Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

MINIMAL RESIDUAL DISEASE IN CANCER OF THE OESOPHAGUS AND GASTRO-OESOPHAGEAL JUNCTION

DOCTOR IN MEDICINE (M.D.)

2009

Dr. Martin James MacGuill

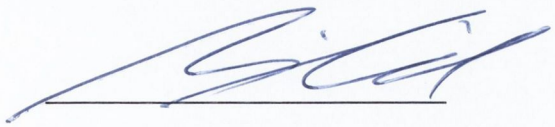
DECLARATION

I declare that

a) the thesis has not been submitted as an exercise for a degree at this or any other University,

b) this is entirely my own work

c) I agree that the Library may lend or copy the thesis upon request.



Dr. Martin James MacGuill

SUMMARY

Epithelial tumours are the most common form of cancer and are responsible for the majority of cancer-related deaths in Western industrialized countries. Minimal residual disease following neo-adjuvant therapy or primary surgical therapy for epithelial tumours is believed to underlie therapy failure. We chose to study Oesophageal cancer in particular because it presents unique challenges. Despite improvements in surgical therapies and the advent of multimodality regimens, the overall outlook remains generally bleak, and only 8-20% of patients are alive at 5 years.

We examined first the phenomenon of residual disease following neo-adjuvant chemotherapy and radiotherapy. Using a prospectively compiled database we collected and analyzed a large volume of clinical and pathological data in patients who received neo-adjuvant therapy. We identified larger tumor size to be an important predictor of residual disease following adjuvant therapies. This is an important finding because a possible reason for this relationship between tumor size and response may simply be that the currently standard dosages of chemotherapy and radiotherapy are inadequate in patients with larger tumors. This will require further investigation.

We then turned our attention to residual disease following surgical therapy. We examined a larger cohort of pN0 (pathological node negative) oesophageal

cancer patients than any previously reported for evidence of minimal residual disease in lymph nodes (occult lymph node metastasis). We found a significantly lower prevalence of occult lymph node disease than those previously reported. Additionally we found a strong association between minimal residual disease in lymph nodes and poor outcome.

When discussing these findings we concluded that there were two main clinical implications of these findings. Firstly, by adding investigation of lymph nodes for minimal residual disease to the routine pathological "work-up" of oesophagectomy specimens one might be able to provide patients with more accurate diagnoses. Secondly, in patients whose primary treatment was surgical and who were demonstrated to have minimal residual disease following surgery, there would be a strong argument for the trialing of adjuvant therapies in these patients. However the quality of the existing evidence regarding the relevance of minimal residual disease in lymph nodes was simply not high enough to even consider making real clinical decisions of their basis. Accordingly we undertook a systematic review of the existing evidence in order to better inform the on-going debate into the clinical relevance of minimal residual disease following surgery. We found enough studies of sufficient quality to warrant a statistical analysis and our analysis showed a significant association between minimal residual disease in gastro-oesophageal or oesophageal cancer and outcome.

ACKNOWLEDGEMENTS

My thanks to:

Jamie, Fraser and Seamus for taking an interest and providing ideas; Ravi who always had time for a quiet word of encouragement and a different perspective; Suzanne for providing all the patient data; Tony, Ludmilla, Antoinette, Lisa, Martin, Laure and everyone else in Mark Lawlor's lab for tea, coffee, a scientist's opinion and reassurance that there was light at the end of the tunnel; all the staff of the Surgery Lab for your technical help; Ronan and everybody else in the CPL for the hours of help with the blocks.

And a few special words of thanks to:

Paddy for keeping in contact, encouraging and always providing good advice.

Prof Reynolds for setting the example, always being available, the hours of discussion, reading, writing and re-writing and most importantly the continuous encouragement.

And finally to my parents, for your belief in me.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

H&E	Haematoxylin and Eosin staining
ITC	Isolated tumour cells
pN0	Pathological node negative
mm	millimeter
PCR	polymerase chain reaction
RT-PCR	reverse-transcriptase polymerase chain reaction
TNM	Tumour-node-metastasis classification
pN0 (i+)	No lymph node metastasis histologically, positive morphological findings for isolated tumour cells.
pN0 (I-)	No lymph node metastasis histologically, negative morphological findings for isolated tumour cells.
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
CK	Cytokeratin
UICC	International Union against Cancer [l'Union Internationale Contre le Cancer]
MRLD	Minimal residual lymph node disease
HR	Hazard ratio

INTRODUCTION

Epithelial tumours are the most common form of cancer and are responsible for the majority of cancer-related deaths in Western industrialized countries (1). Oesophageal cancer presents unique challenges. Despite improvements in surgical therapies and the advent of multimodality regimens, the overall outlook remains generally bleak, and only 8-20% of patients are alive at 5 years (2). Moreover, there has been a marked increase in incidence of adenocarcinoma of the oesophagus and the oesophago-gastric junction in recent years (2). The presence or absence of metastases to regional lymph nodes is the single most important standard risk factor for patients with oesophagogastric cancers, and even in patients with pT1 tumours the presence of lymph node metastasis has been reported to decrease overall 5-year survival by up to 100 per cent (3). A large sub-group of lymph node negative (pN0) patients recur, either locally or systemically, and usually within two years of surgery. It has been hypothesized that early recurrence following complete resection of an apparently localized primary lesion is attributable to disseminated tumour cells that were not detected by routine staging methods at the time of surgery (4). Better methods for detecting and characterising subclinical metastatic deposits in various compartments of the body could enable us to both refine our estimates of the risk of recurrence for individual patients and perhaps tailor therapy more effectively (5). Two significant shortfalls in studies designed to establish the prognostic significance of micrometastases at any location for any tumour have been: insufficient power and failure to properly codify results. Accordingly the aim of this first part of the study was to perform an adequately powered single

centre retrospective study of ITC in node negative cancer of the oesophagus or gastro-oesophagus. We additionally intended to select out one particular subgroup with robust scientific rationale for further analysis – namely the subgroup of patients with a complete pathological response to neo-adjuvant chemoradiotherapy (pCR). Primarily the aim was to establish the prevalence of ITC in this subgroup and whether it was a causative factor in the few relapses that occur in this patient group. A secondary aim was to determine whether any other clinical or pathological factors apart from lymph node metastasis were predictive of pCR and survival.

COMPLETE PATHOLOGICAL RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY IN OESOPHAGEAL CANCER

Poor results with either surgery or radiotherapy alone have lead to intensive investigation of multi-modality therapy of gastro-oesophageal cancer. Some recent studies have shown that combined modality therapy (combined chemotherapy and radiotherapy) appears to be more effective than single modality therapy in the treatment of oesophageal cancers and cancers of the gastro-oesophageal junction (6, 7). However combination chemotherapy and radiation therapy prior to surgery (8-14) is controversial and meta-analyses (15,16) have yielded equivocal results. Notwithstanding the fact that the largest and most adequately powered studies are negative (17-19) the use of multimodal therapy has increased; the Patterns of Care studies showed that preoperative chemoradiation therapy increased from 10.4% during 1992 to

1994 to 26.6% in 1996 to 1999 (20). It is important to note that the case for combined chemo-radiotherapy and radical surgery is not easily proven. For a phase III trial to be of sufficient power to demonstrate a 5-year survival benefit of 10-15% at least 450 patients would need to be recruited (21). This number of recruits was not achieved by any of the above phase III trials. Regarding chemotherapy alone, there are two adequately powered phase III studies of neoadjuvant 5-fluoruracil and cisplatin: the Intergroup (22) and Medical Research Council (MRC) trials (23). Only the MRC study showed a survival benefit.

Nevertheless these investigations have consistently identified a common subset of patients that received significant prognostic benefit from multi-modality therapy – patients who achieved a complete pathological response following the neo-adjuvant component of the regimen. Phase II and a number of randomized phase III studies have demonstrated that such a complete tumour response can be achieved in 20–30% of cases with three-year survival rates of >60% irrespective of the applied protocol, type of histology and tumour stage (24-31). A recent study from this department reported 5-year survival of 50% and median survival of 56 months in patients achieving a complete pathological response. The overall survival 5-year survival rate in the entire group of 243 patients, based on intention to treat, was 27% and the overall median survival was 18 months (32). Accordingly it seems that there are patients who derive a great deal of benefit from neo-adjuvant therapy and a proportion who do not acquire any survival benefit from the neo-adjuvant regime. In addition to the risks linked to each individual modality there is now some evidence of a possible synergistic effect between the 3 modalities

resulting in increased peri-operative morbidity and mortality following neo-adjuvant therapy in comparison to patients treated with surgery alone (33). This effect appeared to be uniform through all patients including those who showed only a partial or no response at all after preoperative chemoradiotherapy.

The question arises as to why patients who achieve a complete pathological response following neo-adjuvant therapy have survival rates of *only* 50%. It may be that pathological response is an imperfect mirror of tumur biology and may not always be a reliable surrogate marker of chemosensitivity. Alternatively the histopathological response may not be a dependable proxy for nodal status. Where patients have apparent TRG1 there is still the possibility that they have minimal residual nodal disease. Against this background of significantly varying degrees of responsiveness to a neo-adjuvant component with attendant morbidity and mortality, the identification of factors that could reliably predict a response to pre-operative chemo-radiotherapy would be of considerable clinical benefit. The aim of this study was to investigate the potential of various demographic, social, clinical and pathological parameters to predict response to neo-adjuvant chemo-radiotherapy.

MICROMETASTATIC DISEASE

The single most important standard risk factor for patients with most solid tumours, including oesophageal or gastro-oesophageal neoplasia, is the presence or absence of metastasis to regional lymph nodes. The current routinely applied methods for scanning the body and examining lymph nodes

for tumour cells have widely recognised limitations. It has been estimated that the probability of a pathologist, using standard techniques of light microscopy, identifying a small (three-cell diameter) metastatic focus of cancer is in the order of 1 percent (34). Consequently it is presumed that the explanation for recurrence following apparently successful local therapy of a tumour is metastasis, which went undetected by standard staging techniques. Furthermore the information provided by current measures of disease extent, although providing reliable information about populations of patients, does not allow accurate prognoses to be made on an individual patient basis. Accordingly methods, which would enable the detection of subclinical metastatic deposits at regional and systemic sites, could enable us to refine our estimates of the risk of recurrence for individual patients and also to tailor therapy more effectively.

HISTORY

The first known report of "circulating tumour cells" appeared in the 19th century (35). Interest in circulating tumour cells was very high in the mid 1950s after a seemingly groundbreaking paper by Engell, who reported their presence in patients with various types of carcinoma using a cell block technique (36). During this period of intensive investigation several thousand patients were tested for "circulating tumour cells" by over 40 investigative teams using 20 different cytological methods (37). Initial studies reported prevalence rates among cancer patients of as high as 100%. However these techniques were discredited in 1965 when it was discovered that circulating haematopoietic

elements, especially megakaryocytes, had often been confused with tumour cells. When cell preservation techniques were improved allowing a better morphological analysis, the detection of true circulating tumour cells by light microscopy was shown to occur in about 1% of patients and research in the field consequently waned.

In the 1980s interest was re-invigorated when investigators at the Royal Marsden Hospital and the Ludwig Institute first used labelled antibodies to detect ectopic epithelial cells at mesenchymal sites (38). Lymph node and bone marrow preparations were examined using labelled antibodies, which specifically bound proteins unique to epithelium. It was hypothesised that ectopic epithelial cells in lymph nodes and bone marrow in patients with primary epithelial malignancies were by definition metastatic and so the term "micrometastases" arose. "Micrometastases" were found to have a high prevalence in lymph nodes and bone marrow samples that had previously been adjudged to be free of metastasis. The advent of polymerase chain reaction (PCR) in the late 1980s provided an even more sensitive technique and a variety of PCR based techniques have been used to detect disseminated tumour cells in all major cancer types (39). Other techniques used to identify micrometastases include both fluorescence and magnetic activated cell sorting, and enzyme-linked immunoadsorbent assay.

WHERE ARE THEY DETECTED?

Bone marrow can be relatively easily and safely collected from the iliac crest and so it has been extensively studied as a site for detecting disseminated epithelial tumour cells. In the case of epithelial tumours that commonly develop skeletal metastases, individual tumour cells are frequently detected among bone marrow cells aspirated from the iliac crest. Occult tumour cells are also detected in the bone marrow of patients who have cancers that generally do not metastasize to the bone (e.g., colon cancer). Consequently bone marrow is a particularly good site for the detection of occult tumour cells. Detection rates of 20-50% of have been quoted for bone marrow for various solid tumours (40-42).

Tumour cells have been identified in the lymph nodes of patients with a broad range of solid tumours including renal, breast, gastric, colorectal, prostate, non-small cell lung, pancreatic and oesophageal. The reported prevalence rates of disseminated cells in lymph nodes vary widely across and within these groups (9-90%) (43).

