective prognostic evaluation of patients with gastro-oesophageal malignancy.

Methods

All patients diagnosed with gastric or oesophageal cancer within the Lothian and Borders regions between March 2002 and June 2004 were eligible for inclusion in the study. Patients were recruited within 2 weeks of diagnosis and all subjects provided written informed consent. The study received permission from the Lothian Research Ethics Committee. No patients was excluded or refused to give consent. All patients had histological confirmation of cancer following endoscopic biopsy. Data were collected prospectively.

Patients were staged with a combination of CT, EUS and laparoscopy/laparoscopic ultrasonography according to the International Union Against Cancer (UICC) system[7]. Tumours located around the OGJ were classified according to Siewert and Stein[8]; type I and II tumours were staged as oesophageal cancers and type III as gastric cancers. In all instances a final clinical stage (clinical tumour node metastasis; cTNM) was agreed at the unit MDT meeting and management strategies were decided. Patients with no evidence of metastatic disease (stages I–III) and who were medically fit were offered surgical resection. Those staged as T3 N0 or T2/3 N1 were considered for preoperative chemotherapy. The remaining patients received various palliative treatments, either alone or in combination. Survival time, defined as the interval from histological confirmation of disease until death, was recorded for all patients.

Performance status was assessed at the time of diagnosis by a single researcher using the Karnofsky scoring system[9]. All patients underwent an assessment of nutritional status at the time of diagnosis. Body mass index (BMI) was calculated following height and weight measurements. Weight before illness was recalled by the patient and confirmed from medical records where possible. Total bodyweight loss was calculated and expressed as a percentage of stable weight before illness. The rate of weight loss was calculated by dividing the total weight lost by the number of months over which symptoms had been experienced. Anthropometry in each patient included measurement of mid-arm circumference and triceps skinfold thickness. Mid-arm muscle circumference was calculated by means of Jelliffe's equation[10]. Measurements were corrected for age and sex, and normalized using standardized reference tables[11]. The severity of dysphagia was scored according to Knyrim and colleagues[12]. Dietary intake was estimated by all patients and described as normal, reduced compared with normal, or poor/minimal.

Blood was collected at the time of diagnosis and before initiation of treatment. C-reactive protein (CRP) was used as a surrogate marker of systemic inflammation[13]. Serum CRP concentrations were measured using an automated immunoturbidimetric assay; a concentration of 10 mg/l represented the upper limit of the normal range.

Variables that could not be used prospectively to aid management, such as number of lymph nodes with metastatic involvement and final histopathological stage, were excluded from prognostic modelling.

Statistical analysis

The Cox proportional hazards model was used to determine the effects of clinical and investigative variables on survival time, and probability of death within 12 and 24 months following diagnosis in both univariable and multivariable analysis. Stepwise regression (using an entry value of $P \leq 0{\cdot}050$ and a removal value of $P \geq 0{\cdot}100$) was used to determine the factors predictive of death. Models were developed separately for clinical and investigative variables, and then for a combination of the two. Receiver–operator characteristic (ROC) curves were used to select cut-off values for continuous variables. Values with the best combination of sensitivity and specificity were chosen. The performance of the prognostic model was assessed by determining the area under the ROC curve. The estimates of probability of death at 12 and 24 months were calculated from survival tables.

Results

Details of the 220 patients recruited between March 2002 and June 2004 are shown in *Table 1*. Approximately two-thirds (68·6 per cent) of the cancers occurred at or close to the OGJ. Histological confirmation of disease was obtained in all cases and 70·3 per cent of oesophageal tumours were adenocarcinomas. Only three patients had well differentiated tumours and almost two-thirds (64·1 per cent) had poorly differentiated cancer. Seventy-five patients (34·1 per cent) had metastatic disease (stage IV) at the time of presentation and most others had locally advanced (stage III) disease. Only one-quarter of patients had early disease (stage I/II).