Pierga et al (44) have shown a correlation between the presence of cytokeratin-positive cells in peripheral blood and the presence of similar cells in bone marrow. However only the presence of cytokeratin-positive cells in the bone marrow could be correlated with metastatic relapse. Peri-operative analysis by molecular methods of blood samples taken from cancer patients undergoing curative surgery clearly points to a temporary intra-operative

dissemination of tumour cells (45,46). Detection of occult tumour cells in the peripheral blood of patients with early stage cancer is much more difficult because of the low frequency of these cells (47). A practical method to detect disseminated tumour cells in the peripheral blood would offer a number of advantages, such as the ability to evaluate serial samples. It seems likely that a method of enrichment of the tumour cells in the blood sample would be required before this can become feasible. Currently available strategies for the physical separation of tumour cells from background rely on either density centrifugation (48) or the application of magnetic labels (49, 50, 51).

HOW ARE THEY DETECTED?

The methods used to detect occult carcinoma cells may be conveniently divided into morphological and non-morphological methods. With morphological methods (immunocytochemistry or immunohistochemistry) there is the opportunity to improve specificity by including malignant morphology (increased cell size, increased nuclear/cytoplasmic ratio) as a criterion for a positive result. With non-morphological methods (flow cytometry, PCR), reverse transcriptase – polymerase chain reaction (RT-PCR)) this is not possible.

NOMENCLATURE

A note on nomenclature is important at this point. Most of the existing research effort has been conducted by dividing patients into two comparison sets based

on the method of identification of cancer cells: either by routine histology or special techniques. Generally there were two possible results of these special procedures: a positive or negative finding. Positive findings were occasionally further subdivided based on number or microscopic appearance (52). As experience was acquired many authors became unhappy with the term "micrometastasis" and consequently the nomenclature for a positive finding has included *inter alia*: "disseminated tumour cells", "minimal residual disease", "micrometastases", "subclinical metastases", "occult metastases", "isolated tumour cells", "tumour cell micro-involvement" or "minimal solid tumour involvement of regional and distant sites". Against this background the International Union against Cancer [l'Union Internationale Contre le Cancer] (UICC) (53) attempted to clarify the terminology. The consensus recommendations for reporting metastases in lymph nodes or other tissues set a size threshold of 2mm or greater in largest diameter on haematoxylin and eosin (H&E) staining. A micrometastasis is defined as being no less than 0.2mm but no more than 2mm in greatest diameter. This suggestion is based on evidence derived from one large study where the majority of metastases necessitating detection by special studies were < 0.2mm in greatest dimension (54). These micrometastatic lesions may demonstrate contact with a blood vessel or lymph sinus wall, invasion and penetration of the vessel or sinus wall, extravascular or extrasinusoidal proliferation, and often an associated stromal reaction (53). According to this definition the diagnosis of "micrometastasis" can only be made on the basis of standard light microscopy. Such lesions are recognised as clinically relevant and classified as pN1 even in the absence of larger nodal metastases. The recommended practice has been to verify the

metastatic potential of lesions identified by special techniques (meaning immunohistochemistry) using H&E staining on a contiguous slice. This is preferred because of the more detailed morphological data, which can be obtained. Smaller lesions are now termed "isolated tumour cells" (ITC) and are defined as tumour cell deposits no larger than 0.2mm in diameter with or without histological features of malignancy. They are classified as pN0 for the moment and are essentially always detected by special techniques such as immunohistochemistry or polymerase chain reaction (PCR). The additional TNM shorthand notation for these metastases first proposed by Hermanek et al (43) is summarised in (Table 1). This UICC shorthand for ITC currently exists purely so that findings can be documented according to uniform criteria.

Table 1.

The TNM shorthand notation for occult lymph node metastases first proposed by Hermanek et al (43).

Shorthand Notation	Definition
pN0	No regional lymph node metastasis histologically, no examination for isolated tumour cells.
pN0 (i+)	No regional lymph node metastasis histologically, positive morphologic findings for ITC.
pN0 (i-)	No regional lymph node metastasis histologically, negative morphologic findings for ITC.

Henceforth we will use the term 'occult lymph node metastasis' (OLNM) in order to avoid confusion with the terms defined by the UICC and to reflect the range of definitions of occult disease used by the many studies referenced within this thesis. In reality most of the studies referenced actually predated the UICC communication and of those which came after it, few have adopted the new terminology.

DETECTION TECHNIQUES

As mentioned above there are a number of experimental techniques available for the detection of sub-clinical metastatic deposits, which include fluorescence activated cell sorting and magnetic activated cell sorting (FACS and MACS). However the two most widely used techniques and the techniques most likely to be seen in routine clinical practice are based on immunochemistry or polymerase chain reaction.

IMMUNOCHEMISTRY

To date the most widely used techniques are immunocytochemistry and immunohistochemistry. Both are antibody-based techniques. Cytokeratins are currently the most widely used cellular targets and belong to a large multigene family of more than 30 known members. They are integral components of the cytoskeleton of epithelial cells and are dependably expressed in cancer cells (55). Most researchers use a combination of several antibodies that recognise

various cytokeratin antigens. This is because individual cytokeratin proteins can be downregulated in epithelial tumours. Broad-spectrum anti-cytokeratin antibodies that recognise a single epitope that is common to most cytokeratins have been less frequently used. In general, the use of anti-cytokeratin antibodies appears to be a reliable and effective method for tumour cell detection. However the choice of antibody used can be a cause of inconsistency between studies. For instance, 'mucin-like tumour-associated cell membrane proteins' and 'epithelial membrane antigen' were widely used in early studies but it is now recognised that they are expressed by various non-transformed haematopoietic cells and so may not be suitable (56-58). The most widely used non-cytokeratin antibodies against epithelial antigens are BerEP4 (59) and an antibody against carcinoembryonic antigen (CEA) (60) .

POLYMERASE CHAIN REACTION (PCR)

Since 1987 a variety of PCR-based techniques have been devised for the identification of micrometastases in leukaemias, lymphomas and various types of solid malignancies (61, 62). In principle either DNA (PCR) or RNA (reverse transcriptase-PCR) can be used. The majority of investigations performed to date have been DNA-based however the most reliable results have been achieved with RT-PCR.

One strategy for the detection of occult tumour cells by PCR is the amplification of tumour-specific abnormalities present in the DNA of micrometastatic cells. This approach was first applied to the detection of the t(14:18) translocation associated with follicular lymphomas (63). Other tumour DNA based markers, such as mutations in the p53 gene or K-ras gene have been used in patients with colorectal, lung or head and neck cancers to detect single tumour cells against a background of thousands of normal lymph node cells (64). Additionally changes of methylation status of a defined gene (65), microsatellite instability (66) or even sequences of carcinogenic viruses (67) have been used. In short any sequence, which is only present in transformed tissue can be used. The other main PCR strategy for the detection of occult tumour cells involves amplification of tissue specific messenger ribonucleic acid (mRNA) by RT-PCR. This approach is based on the fact that malignant cells often continue to express markers that are characteristic of, or specific to, the normal tissue from which the tumour originates or with which the tumour shares histotype. It is the appearance of these tissue-specific mRNAs at a body site where these transcripts are not normally present that implies tumour spread (for example prostate specific antigen (PSA) mRNA in bone marrow).

PCR assays are applicable not only to bone marrow and lymph nodes but also to body tissues such as peripheral blood and cerebrospinal fluid (CSF). It is still however the case that for most cancers (and solid tumours in particular) truly tumour specific mRNA transcripts have not yet been characterized. To date the best strategy available to improve specificity is to use multiple genetic markers.

SENSITIVITY OF DETECTION TECHNIQUES

Techniques for detecting occult tumour cells must be extremely sensitive and ideally well beyond the limits of sensitivity of standard histopathologic analysis. Immunocytochemical methods are exquisitely sensitive and can detect as few as one to two tumour cells in 1 x 10^6 bone marrow mononuclear cells (68). Whether this level of sensitivity is adequate is not known. Enrichment methods are now available that can increase the sensitivity by at least one order of magnitude (69). mRNA based techniques are even more sensitive and can detect one cancer cell in about 10ml of blood (10^7 normal cells) (34). Additionally it is possible to analyse an entire lymph node when using PCR-based techniques. This would not be logistically possible using immunochemistry.

CLINICAL SIGNIFICANCE OF MICROMETASTASIS

It was originally hypothesised that single and small clumps of ectopic tumour cells in lymph nodes and bone marrow samples of cancer patients were by definition metastatic cells and antecedents of gross metastasis. The great majority of the efforts to prove this theorem have been observational cohort studies comparing outcome in those with and without occult metastatic disease. Allied to this is a smaller body of experimental work designed to demonstrate both the malignant and metastatic nature of these cells.

Single cells or small clusters of tumour cells in lymph nodes usually do not exhibit overt metastatic morphologies such as stromal reaction, lymphatic or vascular invasion. However a number of tumour specific molecular characteristics have been identified in cytokeratin positive cells identified in bone marrow and lymph nodes. In double staining studies disseminated tumour cells in bone marrow were found to express proliferation markers (Ki-67 or p120) and urokinase plasminogen activator complex, to overexpress erbB2 and to underexpress major histocompatibility complex class I molecules (71-75). Fluorescence in situ hybridization (FISH) and comparative genomic hybridization analyses have illustrated many genomic aberrations in these cells including the amplification of the erbB2 gene and K-ras mutations (76,77). Genomic analyses of single disseminated tumour cells demonstrated that most contained genetic aberrations consistent with malignancy (42).

Experiments designed to determine whether these cells were actually capable of generating a gross metastasis have also been performed. Extensive cell culture experiments by a German group have shown that short-term culture of cells disseminated to the bone marrow was possible (78). Additionally one Irish group has reported generation of a cell line from rib micrometastases of upper gastrointestinal cancers (79). The cell line was tumorigenic when transferred to athymic immunodeficient mice.

On the other hand, attempts to test the clinical utility of these various detection strategies have yielded mixed results. A large number of studies have purported to show a statistically significant association between the detection of occult metastases in the lymph nodes of patients with node-negative cancer and prognosis at a number of different sites including breast cancer (80,81), colon cancer (82), gastric cancer (83), non-small-cell lung cancer (59), oesophageal cancer (discussed in detail below), prostate cancer (84) and melanoma (85).

The prognostic impact of occult dissemination to bone marrow has been most extensively studied in breast cancer. The last decade has seen 5 large clinical trials all of which have shown a significant correlation between the presence of immunostained tumour cells in bone marrow and unfavourable clinical outcome (42, 86-89).

However some investigators (90-92) have found that such tumour deposits are not associated with clinical outcome. In a large single centre trial with careful case definition no survival difference was found for patients with and without minimal disease detected by immunohistochemistry in axillary lymph nodes of breast cancer patients (93). In a large German multi-centre trial on colorectal carcinoma it was found that when Stage I, II and III patients with bone marrow occult metastases were re-assigned as Stage IV patients the actual 5-year

survival rate increased from 16% to approximately 40% for stage IV patients (43).

SOURCES OF ERROR IN THE DETECTION TECHNIQUES

IMMUNOCHEMISTRY

There are a number of scientifically sound reasons why "tumour cells" identified at metastatic sites may either not be tumour cells or may not be clinically relevant. Benign thyroid follicular cells (94), benign breast epithelium (95), benign renal tubule cells (96) and benign colonic epithelium (97) have all been demonstrated to have "metastasised" to lymph nodes. Many pathologists report as relatively common a finding of single epithelial cells identified by immunohistochemistry that on careful analysis were obviously artefact of overlay of cells from the staining solutions, water bath etc. (even keratinocytes from the skin of the hands of the technician) (52).

Theoretically ectopic expression of cytokeratin by non-transformed mesenchymal cells is possible (98,99). For instance normal lymph node (reticulum) cells can in theory express cytokeratins (e.g., CK19) (100). However studies on patients without malignant disease suggests that ectopic expression of cytokeratins by normal cells of the bone marrow is extremely rare and usually not detectable by immunochemistry. Braun et al analysed bone-

marrow cells from almost 200 individuals without cancer and more than 550 patients with breast cancer. They detected single cytokeratin-positive cells in only 1% of control individuals, whereas 30-40% of bone-marrow cells from patients with breast cancer were cytokeratin positive (42). It remains unclear whether cytokeratin-positive cells found in the bone marrow of control patients are normal epithelial cells or tumour cells derived from an unknown primary carcinoma.

The choice of antibodies, staining methods and the level of technical skill of those involved in performing the procedures and interpreting the results is known to introduce variation. Indeed there are very wide variations in the reported prevalence rates of occult lymph node metastasis even for cohorts of relatively high pathological homogeneity. For instance in early stage breast cancer, occult tumour cell detection rates of 4%-45% have been reported (101).

At the same time false negative results have also been recognised as a source of error. One study on bone marrow of cancer patients who went on to develop overt metastatic disease demonstrated epithelial cells in the bone marrow that lacked the typical morphological signs of a tumour cell. However they could clearly be demonstrated to harbour malignant molecular characteristics (102). Morphological analysis alone might therefore not be sufficient to identify isolated tumour cells on cytological preparations.

There are a number of reasons why PCR based techniques may be unreliable. Sensitivity and specificity are very dependant on choice of amplification conditions, primers and reagent concentrations. One study reported a wide variability of results from one laboratory to the next using identical coded samples (103). Inhibitors present in some tissues and fluids can diminish PCR sensitivity. Therefore endogenous positive controls must be used with each run. It is as a result of this that *in vitro* sensitivity is not always an accurate reflection of *in vivo* sensitivity. The presence of poorly differentiated subclones that do not express the tissue-specific marker being tested is a major limit on the sensitivity of PCR based techniques. It is hoped that the use of multiple marker PCR assays may help to prevent loss of sensitivity due to this phenomenon.

Conversely ectopic or illegitimate CK messenger RNA (mRNA) by hematopoietic cells (104-108) has been demonstrated to cause false positive results. Specifically, cytokeratin 20 mRNA was used as a marker for colorectal carcinoma cells in lymph nodes until it was demonstrated that it could be detected by RT-PCR in 72% of blood samples and all bone marrow specimens from healthy individuals (109).

Carcinoembryonic antigen (CEA) has been widely used as a genetic marker. Several authors have reported the detection of CEA mRNA in the peripheral blood, bone marrow and lymph nodes of patients with gastric, colorectal and pancreatic carcinomas but in none of their control subjects (110-112). However others have reported detecting CEA mRNA by RT-PCR in lymph nodes, blood and bone marrow samples from individuals without epithelial malignancies (106, 113, 114). One strategy to combat this is to optimise cycling conditions. Concretely, the number of PCR cycles should be high enough to detect occult tumour cells but low enough to avoid amplification of illegitimate transcripts.

One limitation specific to PCR as opposed to RT-PCR techniques is the fact that DNA fragments could originate from decaying tumour cells. It is argued that DNA is so stable in human tissues that detection of mutant sequences should only be inferred to mean that tumour cells are present somewhere in the organism (115-119). As a result DNA based assays may be best suited to monitoring tumour burden or the detection of early cancer rather than the location of small metastases.

SUMMARY

PCR amplification of tissue-specific (versus tumour specific) mRNA offers several advantages over protein-based assays. Firstly RNA is very unstable in

the extracellular environment; its detection should therefore indicate the presence of tumour cells in the examined body tissue. Much fewer tumour cells are required for a positive result. The mRNA may be present but the protein may not be expressed, which has previously been observed in poorly differentiated prostate carcinomas (120). Immunohistochemistry requires expert interpretation while RT-PCR assays may in general be more straightforward to interpret. The principle advantages of immunohistochemistry are that it is widely available in clinical pathology laboratories and that it allows evaluation of morphology of the suspect cells. PCR affords potentially higher sensitivity, but current probes for disseminated disease do not yet provide the desired level of specificity for the majority of solid tumours.

SYSTEMATIC REVIEW AND META-ANALYSIS OF OBSERVATIONAL STUDIES.

Numerous studies have reported that it is possible to detect disseminated tumour cells in lymph nodes previously thought to be metastasis free when immunochemical techniques are used. Attempts to define the clinical significance of these cells in oesophageal cancer have however yielded mixed results. We conducted a systematic review of the literature addressing the prognostic significance of occult lymph node metastasis in cancer of the oesophagus or gastro-oesophagus because it provides higher statistical power and improved precision relative to individual studies. Specifically we set out to quantify the impact on disease-free survival of occult lymph node metastasis in cases staged by conventional means as lymph node metastasis free (pN0) and in so doing to improve the quality of the data informing the on-going debate on the prognostic significance of occult lymph node metastasis in pN0 carcinoma of the oesophagus and gastro-oesophagus.

PATIENTS AND METHODS

CLINICOPATHOLOGIC FACTORS PREDICTING COMPLETE PATHOLOGICAL RESPONSE

All patients who underwent multimodality therapy, comprising neoadjuvant chemoradiotherapy and radical surgery, for cancer of the oesophagus or gastro-oesophageal junction between January 1990 and June 2003 were identified from the prospectively compiled upper gastrointestinal cancer database at this institution. The following factors were selected for analysis: age, sex, tobacco and alcohol history, duration of symptoms before diagnosis, pre-treatment body mass index, past medical history, pre-treatment forced volume in the first second of expiration (FEV_1), presence or absence of Barrett's oesophagus or dysplasia on pre-treatment endoscopic examination, pre-treatment tumour length, histologic type, site and degree of differentiation. As in a previous report (129), we classified cases as either complete pathological responders (pCR) or incomplete pathological responders according to the tumour regression classification system of Mandard *et al* (130) (Table 2).

Tumour Regression Grade	Definition
TRG 1	No residual cancer
TRG 2	Rare residual cancer cells
TRG 3	Fibrosis outgrowing residual cancer
TRG 4	Residual cancer outgrowing fibrosis
TRG 5	Absence of regressive changes

Table 2.

Tumour regression classification system of Mandard et al.

In those cases where no residual tumour cells were identified on haematoxylin and eosin slides, immunohistochemistry by the ABC method for MNF116 (Dako, Glostrup, Denmark; 1/50 dilution; pronase digestion) was performed on the area where the tumour had been located endoscopically. Where viable residual tumour cells were identified following immunochemistry, the case was upgraded to a tumour regression grade 2 (TRG 2) or in-complete pathological response. Statistical analysis of the predictive value of variables was performed using Kruskal-Wallis test for continuous variables and Chi-squares with Pearson test for categorical data. Additionally multivariate analysis (Cox procedure) was performed to prove independence of investigated variables. All calculations were performed using SPSS 11.0 ® for windows software.

IMPACT OF ISOLATED TUMOUR CELLS IN PATHOLOGICAL NODE-NEGATIVE LYMPH NODES (PN0) ON PROGNOSIS IN CANCER OF THE OESOPHAGUS OR OESOPHAGO-GASTRIC JUNCTION.

All patients with pN0 tumours who underwent curative therapy for oesophageal or gastro-oesophageal cancer between January 1990 and September 2002 were identified from the prospectively compiled upper gastrointestinal cancer database at this institution. All patients with overt metastatic (M1) disease were excluded and only adenocarcinomas or squamous cell carcinomas (SCCs) were included. The study group consisted of 146 patients, 76 (52%) of whom underwent surgery alone and 70 (48%) who underwent a multimodality regimen. Patients in the neoadjuvant treatment cohort were given a standard protocol of chemoradiotherapy consisting of either 40 Gy in 15 fractions or 44 Gy in 22 fractions on days 1 to 5, 8 to 12, and 15 to 19, and concurrent chemotherapy of 5-fluorouracil (15 mg/kg) on days 1 to 5 and cisplatin (75 mg/m2 body surface area) on day 7. Chemotherapy was repeated on week 6. Patients were restaged by CT and OGD at week 8 and scheduled for surgery on week 9. Surgery took place if the neutrophil count was >2 x 10^6/mL, if performance status had not significantly deteriorated, and if there was no evidence of local or systemic progression of disease on imaging.

All patients had a thoracotomy as a component of their surgical management, either combined with an abdominal and neck exploration (3-stage) for mid- and upper-oesophageal cancers, or cancer arising in long-segment Barrett oesophagus, or with an abdominal exploration (2-stage) for most lower third

and junctional tumours, or combined with a total gastrectomy for junctional tumors with significant gastric extension. A 2-field lymphadenectomy (abdominal and thoracic) was performed in all cases. In the abdomen, nodal dissection routinely involved resection of N1 nodes as well as nodes along the left gastric artery, common hepatic artery, and splenic artery. In the thorax, clearance was obtained of nodes up to and including subcarinal nodes in all cases, and in selected cases paratracheal nodes were resected. Dissection of cervical lymph nodes was not performed. as described in a previous report from the department (9).

This study was approved by the local hospital ethics committee (St. James's Hospital and Federated Dublin Voluntary Hospital joint research ethics committee). For the purpose of the study an additional (4µm) section of each resected node was taken off the surface of paraffin embedded lymph node tissue. Immunostaining was performed as follows: each section was baked at 60° C overnight, deparaffinized and rehydrated through xylenes and graded alcohol series. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide (Sigma-Aldrich Ireland Ltd.) in water for 10 minutes. The slides were washed in de-ionised water for 5 minutes and digested for 5 minutes at room temperature with 0.05% proteinase (Sigma-Aldrich Ireland Ltd.) made in 0.005 M Tris-buffered saline (pH 7.6) (TBS). The slides were then washed in TBS for 5 minutes. The tissue sections were blocked with 5% bovine serum albumen (Sigma-Aldrich Ireland Ltd.) for 10 minutes, blotted dry and then incubated with mouse anti-human cytokeratin (Clone MNF 116, DakoCytomation Ltd. UK) (1:300 in 5% bovine serum albumen) in a humid chamber for one hour at room temperature. The slides

were washed in TBS for 5 minutes and incubated with a biotinylated secondary antibody (1:300 in TBS) (rabbit anti-mouse immunoglobulin DakoCytomation Ltd. UK) for thirty minutes and then avidin-horseradish peroxidase (Vectastain Elite ABC Kit; Vector Laboratories Ltd. UK) for thirty minutes. The slides were washed with TBS for 5 minutes after each incubation. The stain was developed by covering each specimen with a 3'3'-diaminobenzidine solution (DakoCytomation Ltd. UK) for 5 minutes or until desired stain intensity was achieved. Finally each slide was counterstained with haematoxylin, cleared and mounted. The immunostained slides were evaluated by an experienced pathologist, who was blinded to patient information, and were scored as positive for ITC if they contained single or small clusters of strongly immunoreactive epithelial cells in the subcapsular sinus or in the cortex of the lymph node. Patients were pathologically staged as per the 6th AJCC Cancer Staging Manual (131) with the addition of TNM shorthand notation for isolated tumour cells first proposed by Hermanek and colleagues (43). Positive cases were designated as pN0(i+) and negative as pN0(i-). With each run, sections of primary tumours were used as positive controls and a negative control (primary antibody omitted) was always included.

STATISTICAL ANALYSIS – ISOLATED TUMOUR CELLS

Statistical calculations were performed using JMP® software version 5.1.2 for Macintosh (SAS Institute, Cary, North Carolina, USA). The Kaplan-Meier survival model was used to estimate survival. The log rank and Wilcoxon tests were used to determine statistical differences between groups. Analysis of the

predictive value of clinicopathological variables for ITC was performed using the Kruskal-Wallis test for continuous variables and Chi-squares with Pearson test for categorical data. Cox's proportional hazard model was fitted to multivariate analysis. The following variables were controlled for in the model: gender, age, tumour site, tumour morphology, degree of differentiation, treatment modality and presence or absence of isolated tumour cells. All tests were two sided, and the results were considered significant at $p < 0.05$.

THE PROGNOSTIC IMPACT OF OCCULT LYMPH NODE METASTASIS IN CANCER OF THE OESOPHAGUS OR OESOPHAGO-GASTRIC JUNCTION: SYSTEMATIC REVIEW AND META-ANALYSIS OF OBSERVATIONAL STUDIES.

Before embarking on the description of the methods used in the meta-analysis a word on nomenclature is necessary. We have already discussed the UICC's clarification of the terminology (43). Micrometastases are defined as being ≤ 2mm in greatest dimension, in contact with a vessel wall, extravasated, proliferating and usually associated with a stromal reaction. Isolated tumour cells (ITC) in contrast are defined as clusters (<0.2mm) or single tumour cells without any of the above characteristics whose presence can only be determined by immunohistochemistry, immunocytochemistry or molecular methods such as flow cytometry or PCR. We use the term 'occult lymph node metastasis' (OLNM) in order to avoid confusion with the terms defined by the UICC and in order to reflect the range of definitions of "micrometastatic disease" used by studies within this systematic review.

SEARCH STRATEGY

We searched the Medline and Embase databases (1966 – 01 May 2006) using the following terms: micrometastasis, tumour cell microinvolvement, minimal

residual disease, subclinical metastasis, occult metastasis, isolated tumour cells, and oesophagus. Complete specification of the search strategy used is provided in appendix 1. The reference lists of retrieved articles and previous non-systematic reviews were scanned for other potentially relevant articles. Unpublished or in press studies known to the authors were also included.

Any study reporting the use of immunochemistry to detect metastasis in pN0 lymph nodes of oesophageal or gastro-oesophageal cancer patients was potentially included. The only additional methodological criterion was that it should also have been possible to reasonably infer from the report that large lymph node metastases detected by the immunochemical technique, which should have been detectable by conventional staining techniques were reclassified from pN0 to pN1 cases and excluded from the survival analysis. No restrictions were placed on the immunochemical method employed. Nor were any restrictions placed based on study design (studies in which pN0 patients were a subgroup of the population were included), language of publication, geographic location, use of adjuvant therapies, ethnicity, age or sex of patients. Where multiple studies were published on the same or overlapping cohorts, only the last published report was included (unless the data was not suitable for meta-analysis in which case the last suitable report was selected). The outcome parameter of interest was disease-free survival, however if a study only contained "overall survival" outcome data it was also to be included in the meta-analysis because we believed that overall survival would tend to underestimate a detrimental prognostic effect of OLNM.

DATA

Data was entered onto a customized data sheet (appendix 2). The data chosen for extraction was limited to those variables where sound scientific rationale for their inclusion existed. The data from each study was reviewed twice to minimize the probability of data-entry error.