Ninety-five patients (43·2 per cent) underwent surgical resection. In 90 patients the resection was 'curative' (R0 resection). Three patients had unexpected metastatic disease identified at laparotomy and did not proceed to resection, and two patients had residual macroscopic

Table 1 Patient demographics

	No. of patients ($n = 220$)*
Age (years)‡	71 (62–78)
Sex	
M	145 (65·9)
F	75 (34·1)
Karnofsky score†	
30	4 (1·9)
40	1 (0·5)
50	5 (2·4)
60	17 (8·2)
70	25 (12·0)
80	36 (17·3)
90	50 (24·0)
100	70 (33·7)
Unknown	12
Tumour site	
Oesophagus	101 (45·9)
Proximal third	2
Middle third	13
Distal third	86
Oesophagogastric junction	40 (18·2)
Stomach	79 (35·9)
Proximal	25
Body	26
Distal	28
Histology	
Adenocarcinoma	185 (84·1)
Squamous cell carcinoma	30 (13·6)
Small cell	2 (0·9)
Indeterminate	3 (1·4)
Tumour grade†	
Well differentiated	3 (1·6)
Moderately differentiated	63 (34·2)
Poorly differentiated	118 (64·1)
Unknown	36
Treatment	
Surgery alone	70 (31·8)
Preoperative chemotherapy/surgery	25 (11·4)
Chemoradiotherapy with curative intent	7 (3·2)
Palliative chemotherapy	28 (12·7)
Palliative radiotherapy	6 (2·7)
Stent/dilatation/laser/symptomatic	84 (38·2)
UICC stage	
I	25 (11·4)
II	34 (15·5)
III	86 (39·0)
IV	75 (34·1)
Status	
Alive	73 (33·2)
Dead	147 (66·8)

*Values are number of patients with percentages in parentheses unless indicated otherwise; †percentages based on number of patients with known results; ‡values are median (interquartile range). UICC, International Union Against Cancer.

disease (R2 resection). Twenty-five of the 95 patients who underwent surgery received preoperative chemotherapy. Oesophageal and OGJ tumours were treated by Ivor Lewis oesophagogastrectomy (46 patients) or thoracoabdominal resection (16). Distal gastric tumours were resected by either partial (16) or total (17) gastrectomy. Seven patients (3·2 per cent) with squamous cell carcinoma of the oesophagus received only chemoradiotherapy with curative intent. The remaining 118 patients (53·6 per cent) were deemed unsuitable for curative therapy and received palliative chemo/radiotherapy or alternative palliative treatments, including endoscopic stenting, dilatation or laser therapy.

Median follow-up was 32 (range 18–45) months. At the time of censoring, 147 patients (66·8 per cent) had died. Three patients died in the postoperative period and one from injuries sustained following a fall several months after discharge. Information obtained from death certificates indicated that all other deaths were disease related. One hundred and eight patients (49·1 per cent) died within 12 months of diagnosis and 136 (61·8 per cent) died within 24 months. The median survival for patients who underwent surgical resection was 30 months and that for patients treated with palliative intent was 7 months. Overall median survival was 13 months.

Seventy patients (33·7 per cent) had a normal performance status at the time of diagnosis (Karnofsky score 100) and 86 (41·3 per cent) were able to undertake normal activities but with some degree of effort (Karnofsky 80 or 90). Forty-two patients (20·2 per cent) were unable to carry out normal activity or work and required occasional assistance with care (Karnofsky 60 or 70), and ten patients (4·8 per cent) required considerable assistance and frequent medical care or admission to hospital (Karnofsky 30–50) (*Table 1*).

Median total bodyweight loss by the time of diagnosis was 7·1 per cent, equivalent to a median rate of weight loss of 2·5 per cent total bodyweight per month of illness (*Table 2*). Median BMI fell from 26·4 to 24·6 (mean reduction 2·4 (95 per cent confidence interval 2·0 to 2·7) kg/m^2; $P < 0·001$, paired t test). Only 38 patients (17·3 per cent) remained weight steady at the time of diagnosis, whereas 97 (44·1 per cent) had lost up to 10 per cent of their total bodyweight and 85 (38·6 per cent) had lost more than 10 per cent. Anthropometric values for mid-arm circumference, triceps skinfold and arm muscle circumference, dietary intake and dysphagia scores are summarized in *Table 2*. Of the 166 patients with oesophageal, junctional or proximal gastric tumours, 75·3 per cent had some degree of dysphagia.

The median serum CRP concentration was 7 (interquartile range 2–25) mg/l. One hundred and twenty-one patients (55·0 per cent) had a CRP level greater than 5 mg/l at the time of diagnosis.