QUALITY ASSESSMENT

Our assessment of study quality was based on recently published guidelines for the evaluation of the quality of prognosis studies (132). In summary, six quality items (study participation, study attrition, prognostic factor measurement, outcome measurement, confounding measurement and analysis) were used. Each quality item was described or "operationalized" on a quality assessment sheet (appendix 3). For each quality item a score of "high risk of bias", "low risk of bias" or "unclear" was given. If a study scored "high risk of bias" for any item it was given an overall quality assessment of "high risk of bias". The only exception was where there was a high risk of attrition bias. Studies at high risk of attrition bias were not given an overall assessment of "high risk of bias" as discussed in the Cochrane Handbook (133). Studies that

reported overall survival data rather than disease free survival data were classified as "high risk" under "outcome measurement". The quality assessment data was incorporated into the sensitivity analysis.

STATISTICAL ANALYSIS

The most appropriate way of summarising time-to-event data is to use methods of survival analysis and express the treatment effect as a hazard ratio. Hazard is similar in notion to risk, but is subtly different in that it measures instantaneous risk and may change continuously (for example, your hazard of death changes as you cross a busy road). A hazard ratio is interpreted in a similar way to a risk ratio, as it describes how many times more (or less) likely a participant is to suffer the event at a particular point in time if they are exposed to the factor under study or not exposed. The hazard ratio is the only summary statistic that allows for both censoring and time to an event. Accordingly the first stage of the meta-analysis was to obtain a numerical estimate of the log hazard ratio and its standard error for each trial. The values of ratio treatment effects (such as the odds ratio, risk ratio, rate ratio and hazard ratio) always undergo log transformations before being analysed, and they may occasionally be referred to in terms of their log transformed values. The natural log (ln) transformation is used.

Conducting a meta-analysis using summary information from published papers with time-to-event outcome data is known to be problematic as the most appropriate summary statistics are typically not explicitly presented (134). My approach is summarised below and is based on the methods described by Parmar and colleagues (135).

If a Cox proportional hazards model was used to analyse the data the coefficient for the OLNM positive versus OLNM negative comparison was used as a direct estimate of the hazard ratio. Where a 95% confidence interval was quoted for the comparison coefficient the standard (SE) was calculated using equation 1.

(1) SE [ln(HR)] = [ln(upper confidence limit) – ln(lower confidence limit)]/3.92

When only a p-value was quoted with the coefficient, the *Z*-value corresponding to the reported *P*-value was first calculated. The standard error of the ln(HR) was then calculated using equation 2:

(2) SE [ln(HR)] = ln(HR) / Z

Where a Cox proportional hazards ratio was not used to analyse the data, the natural log of the hazard ratio - ln(HR) - and its standard error were extracted from quoted statistics or survival curves using the methods described by Parmar, Torri and Stewart (135) and are detailed below and in Appendix 4.

(3) $O_{ri} - E_{ri} = \{[\sqrt{(O_i R_{ri} R_{ci})}]/(R_{ri} + R_{ci})\} \times \Phi^{-1}(1 - p_i/2)$

(4) $V_{ri} = O_i R_{ri} R_{ci}/(R_{ri} + R_{ci})^2$

(5) $\ln(HR) = [(O_{ri} - E_{ri}) / V_{ri}]$

where:

O_{ri}= observed number of deaths in the OLNM positive group

O_{ci} = observed number of deaths in the OLNM negative group

E_{ri} = logrank expected number of events in OLNM positive group

E_{ci} = logrank expected number of events in OLNM negative group

V_{ri} = variance of the log hazard ratio

O_i = total number of deaths between the two groups

p_i = the reported p-value associated with the quoted logrank statistic

Φ = the cumulative distribution function of the Normal distribution.

R_{ri} = the number of patients in the OLNM positive group

R_{ci} = the number of patients in the OLNM negative group

Where neither of the above approaches could be used to estimate the log hazard ratio and its variance it was possible to use the method outlined below to obtain them from the published survival curves.

For each curve, the time axis was split into T non-overlapping intervals. The time intervals were chosen such that the event rate within each time interval was, if possible, less than 20% of those at the beginning of the time interval. The final interval was chosen such that the end of the interval was equal to the maximum estimated follow-up time (F_{max}). The survival probability was read

from the Kaplan-Meier curves at T prespecified time points. From reading the manuscript, the minimum and maximum follow-up of patients was estimated. The number alive and at risk was calculated for each of the T time intervals. The model for censoring during each time interval assumed that patients were censored at a constant rate during each time interval. The number of patients at risk of death in the OLNM positive group during the time interval (t-1, t) is given by:

(6) $R_{ri}(t) = R_{ri}(t-1) - D_{ri}(t-1) - C_{ri}(t)$

where:

$R_{ri}(t-1)$ = effective number of patients at risk in OLNM positive group during the time interval $(t-2, t-1)$.

$D_{ri}(t-1)$ = the effective number of deaths in OLNM positive group during the time interval $(t-2, t-1)$

$C_{ri}(t-1)$ = the effective number of patients censored in the OLNM positive group during the time interval

$(t-2, t-1)$.

Corresponding equations and definitions for the OLNM negative group were used.

The following conventions were used:

$R_{ri}(0)$= total number of patients in the OLNM positive group

$R_{ci}(0)$ = total number of patients in the OLNM negative group

$D_{ri}(0)= D_{ci}(0) = C_{ri}(0)= C_{ci}(0) = 0$

To calculate the effective number censored during a particular time interval (t – 1, t) the effective number of patients alive and at risk at the start of the interval was needed. For the OLNM positive group this was given be:

(8) $R_{ri}(t_s) = R_{ri}(t - 1) – D_{ri}(t - 1)$

The effective number of patients censored during the time interval (t – 1, 1) was then estimated using the following model for censoring: $C_{ri}(t)$ =

(9) $= R_{ri}(t_s)\left\{\frac{1}{2}\frac{(t_c - t_s)}{(F_{min} - t_s)}\right\}$.

Where:

N = total number of patients in the OLNM+ group

F_{min} = minimum follow-up in the trial

F_{max} = maximum follow-up in the trial

t_s = is start of time interval (t – 1, t)

t_e = end of time interval (t – 1, t)

$R_{ri}(t_s)$ = number at risk on research arm at beginning of time interval (t – 1, t).

The following conventions were used for this model:

If $t_s < F_{min}$ and $t_e < F_{min}$ the number censored = 0

If $t_s < F_{min}$ and $F_{min} \leq t_e \leq F_{max}$ then $t_s = F_{min}$

If $t_s < F_{min}$ and $t_e > F_{max}$, then $t_s = F_{min}$ and $t_e = F_{max}$

If $t_s > F_{min}$ and $t_e > F_{max}$, then $t_e = F_{max}$

Using the above calculations the effective number at risk in the OLNM+ group during the time interval (t – 1, t) could be calculated using:

(10) $$R_{ri}(t) = R_{ri}(t_s) - C_{ri}(t)$$

The effective number of deaths during the time interval (t – 1, t) in the OLNM+ group was then calculated as follows:

(11) $$D_{ri}(t) = \left[R_{ri}(t) \times \left(\frac{S_{ri}(t_s) - S_{ri}(t_e)}{S_{ri}(t_s)} \right) \right]$$

Where $S_{ri}(t_s)$ is the estimate of the survival probability in the OLNM+ group read from the Kaplan-Meier curve at the start of time interval (t – 1, t) and $S_{ri}(t_e)$ is the estimate of the survival probability in the OLNM+ group read from the curve at the end of the time interval (t – 1, t).

The log hazard ratio during the time interval (t – 1, t) was estimated using the equation:

(12) $$\ln(\mathbf{HR}_i(t)) = \ln\left(\frac{D_{ri}(t)/R_{ri}(t)}{D_{ci}(t)/R_{ci}(t)} \right)$$

The variance of this estimate was approximated using the equation:

(13) $$\mathrm{var}[\ln(\mathbf{HR}_i(t))] = \frac{1}{D_{ri}(t)} - \frac{1}{R_{ri}(t)} + \frac{1}{D_{ci}(t)} - \frac{1}{R_{ci}(t)}.$$

Where $D_{ri}(t)$ or $D_{ci}(t)$ were equal to 0, the zero was replaced by 10^{-6}.

An estimate of the overall log hazard ratio for the trial was given by a weighted sum of the individual extimates of the log hazard ratio during each time interval (t – 1, t) where the weights were inversely proportional to the variance of the estimate:

$$\ln(\mathrm{HR}_i) = \frac{\sum_{t=1}^{T} \frac{\ln(\mathrm{HR}_i(t))}{\mathrm{var}[\ln(\mathrm{HR}_i(t))]}}{\sum_{t=1}^{T} \frac{1}{\mathrm{var}[\ln(\mathrm{HR}_i(t))]}}.$$

(14)

An estimate of the variance of this estimate was given by:

$$\mathrm{var}[\ln(\mathrm{HR}_i)] = \left[\sum_{t=1}^{T} \frac{1}{\mathrm{var}[\ln(\mathrm{HR}_i(t))]}\right]^{-1}.$$

(15)

I designed a Microsoft Excel spreadsheet to perform these calculations (the entire spreadsheet for the indirect method from logrank statistics and an example of the spreadsheet used to estimate summary statistics from survival curves are reproduced in appendices 5 and 6 respectively).

In the second stage of the meta-analysis, a summary (pooled) treatment effect estimate and confidence interval were calculated. Additionally a p-value reflective of the strength of the evidence against the null hypothesis of no prognostic effect was derived. In accordance with the Cochrane Handbook's guidelines (136) the generic inverse variance approach to meta-analysis was adopted. It is so named because the weight given to each study is chosen to be the inverse of the variance of the effect estimate (the reciprocal of the square root of its standard error). A random effects meta-analysis (the DerSimonian and Laird version) was also performed as part of the sensitivity analysis.

The meta-analysis was performed on RevMan version 4.2 software. I obtained pooled estimates using a fixed effects model. Where heterogeneity is believed to be due to clinical diversity, the pooled estimate for random effects analyses should be interpreted differently from the fixed effect estimate since it relates to a different question. The random effects estimate and its confidence interval address the question 'what is the average prognostic effect?' while the fixed effect estimate and its confidence interval addresses the question 'what is the best estimate of the prognostic effect?' The answers to these questions coincide either when no heterogeneity is present (which is very unlikely to be the case in this study), or when the distribution of the treatment effects is roughly symmetrical (however it is difficult to establish the validity of any distributional assumption). When the answers do not coincide, the random effects estimate may not reflect the actual effect in any particular population being studied. Consequently the pooled effect estimate from a fixed effect meta-analysis is normally interpreted as being the best estimate of the treatment effect (137). Care must be taken that random effects analyses are not applied when the idea of a 'random' distribution of treatment effects cannot be justified. In particular, if results of smaller studies are systematically different from those of larger studies, which can happen as a result of publication bias or methodological quality bias, then a random effects model will exacerbate the effects of the bias.

We assessed publication bias visually using a funnel plot.

SENSITIVITY ANALYSIS

Variability in the treatment effects being evaluated in the different trials is known as statistical heterogeneity and is a consequence of clinical and/or methodological heterogeneity. Henceforth I will refer to statistical heterogeneity simply as heterogeneity.

A chi-squared test for heterogeneity was used. It assesses whether observed differences in results are compatible with chance alone. A low p-value (or a large chi-squared statistic relative to the degree of freedom) would provide evidence of heterogeneity of treatment effects. The chi-squared test for heterogeneity was used with a p-value of 0.1 rather than 0.05 to determine statistical significance (138). The I^2 statistic (equation 16) was used to quantify the variability across studies attributable to heterogeneity rather than to random variation.

(16) $I^2 = [(Q - df) / Q] \times 100\%$

Q = chi-squared statistic

Df = degrees of freedom

A value greater than 50% was considered significant.

We used sub-group analysis to explore inter-study heterogeneity. Three subgroup analyses with robust clinical justification were defined *a priori*. The first was defined by the number of sections examined per lymph node (1 or more than 1); the second was defined by the mean number of lymph nodes examined per case (≤20 or >20) and the third was based on methodological quality (studies at high risk of bias versus the remaining studies). I used the significance test described by Deeks (139) to investigate differences within subgroup analyses. It is described below:

If

Q_{all} is the chi squared statistic for all the trials

Q_1 is the chi squared statistic for trials within the first subgroup

Q_2 is the chi squared statistic for trials within the second subgroup

Then:

$Q_{int} = Q_{all} - (Q_1 + Q_2)$ with 1 degree of freedom tests for a difference between the first and second subgroups. A p-value was then derived from the chi-squared statistic.

RESULTS

CLINICOPATHOLOGIC FACTORS PREDICTING COMPLETE PATHOLOGICAL RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY IN OESOPHAGEAL CANCER

176 patients underwent multi-modal therapy at this institution between 1990 and 2003. There were 118 men (67%) and 58 (33%) women (male : female = 2.0 : 1). The median age at diagnosis was 62 (upper – lower quartiles : 48 – 76). The series included 53 (30%) squamus cell carcinomas and 121 (69%) adenocarcinomas. All patients underwent multimodality therapy comprised of neo-adjuvant chemoradiotherapy followed by radical surgery.

The summary of demographic, social, clinical, nutritional and histopathological features as assessed before neo-adjuvant therapy for the whole study group and their relation to response to chemo-radiotherapy is shown in the table below (Table 3).

Variable	Incomplete Pathological Response n (%)	Complete Pathological Response n (%)	Total n	*p*
Total number of patients	122 (77%)	36 (23%)	158	n.s.
Sex Male Female	86 (79%) 36 (73%)	23 (21%) 13 (27%)	109 49	n.s.
Median Age at diagnosis Median (upper and lower quartiles)	62 (53-66)	64 (57-67)	-	n.s.
History of cigarette smoking	62 (76%)	20 (24%)	82	n.s
History of heavy alcohol intake	20 (80%)	5 (20%)	25	n.s.
Duration of symptoms (months) Median (upper and lower quartiles)	12 (8-25)	11 (8-26)	-	n.s.
Co-morbid disease	41 (76%)	13 (24%)	54	n.s.
Pre-treatment body mass index Median (upper and lower quartiles)	26.2 (22.7 Š 28.8)	25.7 (24.9 Š 27.1)	-	n.s.
Pre-treatment FEV_1 Median (upper and lower quartiles)	3.1 (2.4 Š 3.8)	2.6 (2.1 Š 3.4)	-	<0.05
History of BarrettÕ Oesophagus	42 (78%)	12 (22%)	54	n.s.
Dysplasia	26 (74%)	35 (26%)	61	n.s.
Squamus cell carcinoma	17 (59%)	12 (41%)	29	p < 0.05
Adenocarcinoma	89 (79%)	23 (21%)	112	n.s.
Site Proximal Stomach Lower Oesophagus Upper / Middle Oesophagus	 18 (86%) 79 (75%) 9 (56%)	 3 (14%) 26 (25%) 7 (43%)	 21 105 16	 n.s.
Differentiation Well Moderate Poor Undifferentiated	 3 52 47 20	 1 (25%) 13 (20%) 10 (18%) 8 (29%)	 4 65 57 28	 n.s.
Clinical T stage T_0 T_1 T_2 T_3 T_4	 5 4 5 29 2	 0 (0%) 1 (20%) 3 (38%) 22 (43%) 0 (0%)	 5 5 8 51 2	
Clinical N stage N_1	 36	 9 (25%)	 45	
Pre-treatment tumour length (cm) Median (upper and lower quartiles)	3.0 (2.0 Š 5.0)	2.0 (1.0 Š 5.0)		p < 0.05
Pre-treatment tumour width Median (upper and lower quartiles)	2.0 (1.1 Š 3.0)	0.7 (0.0 Š 1.5)		P < 0.05

Table 3.

Table 3: Summary of demographic, social, clinical, nutritional and histopathological features as assessed before neo-adjuvant therapy for the whole study group and their relation to response to chemo-radiotherapy.

There were 40 (23%) cases of complete pathological response. There was no significant difference between responders and non-responders with respect to demographic and social variables. No pre-operative clinical or nutritional parameters, namely duration of symptoms before diagnosis, co-morbidity or body mass index were found to be significantly associated with response. Of the pathological variables, tumour length was significantly correlated with response. The median pre-treatment tumour length among pCR cases was 2.0cm (range 1.0 – 5.0cm) compared to 3.0cm (2.0 – 5.0cm) in the non-responders ($p<0.05$). No other pathological variables, namely location, differentiation, histological type or presence of Barrett's Oesophagus or dysplasia were predicitive of reponse.

IMPACT OF ISOLATED TUMOUR CELLS IN PATHOLOGICAL NODE-NEGATIVE LYMPH NODES (PN0) ON PROGNOSIS IN CANCER OF THE OESOPHAGUS OR OESOPHAGO-GASTRIC JUNCTION

Demographic and clinical details of the study group (all patients with pN0 tumours who underwent curative therapy for oesophageal or gastro-oesophageal cancer – with or without neoadjuvant therapy - between January 1990 and September 2002) were identified from the prospectively compiled

upper gastrointestinal cancer database at this institution are displayed in (Table 4). The median age of the patients was 63 (range 29 – 84). There were 101 (69%) men and 45 (41%) women. A total of 1,694 lymph nodes were dissected from the 146 oesophagogastrectomy specimens with a mean of 11 nodes per patient. Follow-up data was obtained for all 146 patients. The median follow-up time was 28 months (range 0 to 160 months) and the median actuarial overall survival time was 21 months (95% quartiles: 34 – 64 months) for the entire study group. Positive MNF 116 staining with malignant morphology was found in 24 of 1,694 lymph nodes (1%) and in 12 of 146 patients (8%) studied. ITC was predominantly identified in the subcapsular sinuses either as a single cell (Figure 1) or as small clusters of tumour cells. At a median follow-up of 28 months (range 0 – 160) 59 patients were alive (four with evidence of relapse) and 87 patients had died. The overall and relapse free actuarial survival rates were significantly worse among patients who were pN0(i+). The actuarial median overall survival in the pN0(i-) patients was 53 months (95% quartiles: 27 – 69 months) versus 21 months (9 – 28) for the pN0(i+) patients ($p < 0.05$) (Figure 2). The relapse-free actuarial survival rate was 40 (27 – 56) months for the pN0 (i-) patients versus 21 (9 – 28) months for the pN0 (i+) patients ($p < 0.05$) (Figure 3).

Variable	Number of patients (%)
Male	101 (69%)
Female	45 (31%)
Male:Female	2.2:1
Tumour site	
Upper oesophagus	3 (2%)
Middle oesophagus	21 (14%)
Lower oesophagus	57 (39%)
OG junction	65 (45%)
Histology	
Squamous cell carcinoma	51 (35%)
Adenocarcinoma	95 (65%)
Differentiation	
Well	18 (12%)
Moderately	69 (46%)
Poorly	37 (27%)
Undifferentiated	22 (15%)
Pathological tumour stage	
Tis	5 (3%)
T0	30 (21%)
T1	29 (20%)
T2	19 (13%)
T3	60 (41%)
T4	3 (2%)

Abbreviations:
(OG – oesophagogastric; Tis – tumour in situ)

Table 4.

Demographic and pathological data for patients with pN0 tumors who were treated with either surgery alone or surgery plus neoadjuvant therapy.

Figure 1. ITC was predominantly identified in the subcapsular sinuses. Example of a single cell.

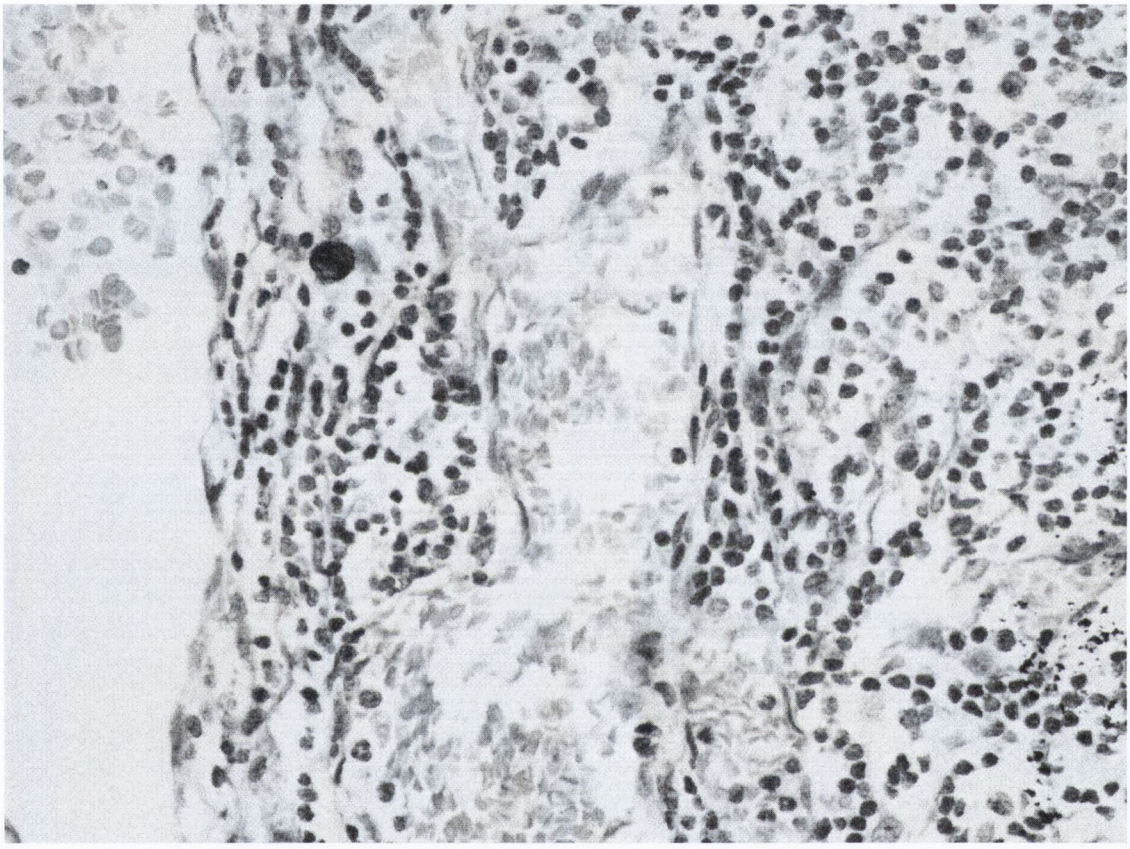


Figure 2. Overall survival - Kaplan-Meier survival curve.

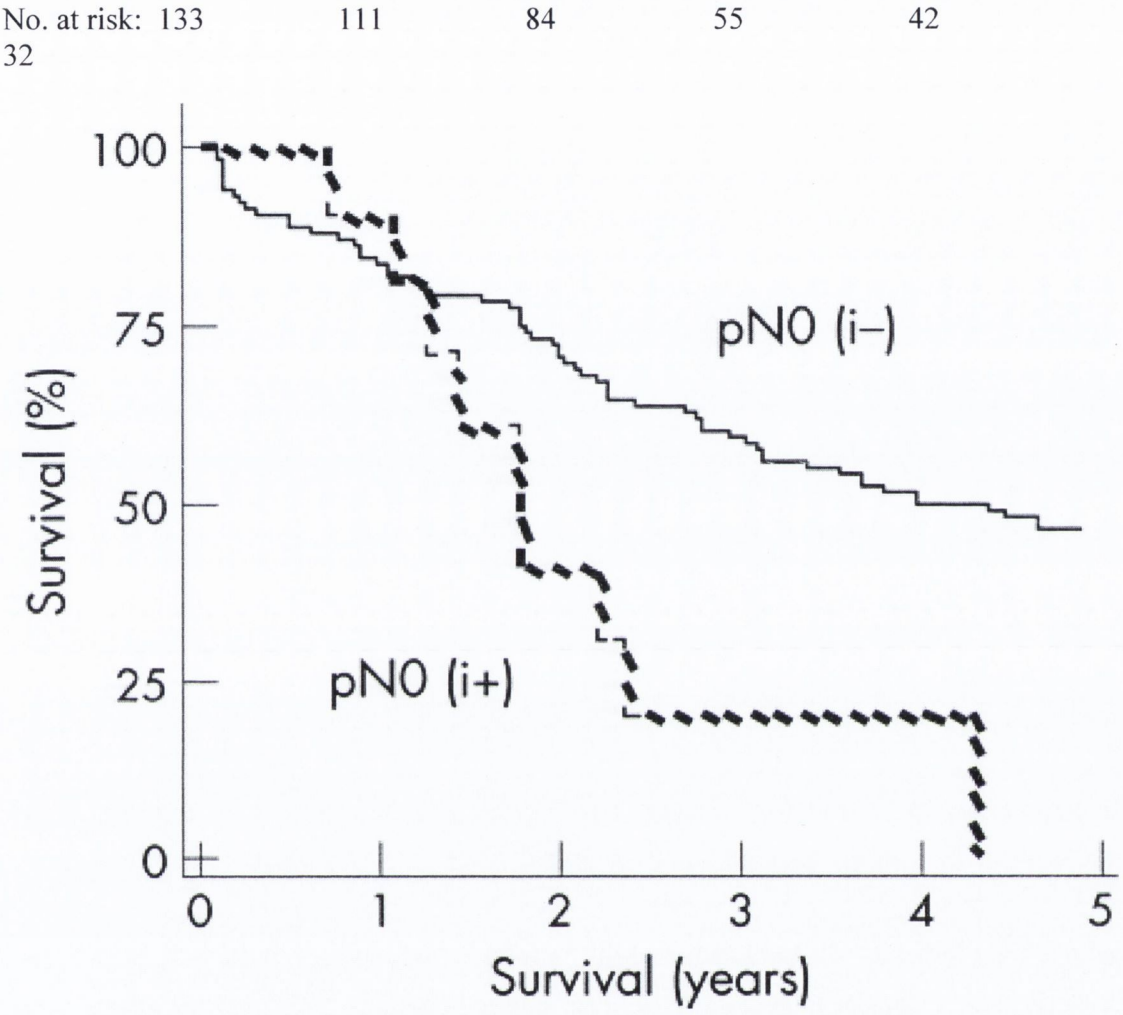
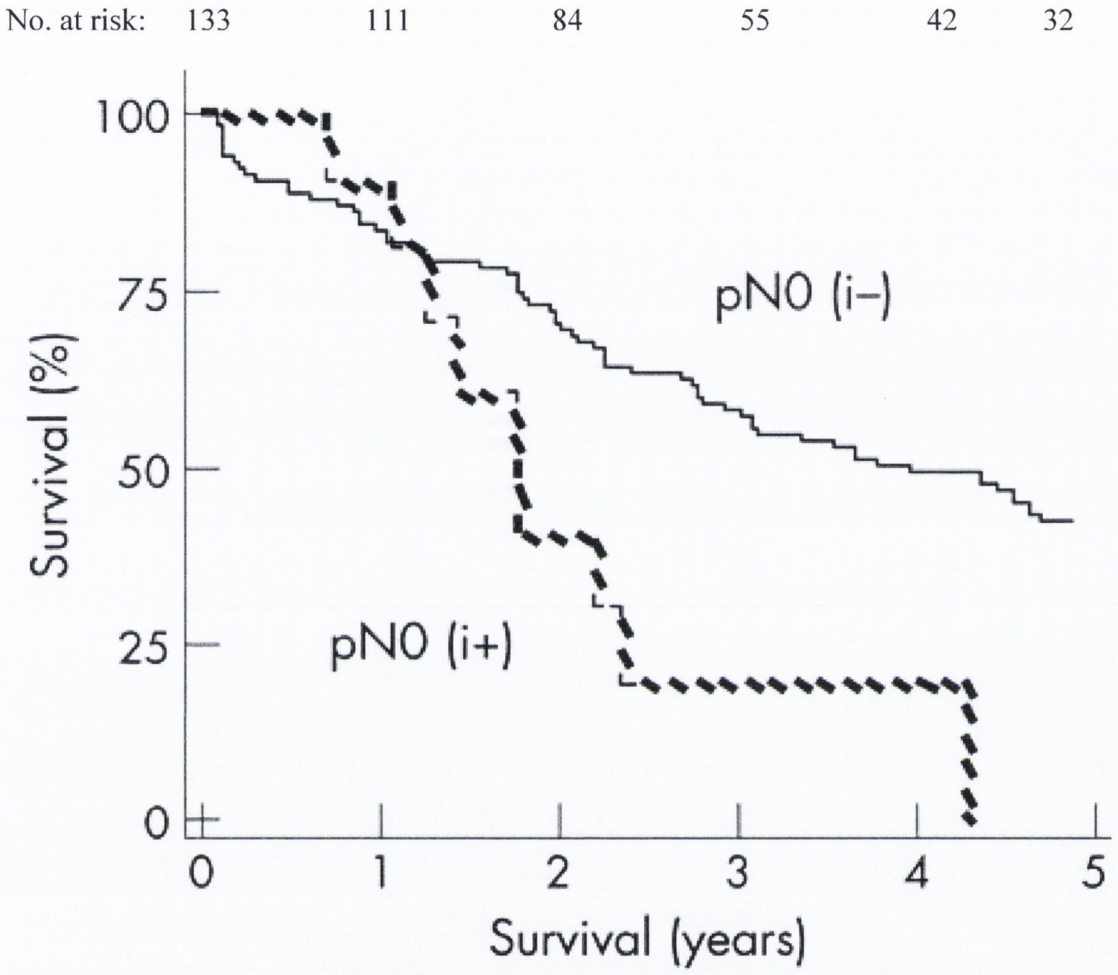