Table 2 Nutritional variables measured at the time of diagnosis

	No. of patients ($n = 220$)*
Body mass index (kg/m^2)†	
Before illness	26·4 (24·1–30·1)
At diagnosis	24·6 (21·4–28·0)
Total bodyweight loss (%)†	7·1 (1·2–14·2)
Rate of weight loss (% per month)†	2·5 (0·3–6·5)
Mid-arm circumference (percentile group)†	10 (1–25)
Triceps skinfold thickness (percentile group)†	25 (10–50)
Arm muscle circumference (percentile group)†	10 (1–25)
Dietary intake	
Normal	85 (38·6)
Reduced	103 (46·8)
Poor or minimal	32 (14·6)
Dysphagia score	
0	89 (40·5)
1	52 (23·6)
2	43 (19·5)
3	32 (14·5)
4	4 (1·8)

*Values are number of patients with percentages in parentheses unless indicated otherwise; †values are median (interquartile range).

Table 3 Hazard ratio for risk of death associated with baseline clinical characteristics and results of investigations for the whole patient group with gastro-oesophageal cancer

	Hazard ratio	P*
Age (years)	1·02 (1·00, 1·04)	0·015
Body mass index at diagnosis (kg/m^2)	0·95 (0·92, 0·99)	0·005
Ln total weight loss at diagnosis (%)	1·44 (1·20, 1·73)	< 0·001
Ln rate of weight loss (% per month)	1·43 (1·20, 1·71)	< 0·001
Dysphagia score	1·28 (1·10, 1·49)	0·002
Dietary intake	1·86 (1·38, 2·31)	< 0·001
Karnofsky score	0·96 (0·95, 0·97)	< 0·001
Ln C-reactive protein (mg/l)	1·40 (1·25, 1·56)	< 0·001
Tumour grade	1·66 (1·14, 2·51)	0·009
Stage of disease (cTNM)	2·60 (2·01, 3·29)	< 0·001

Values in parentheses are 95 per cent confidence intervals. Ln, natural log; cTNM, clinical tumour node metastasis. *Cox univariable analysis.

Table 3 shows the risk of death from gastro-oesophageal cancer associated with baseline clinical characteristics and results of investigations. Age, BMI at the time of diagnosis, total body weight loss at diagnosis, rate of weight loss, Karnofsky performance score, serum CRP concentration, dysphagia score, level of dietary intake, tumour grade and clinical stage were all associated with outcome for the whole patient group.

On the basis of ROC curves, cut-off values with the best discriminatory power for each continuous variable were: age at least 70 years, BMI less than or equal to 25 kg/m^2, weight loss at least 6 per cent, rate of weight loss greater than 2·75 per cent per month, Karnofsky score 80 or less, and CRP concentration greater than 5 mg/l. The risk of death for the cut-off point selected for each variable is shown in *Table 4*.

Because of the large number of prognostic variables, clinical and investigative variables were initially included in separate multivariable analyses. Rate of weight loss and Karnofsky score were identified as independent prognostic clinical variables, and serum CRP concentration and clinical stage as independent investigative variables. These four variables were then analysed together and each retained independent prognostic value (*Table 5*).

A prognostic scoring system was devised by logarithmically transforming the hazard ratio for each of these variables and multiplying by 100 (Reference[14]). For each patient a cumulative risk score could be calculated (maximum score 202) (*Table 6*). The prognostic risk score could then be used to estimate the probability of death within 12 and 24 months (*Fig. 1*). Applying the model gave an area under the ROC curve of 0·84 and 0·85 for prediction of death at 12 and 24 months respectively (both $P < 0·001$).

Table 4 Hazard ratio for risk of death for each new cut-off point chosen following discrimination analysis by use of receiver–operator characteristic curves

	Hazard ratio	P*
Age (≥ 70 years)	1·60 (1·15, 2·24)	0·005
Body mass index (≤ 25 kg/m^2)	1·53 (1·04, 2·02)	0·028
Weight loss (≥ 6%)	2·54 (1·79, 3·60)	< 0·001
Rate of weight loss (> 2·75% per month)	2·64 (1·86, 3·75)	< 0·001
Dysphagia score		< 0·001
1 *versus* no dysphagia	0·68 (0·42, 1·11)	
2 *versus* no dysphagia	1·48 (0·95, 2·30)	
3 *versus* no dysphagia	1·68 (1·03, 2·75)	
4 *versus* no dysphagia	28·88 (8·19, 101·84)	
Dietary intake		< 0·001
Reduced *versus* normal	1·59 (1·08, 2·34)	
Poor *versus* normal	3·64 (2·21, 6·00)	
Karnofsky score		< 0·001
80–90 *versus* normal	1·49 (0·96, 2·29)	
60–70 *versus* normal	4·37 (2·73, 6·99)	
< 60 *versus* normal	9·23 (4·53, 18·83)	
C-reactive protein (> 5 mg/l)	2·58 (1·83, 3·64)	< 0·001
Clinical stage of disease (cTNM)		< 0·001
Stage II *versus* stage I	1·87 (0·63, 5·57)	
Stage III *versus* stage I	3·04 (1·11, 8·35)	
Stage IV *versus* stage I	11·16 (4·02, 30·96)	
Tumour grade		
Poorly differentiated *versus* moderate	1·66 (1·12, 2·46)	0·012

Values in parentheses are 95 per cent confidence intervals. cTNM, clinical tumour node metastasis. *Cox univariable analysis.

Table 5 Clinical and investigative variables independently predictive of death, determined by using stepwise selection procedures in a general population of patients with gastro-oesophageal cancer

	Hazard ratio	*P**
C-reactive protein (> 5 mg/l)	1·58 (1·04, 2·34)	0·031
Rate of weight loss (> 2·75% per month)	1·61 (1·05, 2·28)	0·026
Karnofsky score		
80–90 *versus* normal	1·01 (0·64, 1·56)	0·996
60–70 *versus* normal	2·08 (1·30, 3·54)	0·003
< 60 *versus* normal	4·83 (2·23, 10·16)	< 0·001
Clinical stage of disease (cTNM)		
Stage II *versus* stage I	2·02 (0·57, 6·96)	0·277
Stage III *versus* stage I	2·88 (0·89, 9·36)	0·076
Stage IV *versus* stage I	8·83 (2·68, 28·60)	< 0·001

Values in parentheses are 95 per cent confidence intervals. cTNM, clinical tumour node metastasis. *Forward conditional method of Cox proportional hazards model.

Table 6 Risk score based on four prognostic variables

	Risk score*
C-reactive protein (mg/l)	
≤ 5	0
> 5	20
Rate of weight loss (% per month)	
≤ 2·75	0
> 2·75	20
Karnofsky score	
80–100	0
60–70	32
< 60	68
Clinical stage of disease (cTNM)	
I	0
II	30
III	46
IV	94

*Log (hazard ratio) multiplied by 100. cTNM, clinical tumour node metastasis.

Applying the risk score to the cancer subsites gave areas under the ROC curves for survival at 12 and 24 months of 0·84 and 0·89 for oesophageal cancer, 0·86 and 0·89 for gastric cancer, and 0·78 and 0·67 for tumours at the OGJ. The score was valid for adenocarcinoma at 12 and 24 months (area under ROC curve 0·86 and 0·87) and squamous cell carcinoma (0·78 and 0·68). Analysis by treatment type gave areas under the curve of 0·78 and 0·79 for survival at 12 and 24 months in patients who underwent surgery with curative intent, and 0·77 and 0·79 for those treated by palliative methods alone.

Discussion

This study identified clinical disease stage, Karnofsky performance score, rate of weight loss and serum CRP concentration as independent prognostic indicators for patients with gastro-oesophageal cancer. The development of a risk prediction score based on these four variables allows improved prognostic accuracy and risk prediction of death within 12 and 24 months after diagnosis.

Despite advances in clinical staging accuracy, patients are still not stratified adequately, particularly those with disease involving the OGJ[15]. When compared with

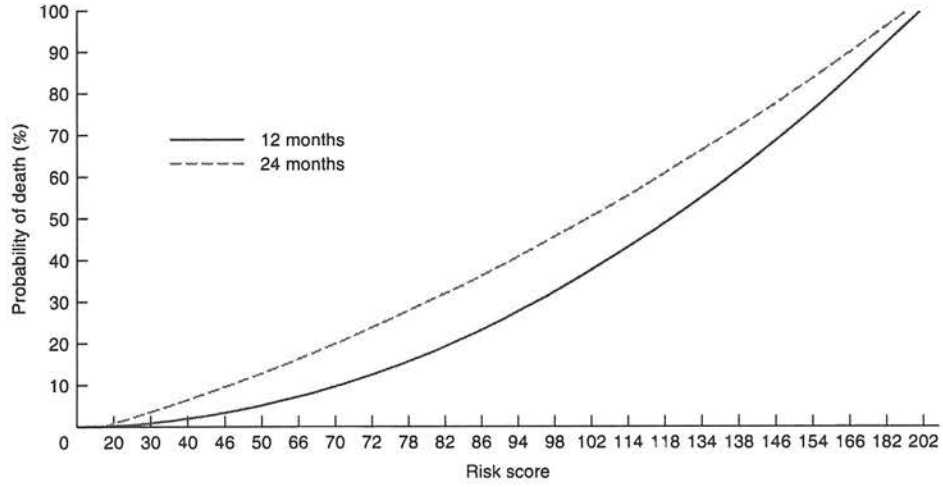

Fig. 1 Plot with which to assign estimated probability of death within 12 or 24 months after diagnosis according to prognostic risk score. Values along the horizontal axis represent the total risk score attainable through application of the individual risk scores outlined in *Table 6*. Total scores are not linear as patients' scores may not fall between these values