Figure 3. Relapse free survival curves.



No significant correlation was found between isolated tumour cells and a number of clinico-pathological parameters including age, gender, tumour location, treatment modality, tumour stage, tumour grade, tumour length or mean number of lymph nodes examined (Table 5). Multivariate analysis revealed that degree of differentiation and the presence of isolated tumour cells were independent prognostic factors for both relapse-free and overall survival (Table 6).

Variable	Positive (%)	Negative (%)	P-value
All patients	12 (8)	134 (92)	
Male	7 (7)	94 (93)	n.s.
Female	5 (11)	40 (89)	
Age Mean (s.d.)	60 (9)	61 (10)	n.s.
pTis	0 (0)	4 (100)	
pT0	1 (4)	26 (96)	
pT1	1 (4)	25 (96)	n.s.
pT2	2 (11)	17 (89)	
pT3	5 (9)	49 (91)	
pT4	2 (100)	0 (0)	
Squamous cell carcinomas	1 (2)	48 (98)	
Adenocarcinoma	11 (12)	82 (88)	n.s.
Other	0 (0)	4 (100)	
Well	1 (6)	17 (94)	
Moderately	4 (6)	65 (94)	n.s.
Poorly	6 (16)	31 (83)	
Undifferentiated	1 (5)	20 (95)	
Upper	0 (0)	3 (100)	
Middle	0 (0)	21 (100)	n.s.
Lower	5 (9)	52 (91)	
OGJ	7 (11)	58 (89)	
Surgery alone	9 (12)	67 (88)	n.s.
Multi-modal	3 (4)	67 (96)	
Barrett's Oesophagus:			
Yes	5 (12%)	37 (88%)	n.s.
No	7 (7%)	96 (93%)	
No. of dissected lymph nodes Mean (s.d.)	16 (9)	11 (13)	n.s.
Tumour length (cm)	2.7 (0.9)	3.5 (1.9)	n.s.

Table 5.

Relationships between various clinicopathological parameters and findings of ITC.

Factor	Risk Ratio	(95% confidence limits)
ITC (Yes / No)	2.92	1.32 – 5.91
Gender	0.71	0.52 - 0.96
Age at diagnosis (≥63 years, <63 years)	1.05	0.81 - 1.08
Tumour site (Upper / Lower / Middle / OGJ)	0.79	0.49 - 1.39
Morphology (Adenocarcinoma / Squamous Cell Carcinoma)	0.33	0.16 - 0.94
Differentiation (Well, moderate, poor)	1.84	1.28 - 2.69
Treatment (Multi-modal / surgery)	1.11	0.88 - 1.41

Table 6.

Multivariate analysis for prognosis after resection for pN0 gastro-oesophageal carcinoma.

THE PROGNOSTIC IMPACT OF OCCULT LYMPH NODE METASTASIS IN CANCER OF THE OESOPHAGUS OR OESOPHAGO-GASTRIC JUNCTION: SYSTEMATIC REVIEW AND META-ANALYSIS OF OBSERVATIONAL STUDIES.

Our search strategy yielded 762 articles, of which 14 were non-overlapping and met the inclusion criteria. 2 (122, 140) of these 14 studies were excluded from the meta-analysis (see on-line addenda for details of excluded studies) as it was not possible to obtain sufficient data to accurately estimate effect. The remaining 12 cohort studies (141-132) included information on 741 patients (192 positive for occult lymph node metastases). Key features of the included and excluded studies are compared in the table below (Table 7).

Study	N	Positive	Histology	% Adjuvant	Location	Antibody	Sections	Nodes	Risk of bias
Included Studies									
Natsugoe 1998	48	27%	SCC	0	Oesophagus	AE1/AE3	1	24	High
Mueller 2000	75	17%	adeno	0	OGJ	AE1/AE3	1	28	High
Heeren 2005	60	30%	adeno	0	OGJ	AE1/AE3	>1	11	High
Matsumoto 2000	59	56%	SCC	0	Oesophagus	AE1/AE3	1	50	Low
Sato 2001	50	40%	SCC	0	Oesophagus	AE1/AE3	1	37	Low
Vazquez 2002	124	11%	Adeno & SCC	0	Oesophagus	AE1/AE3	1	16	Low
Nakamura 2002	53	26%	SCC	0	Oesophagus	AE1/AE3	1	47	Low
Hosch 2000	54	56%	Adeno & SCC	0	Oesophagus	BerEP4	3	17	High
Laso 2004	21	24%	Adeno & SCC	N/A	Oesophagus	AE1/AE3	1	N/A	High
Komukai 2000	37	37%	SCC	0	Oesophagus	AE1/AE3	5	75	High
MacGuill 2006	146	8%	Adeno & SCC	48%	OGJ & Oes.	MNF116	1	11	Low
Excluded Studies									
Glickman 1999	78	26%	Adeno & SCC	64%	Oesophagus	AE1/AE3	5	7	Low
Xiao 2002	42	62%	Adeno & SCC	0	Oesophagus	AE1/AE3	3	9	High

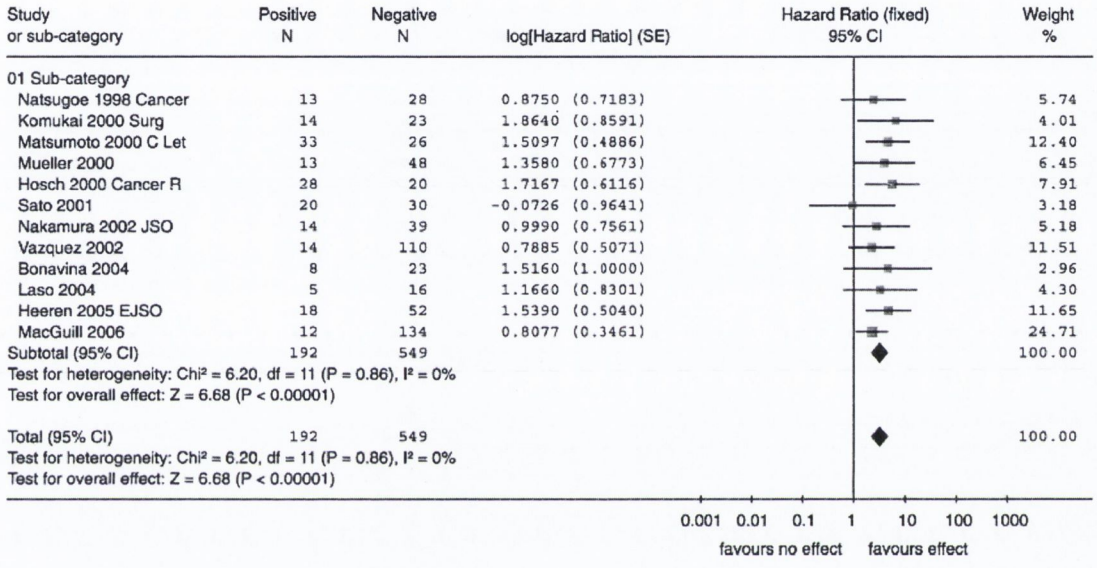
Table 7.

Selected characteristics of included and excluded studies.

N = number of cases; Positive = % of OLNM positive cases; SCC = squamous cell carcinoma, adeno = adenocarcinoma; % adjuvant = % of patients who received neo-adjuvant chemoradiotherapy; N/A = not available; OGJ = oesophagogastric junction; Oes. = oesophagus; sections = number of sections examined per block/lymph node; Nodes = mean number of lymph nodes examined per case.

A fixed effect meta-analysis produced a pooled hazard ratio for disease relapse in positive patients relative to negative patients of 3.16 (95% confidence interval 2.25 to 4.42) (Figure 4). The P-value obtained from the fixed effect meta-analysis of overall effect was <0.00001. When the 7 trials judged to be at high risk of bias were excluded from the meta-analysis, the hazard ratio was reduced to 2.52 (1.61 – 3.94).

Figure 4. Forest plot and details of all included studies.



Neither the chi-squared test (p = 0.65) or a visual assessment of overlap of confidence intervals on the Forest plot suggested significant inter-trial heterogeneity. Significance testing using the method described by Deeks et al did not demonstrate that any of the subgroup factors (number of sections examined per node/paraffin block, mean number of lymph nodes examined per case or methodological quality) were significant sources of heterogeneity (Table 8). and (Figures 5&6)

Figure 5. Forrest plots for sub-groupings based on mean number of nodes examined.

Study (sub-group)	Positive N	Negative N	log[Hazard Ratio] (SE)	Harzard ratio (random) 95% CI	Weigth %	Hazard ratio 95% CI
(<20 nodes)						
Hosch 2000	28	20	1.7167 (0.6116)		7.91	5.57(1.68, 18.46)
Vazquez 2002	14	110	0.7885 (0.5071)		11.51	2.20(0.81, 5.94)
Heeren 2005	18	52	1.5390 (0.5040)		11.65	4.66(1.74, 12.51)
MacGuill 2006	12	134	0.8077 (0.3461)		24.71	2.24(1.14, 4.42)
Subtotal	72	316			55.78	2.96(1.09, 4.05)
(>20 nodes)						
Natsugoe 1998	13	28	0.8750 (0.7183)		5.74	2.40(0.59, 9.80)
Komukai 2000	14	23	1.8640 (0.8591)		4.01	6.45(1.20, 34.74)
Matsumoto 2000	33	26	1.5097 (0.4886)		12.40	4.53(1.74, 11.79)
Mueller 2000	13	40	1.3500 (0.6773)		6.45	3.09(1.03, 14.67)
Sato 2001	20	30	-0.0726 (0.9641)		3.18	0.93(0.14, 6.15)
Nakamura 2002	14	39	0.9990 (0.7561)		5.18	2.72(0.62, 11.95)
Bonavina 2004	8	23	1.5160 (1.0000)		2.96	4.55(0.64, 32.33)
Subtotal	115	217			39.92	3.45(2.02, 4.88)
Test for heterogeneity: Chi Sq. = 3.15 (p = 0.79)						
Total (95% CI)	107	533	100.00	3.15(2.23, 4.45)		

Test for heterogeneity Chi Sq. = 6.20 (p = 0.80)
Test for overall effect Z = 6.53 (P < 0.00001)

Review: A systematic review of the prognostic impact of lymph node micrometastasis in cancer of the oesophagus (working draft)
Comparison: 01 Relapse free survival
Outcome: 04 Study Quality

Study or sub-category	Posit N	Negative N	log[Hazard Ratio] (SE)	Hazard Ratio (fixed) 95% CI	Weight %	Hazard Ratio (fixed) 95% CI
01 High risk of bias						
Natsugoe 1998 Cancer	13	28	0.8750 (0.7183)		5.74	2.40 [0.59, 9.80]
Komukai 2000 Surg	14	23	1.8640 (0.8591)		4.01	6.45 [1.20, 34.74]
Mueller 2000	13	48	1.3580 (0.6773)		6.45	3.89 [1.03, 14.67]
Hosch 2000 Cancer R	28	20	1.7167 (0.6116)		7.91	5.57 [1.68, 18.46]
Bonavina 2004	8	23	1.5160 (1.0000)		2.96	4.55 [0.64, 32.33]
Laso 2004	5	16	1.1660 (0.8301)		4.30	3.21 [0.63, 16.33]
Heeren 2005 EJSO	18	52	1.5390 (0.5040)		11.65	4.66 [1.74, 12.51]
Subtotal (95% CI)	99	210			43.02	4.25 [2.54, 7.11]
Test for heterogeneity: Chi² = 1.23, df = 6 (P = 0.98), I² = 0%						
Test for overall effect: Z = 5.52 (P < 0.00001)						
02 Low risk of bias						
Matsumoto 2000 C Let	33	26	1.5097 (0.4886)		12.40	4.53 [1.74, 11.79]
Sato 2001	20	30	-0.0726 (0.9641)		3.18	0.93 [0.14, 6.15]
Nakamura 2002 JSO	14	39	0.9990 (0.7561)		5.18	2.72 [0.62, 11.95]
Vazquez 2002	14	110	0.7885 (0.5071)		11.51	2.20 [0.81, 5.94]
MacGuill 2006	12	134	0.8077 (0.3461)		24.71	2.24 [1.14, 4.42]
Subtotal (95% CI)	93	339			56.98	2.52 [1.61, 3.94]
Test for heterogeneity: Chi² = 2.70, df = 4 (P = 0.61), I² = 0%						
Test for overall effect: Z = 4.06 (P < 0.0001)						
Total (95% CI)	192	549			100.00	3.16 [2.25, 4.42]
Test for heterogeneity: Chi² = 6.20, df = 11 (P = 0.86), I² = 0%						
Test for overall effect: Z = 6.66 (P < 0.00001)						

Figure 6. Forest plots of sub-groupings based on study methodological quality.

	No. of studies	HR (95% CI)	P-value*
No. of nodes			
>20	7	3.45 (2.02, 5.88)	0.099
<20	4	2.96 (1.89, 6.65)	
No. of sections			
1	9	2.68 (1.85, 3.89)	0.079
>1	3	5.23 (2.61, 10.47)	
Risk of bias			
high	7	4.25 (2.54, 7.11)	0.089
low	5	2.52 (1.61, 3.94)	

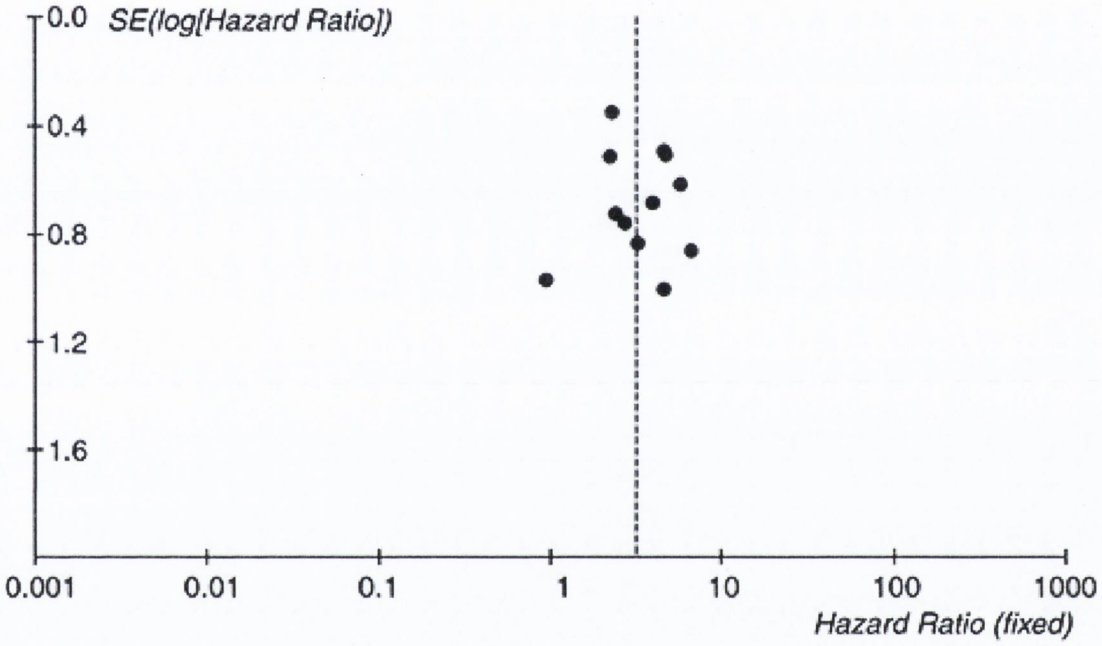
Table 8.

Details of heterogeneity analysis

***p-value associated with test for heterogeneity described by Deeks et al.**

In order to assess the sensitivity of the study we reanalysed the data using a random effects model instead of a fixed effect model (the random effects model pooled hazard ratio was 3.16 with 95% confidence intervals of 2.25 to 4.42). Then we re-examined the data following exclusion of studies deemed to be at high risk of bias. Additionally we reanalysed the data after inputing a reasonable range of values for missing data from excluded studies. These changes did not materially change the results of the meta-analysis. The funnel plot did not suggest marked publication bias (Figure 7).

Figure 7. The funnel plot for all included studies did not suggest marked publication bias.



DISCUSSION

CLINICOPATHOLOGIC FACTORS PREDICTING COMPLETE PATHOLOGICAL RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY IN OESOPHAGEAL CANCER

In this series, in which 176 gastro-oesophageal carcinomas are described, we report a 23% complete pathological response rate after multimodality treatment. Our most significant finding was the relationship between tumour length and complete pathological response. Decreased tumour length was correlated with increased likelihood of complete response to pre-operative chemo-radiotherapy. Tumour size and tumour cell number have long been recognised as important determinants of tumour response to treatment (153). Furthermore tumour length was identified as an important prognostic factor for survival in patients with oesophageal cancer in a recent analysis (n=10,441) of the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) database (154). However it remains unclear why larger neoplasia tend to be less responsive to adjuvant therapies. The most easily remediable explanation for treatment failure in larger tumours is that the existing dose and treatment schedule is inadequate. Although this hypothesis has not been specifically addressed in oesophageal cancer, it has been a focus of considerable research in breast cancer. Following reports that larger breast cancers were less responsive to chemo-radiotherapy it was hypothesised that existing dose and treatment schedules for breast cancer were sub-optimal.

Consequently a number of trials of escalation of adjuvant therapy dosage, up to and including high-dose therapy with bone marrow stem-cell transplantation, were undertaken (155-159). Unfortunately these trials failed to show significant survival benefit associated with dose escalation. However it was reported that certain sub-groups of breast cancers displayed dose-response type behaviour. For instance overexpression of erbB-2 was identified as a reasonably effective predictor of improved response upon increased dose of anthracyclins in the adjuvant setting (160, 161). Although this work did not result in any changes in standard clinical practice, the principle that some breast cancers are susceptible to dose escalation was demonstrated.

The apparent lack of responsiveness among larger tumours can alternatively be attributed to different tumour biology; larger tumours may be inherently more resistant than smaller tumours. As will be discussed, evidence indicates that defective apoptosis may be the basis of chemotherapeutic resistance in many cases. The overall growth or regression of a tumour is dependant upon the balance between proliferation and apoptosis. Despite variations in individual mechanisms of action, chemotherapy and radiotherapy ultimately bring about cancer cell death by activation of the apoptotic cascade (162, 163). Consequently changes in apoptosis and proliferation are thought to be finally involved in the response process. The role of apoptosis and proliferation in the biology of adjuvant therapy failure has been extensively investigated. We have previously reported that spontaneous apoptosis occurs in all cases of oesophageal adenocarcinoma, that patients with a high apoptotic index have a

better response to neo-adjuvant therapy and that apoptosis was significantly induced by chemoradiotherapy (164). Regulators of apoptosis include markers such as p53, the Bcl-2 protein family, caspases and DNA fragmentation factor. Both in vivo and in vitro work has implicated these factors in the biology of chemoresistance. Loss of p53 function has been shown to disrupt apoptosis and accelerate tumour development in transgenic mice (165). in addition, loss of p53 function correlates with multi-drug resistance in many tumour types (166). Bcl-2 proteins are a family of cytoplasmic proteins. Bcl-2 and Bcl XL inhibit apoptosis whereas Bax, Bag-1 and Bad promote it. Residual breast tumour at cessation of chemotherapy has been shown to have increased Bcl-2 concentrations relative to pre-chemotherapy specimens(167). Additional defects in apoptosis implicated in adjuvant therapy failure include functional mutations in many p53 upstream regulators and downstream effectors. Downstream regulators include Apaf-1, PTEN, heat shock proteins, inhibitor of apoptosis proteins (IAPs) and caspase-8. Apaf-1 is downstream to the Bcl-2 family and is involved in activation of caspase-9 activation. In-vitro studies on human leukaemia and ovarian cell lines have shown that loss of Apaf-1 expression is associated with decreased drug-induced apoptosis (168). On the other hand altered function of upstream regulators (e.g. ataxia telangiectasia mutated gene (ATM) or CHk2) also frequently occur in tumours and, in many cases, correlates with drug resistance (169).

Proliferative pathways have been less widely studied. HER2 (c-erbB-2, c-neu) a transemembrane receptor similar to epidermal growth factor, is the most extensively investigated. Activation of HER2 induces activation of ras, leading to a phosphorylation cascade ultimately resulting in cell proliferation. As mentioned above, HER-2 expression has been associated with chemotherapeutic responsiveness in breast cancer.

Future comparisons of the apoptotic and proliferative pathways in responding and non-responding tumours may enable accurate prediction of response to neo-adjuvant therapy and provide the basis for more tailored treatment. The new techniques of comparative genomic hybridisation, gene expression micro-array and proteomics will be useful in performing these comparisons. Additionally, it has recently been reported that serial 18F-labelled deoxygluccose (FDG) positron emission tomography (PET) (FDG-PET) assessment of response to neo-adjuvant therapy is strongly correlated with histopathological findings (170-172). On the basis of these findings, the ability of pre-treatment FDG-PET to predict response to neo-adjuvant chemo-radiotherapy is under assessment at this and a number of other institutions.

In conclusion our most significant observation was that smaller tumour size was predictive of a greater response to chemotherapy and radiation therapy. This may reflect different tumour biology, perhaps with acquired resistance to treatment-induced apoptosis in the larger tumours. A simpler explanation is

that the existing dose and treatment schedule for combination chemoradiotherapy is sub-optimal in patients with larger tumours. Both these hypotheses require further evaluation.

IMPACT OF ISOLATED TUMOUR CELLS IN PATHOLOGICAL NODE-NEGATIVE LYMPH NODES (PN0) ON PROGNOSIS IN CANCER OF THE OESOPHAGUS OR OESOPHAGO-GASTRIC JUNCTION

A variety of techniques have been developed to demonstrate occult tumour cells at sites such as the bone marrow, blood and lymph nodes of patients with breast cancer, colon cancer, non-small-cell lung cancer, prostate cancer, melanoma and oesophagogastric cancers (85, 112, 173, 174, 175). Both *in vivo* and *in vitro* studies have suggested that these cells not only have the phenotype of malignant cells, but also possess malignant molecular characteristics (76, 79, 176, 177). Nevertheless the prognostic significance of these cells remains controversial, and a number of studies and a meta-analysis have failed to verify the presence of occult metastases as an independent prognostic factor in solid tumours (178).

One confounding variable in the interpretation of the literature to date has been the inconsistency in nomenclature for "metastases" detected by these methods. They have been inconsistently classified *inter alia* as micrometastases, sub-clinical metastases, occult metastases, and tumour cell micro-involvement. The UICC has tried to clarify the terminology by making a distinction between "micrometastastasis" and "isolated tumour cells" (43).