final histopathological stage, CT has an accuracy of 68–86 per cent for locoregional assessment (TN stage) and 80–98 per cent for detection of metastatic disease. Comparable rates have been found for magnetic resonance imaging and EUS[16–23]. In addition, not all tumours within the same UICC stage group behave in the same way, and so clinical staging and estimation of prognosis should also allow for biological tumour behaviour.

Both the presence of systemic inflammation and nutritional depletion (cachexia) can be used as indirect measures of biological tumour activity. Systemic inflammation has been found in association with most epithelial malignancies and linked with adverse outcome[13,24–36]. Up to 50 per cent of patients may have a raised acute-phase response at the time of diagnosis of cancer and an increased serum CRP level is an independent adverse prognostic indicator in a number of types of gastrointestinal cancer[13]. In patients with gastric cancer median survival is shortened from 53 to 9 weeks in the presence of a heightened inflammatory response ($P < 0{\cdot}001$)[33]. Similarly, shortened survival has been associated with a raised serum CRP level in patients with oesophageal cancer[34–36].

Cachexia remains an important cause of morbidity and mortality among patients with cancer, affecting up to 85 per cent of those with gastrointestinal malignancy at the time of diagnosis[37]. Weight loss of greater than 10 per cent total bodyweight has been associated with adverse outcome on multivariable analysis among patients with oesophageal cancer who had undergone surgical resection and chemoradiotherapy[38,39].

The relevance of performance status to prognosis is well documented and performance is an important assessment tool in the selection of patients for chemo/radiotherapy. Among patients with gastro-oesophageal malignancy, performance status was identified as an independent prognosticator on multivariable analysis of over 1000 patients, and reduced physical function has been associated with increased probability of death in the first 6 months after surgical resection of gastro-oesophageal cancer[40,41].

A number of prognostic scoring systems have been proposed for patients with gastro-oesophageal cancer. Most include pathological information gained from tumour resection and these data cannot be used at the time of diagnosis to inform management. Other prognostic studies have used sophisticated novel molecular techniques to determine prognosis, for example DNA microarray[42], or involved the development of complex computer models, such as artificial neural networks, for which extensive clinical, investigative and pathological information is required to estimate prognosis[43]. A study in patients with inoperable oesophagogastric cancer devised an inflammation-based prognostic scoring system[36]. The score was based on the presence of a CRP concentration above 10 mg/l and/or an albumin concentration below 35 g/l. Survival time was reduced significantly with an increasing score. A Japanese group similarly found that clinical stage (cTNM), weight loss (more than 2 per cent bodyweight) and serum CRP concentration (greater than 0·5 mg/dl) were associated independently with survival in patients with gastro-oesophageal malignancy[44].

The scoring system developed in this study allows accurate estimation of the probability of death within 12 and 24 months for individual patients. Clearly, the score selected as the cut-off will influence the probability of survival beyond 12 or 24 months. A higher score would have better specificity, but some patients would still be offered surgery without much prospect of longer survival. Selection of a lower score would perhaps deny the opportunity of surgery to some patients who may benefit from resection. The authors believe that it is better to select a higher score and accept that a few patients may have been overtreated. The final cut-off is likely to depend on future validation studies and individual preferences.

This study has shown that the addition of biological (CRP) and host-related (weight loss and performance score) factors to conventional clinical staging modalities can improve prognostic accuracy for individual patients. The novel prognostic scoring system may be used for patients with oesophageal, junctional and gastric tumours with similar accuracy, and for different histological tumour types. The risk score is based on four measurements that are widely available, reproducible, inexpensive and easy to perform. The score may be used prospectively to guide management decisions at the time of diagnosis, and provide more realistic prognostic information for patients and their families. In addition, the risk model can be used to provide quality assurance in the MDT decision-making process. This model now requires prospective validation in other centres before widespread introduction into clinical practice.

Acknowledgements

D.A.C.D. was funded by a fellowship from the Royal College of Surgeons of England. Dr J. A. Ross provided invaluable laboratory advice and assistance.

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