Micrometastases are defined as being ≤ 2mm in greatest dimension, in contact with a vessel wall, extravasated, proliferating and usually associated with a stromal reaction. Isolated tumour cells (ITC) in contrast are defined as clusters (<0.2mm) or single tumour cells without any of the above characteristics whose presence can only be determined by immunohistochemistry, immunocytochemistry or molecular methods such as flow cytometry or PCR. This study is to our knowledge the largest evaluation of the prevalence and prognostic significance of isolated tumour cells in patients reported as pN0 after curative treatment for localized cancer of the oesophagus or oesophago-gastric junction. The study reports a low prevalence of isolated tumour cells in pN0 lymph nodes but a significant impact on overall and relapse-free survival.

We have identified 12 previous studies examining the prevalence and prognostic impact of occult lymph node metastases in oesophogastric cancer (99, 122, 141, 142, 144-147, 179-182). Seven of these studies reported a significant impact on survival (99, 141, 142, 144, 180-182), 4 did not show significance (122, 145-147), and one paper did not specify prognostic impact with respect to pN0 cases (179). This study differs from these earlier studies in a number of respects. We report on a larger series of pN0 patients than previous studies. Patients with pathologically involved nodes (pN1) were excluded, in contrast to other series (99, 122, 141, 142, 179, 180, 181). With the exception of a study by Vazquez-Sequeiros et al (146), which reported a prevalence rate of 9%, the rate of 8% in this series was considerably lower than those quoted in previous studies which ranged from 26 to 62 per cent.

There are a number of potential explanations for this low prevalence rate. Many pathologists report as relatively common a finding of single epithelial cells identified by immunohistochemistry for cytokeratin that on careful analysis was found to be hyalinized cytokeratin particles or artifact of overlay of cells from the staining solutions, water bath, or keratinocytes from the skin of the hands of the technician (52). These were also encountered during the course of this study, but only positively staining material with both cellular and malignant morphology as determined by an experienced histopathologist was classified as positive, consistent with current consensus (38).

A significant proportion of pN0 patients will be reclassified as pN1 when additional lymph nodes are sampled in a systematic manner (183, 184), and it is not unreasonable to infer that the likelihood of finding isolated tumour cells directly correlates with the number of sampled nodes. It is notable that studies such as this from the western world report much smaller nodal yield compared with Japanese series in particular (141, 144, 145, 147, 181). Notwithstanding this possibility, we did not find a significant difference in the average numbers of nodes sampled per case between the positive and negative groups in this study.

There is some experimental evidence that the greater the number of sections sampled per lymph node, the higher is the probability of identifying isolated

tumour cells or micrometastases (179, 185). It is also clear that to serially section and immunostain every resected node after an en bloc oesophagogastrectomy is not a practical proposition for routine pathology laboratories. Accordingly investigators make an empiric decision to sample somewhere between 1 and 5 sections per lymph node. We elected to sample one 4μm section per lymph node because this was standard practice at this institution and would therefore be a procedure that we could expect to be practicable should this investigation suggest itself to be of clinical relevance.

In summary this study shows that the prevalence of isolated tumour cells in pN0 lymph nodes of oesophagogastric cancer may be lower than previously reported, and that the presence of isolated tumour cells has prognostic significance when analysed in a sufficiently large cohort using stringent detection criteria. The integration of ITC detection into the routine staging of oesophagogastric cancers could improve our ability to determine prognosis and bring the realization of the goal of patient-tailored cancer therapy closer.

In summary, the results of this meta-analysis suggest that occult lymph node metastasis has a significant detrimental impact on prognosis in node negative cancers of the oesophagus or gastro-oesophagus. The sensitivity analysis shows that the results of this meta-analysis are robust to the choice of number of sections examined per node or block, to the mean number of lymph nodes examined, to the choice of the statistical method and to the exclusion of trials of poorer quality. It also suggests that publication bias is unlikely to have distorted its findings.

However this meta-analysis is subject to several limitations. Any meta-analysis of observational studies, particularly those of time-to-event data, comes with a number of "health warnings". Being made up of non-randomised trials, there are potentially unknown confounding factors, which could cause selection bias. Clearly all important factors relevant to prognosis in cancer of the oesophagus or gastro-oesophagus are not yet known. I have attempted to control for some of the known factors through the sensitivity analyses.

The quality of procedures for controlling for differences between the OLNM positive and negative groups was an important element of our overall quality assessment. We classified trials, which did not compare their OLNM positive and negative groups with respect to some key pathological parameters (specifically T-staging and tumour grade) as high risk studies. Additionally two trials were classified as high risk despite reporting a comparison of the pathological characteristics of their two groups because the comparison demonstrated significant differences between the two groups.

With respect to our procedures to investigate inter-trial heterogeneity, we sought to avoid "data-dredging" by strictly limiting the number of parameters selected for analysis *a priori*. Consequently it is simply not possible to examine all conceivable sources of inter-trial heterogeneity in this type of study. Indeed it can be seen in table 1 that there are a number of obvious sources of clinical heterogeneity within the included trials for which we did not control. The parameters selected for the sensitivity analysis were those which we deemed to have the most robust clinical and scientific rationale: number of nodes examined, number of sections per node/block examined and methodological quality.

We extracted or estimated summary statistics from each trial using methods described by Parmar *et al.* Clearly such an approach will never be as complete or secure as collecting individual patient data and has a number of intrinsic

sources of error. The smaller the number of events (meaning the total number of relapses and/or disease related deaths in this study population) the greater is the probability of a significant difference between the estimated log hazard ratio and variance and the true values. Relative to most meta-analyses of large intervention trials, the size of and number of events within the cohorts in this study were small. Further, the methods described by Parmar *et al* are intended for use with p-values quoted for two-sided logrank statistics. We thought it a safe assumption that all statistical tests were two-tailed unless it was clearly stated in the text that they were not. Williamson *et al* (186) have pointed out that the method described by Parmar *et al* for estimating the variance of the log hazard ratio from a survival curve tends to underestimate the truth and consequently results in the trial being given too much weight in the meta-analysis.

For these reasons and in order to conform with good practice guidelines for the reporting of meta-analyses of observational studies (187) we have tried to avoid giving our quantitative estimate of effect undue prominence and have concentrated instead on analyzing sensitivity and demonstrating the robustness of our findings. The sensitivity analysis is itself not without limitations. Care must be taken in the interpretation of the chi-squared test, since it has low power in the situation of a meta-analysis of studies with sample size. This means that while a statistically significant result may indicate a problem with heterogeneity, a non-significant result may not necessarily be taken as evidence of no heterogeneity. This is why we chose *P*-value of 0.10, rather than the conventional level of 0.05, to determine statistical significance.

The results of this study are of clinical relevance for a number of reasons. Here we provide a more objective appraisal of the evidence than traditional narrative reviews and a more precise estimate of the prognostic effect of occult lymph node metastases than currently available. In so doing we hope to have better informed the on-going debate regarding their prognostic significance. The considerable expense of resources necessary to perform an individual patient data meta-analysis of this same question could now be justified. The data presented here may help in planning future clinical trials aimed at determining whether the presence or absence of lymph node micrometastasis should guide decisions concerning new or existing adjuvant therapies of oesophageal or gastro-oesophageal cancers. The data from observational studies suggests that occult lymph node metastasis has a clinically significant detrimental effect on disease free survival in node negative cancer of the oesophagus or gastro-oesophagus.

To summarise, we examined the phenomenon of residual disease following neo-adjuvant chemotherapy and radiotherapy. Using a prospectively compiled database we collected and analyzed a large volume of clinical and pathological data in patients who received neo-adjuvant therapy. We identified larger tumor size to be an important predictor of residual disease following adjuvant therapies. This is an important finding because a possible reason for this relationship between tumor size and response may simply be that the currently standard dosages of chemotherapy and radiotherapy are inadequate in patients with larger tumors. This will require further investigation. We then

turned our attention to residual disease following surgical therapy. We examined a larger cohort of pN0 (pathological node negative) oesophageal cancer patients than any previously reported for evidence of minimal residual disease in lymph nodes (occult lymph node metastasis). We found a significantly lower prevalence of occult lymph node disease than those previously reported. Additionally we found a strong association between minimal residual disease in lymph nodes and poor outcome. We concluded that there were two main clinical implications of these findings. Firstly, by adding investigation of lymph nodes for minimal residual disease to the routine pathological "work-up" of oesophagectomy specimens one might be able to provide patients with more accurate diagnoses. Secondly, in patients whose primary treatment was surgical and who were demonstrated to have minimal residual disease following surgery, there would be a strong argument for the trialing of adjuvant therapies in these patients. However the quality of the existing evidence regarding the relevance of minimal residual disease in lymph nodes was simply not high enough to even consider making real clinical decisions of their basis. Accordingly we undertook a systematic review of the existing evidence in order to better inform the on-going debate into the clinical relevance of minimal residual disease following surgery. We found enough studies of sufficient quality to warrant a statistical analysis and our analysis showed a significant association between minimal residual disease in gastro-oesophageal or oesophageal cancer and outcome.

REFERENCES

(1) Pantel, K., von Knebel Doeberitz, M. (2000). "Detection and clinical relevance of micrometastatic cancer cells." Curr Opin Oncol 12(1): 95-101.

(2) Enzinger, P. C., Mayer, R. J. (2003). "Oesophageal cancer." N Engl J Med 349(23): 2241-2252.

(3) Glickman, J. N., Torres, C., Wang, H. H., *et al.* (1999). "The prognostic significance of lymph node micrometastasis in patients with oesophageal carcinoma." Cancer 85(4): 769-778.

(4) Pantel, K., Izbicki, J., Passlick, B., *et al.* (1996). "Frequency and prognostic significance of isolated tumour cells in bone marrow of patients with non-small-cell lung cancer without overt metastases." Lancet 347(9002): 649-653.

(5) Lugo, T. G., Braun, S., Cote, R. J., *et al.* (2003). "Detection and measurement of occult disease for the prognosis of solid tumors." J Clin Oncol 21(13): 2609-2615.

(6) Gaspar, L. E., Winter, K., Kocha, W. I., et al. (2000). "A phase I/II study of external beam radiation, brachytherapy, and concurrent chemotherapy for patients with localized carcinoma of the esophagus (Radiation Therapy Oncology Group Study 9207): final report." Cancer 88(5): 988-995.

(7) Cooper, J. S., Guo, M. D., Herskovic, A., *et al.* (1999). "Chemoradiotherapy of locally advanced oesophageal cancer: long-

term follow-up of a prospective randomized trial (RTOG 85-01). Radiation Therapy Oncology Group." Jama 281(17): 1623-1627.

(8) Le Prise, E., Etienne, P. L., Meunier, B., et al. (1994). "A randomized study of chemotherapy, radiation therapy, and surgery versus surgery for localized squamous cell carcinoma of the esophagus." Cancer 73(7): 1779-1784.

(9) Walsh, T. N., Noonan, N., Hollywood, D., *et al.* (1996). "A comparison of multimodal therapy and surgery for oesophageal adenocarcinoma." N Engl J Med 335(7): 462-467.

(10)Bosset, J. F., Gignoux, M., Triboulet, J. P., et al. (1997). "Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the esophagus." N Engl J Med 337(3): 161-167.

(11)Nygard K, Hagen S, Hansen HS (1992) Preoperative radiotherapy prolongs survival in operable esophageal cancer: a randomised, multicenter study of pre-operative radiotherapy and chemotherapy: the second Scandinavian trial in esophageal carcinoma. World J Surg. 1992;16:1104–1110.

(12)Urba SG, Orriner MB, Turrisi A (2001) Randomised trial of preoperative chemoradiotherapy versus surgery alone in patients with locoregional esophageal carcinoma. J Clin Oncol. 2001;19:305–313.

(13)Law S, Kwong D, Tung H (1998) Preoperative chemoradiation for squamous cell esophageal cancer: a prospective randomised trial. Can J Gastroenterol. 1998;12(suppl B):56.

(14)Burmeister BH, Smithers BM, Gebski V, (2005) Surgery alone versus chemoradiotherapy followed by surgery for respectable cancer of the oesophagus: a randomised controlled phase III trial. Lancet Oncol. 2005;6:659–668.

(15)Urschel JD, Vasan H (2003) A meta-analysis of randomised controlled that compared neo-adjuvant chemoradiation and surgery to surgery alone for respectable esophageal cancer. Am J Surg. 2003;185:538–543.

(16)Fiorica F, DiBona D, Schepis F (2004) Preoperative chemoradiotherapy for oesophageal cancer: a systematic review and meta-analysis. Gut. 2004;53:925–930

(17)Bosset, J. F., Gignoux, M., Triboulet, J. P., et al. (1997). "Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the esophagus." N Engl J Med 337(3): 161-167.

(18)Law S, Kwong D, Tung H (1998) Preoperative chemoradiation for squamous cell esophageal cancer: a prospective randomised trial. Can J Gastroenterol. 1998;12(suppl B):56.

(19)Burmeister BH, Smithers BM, Gebski V, (2005) Surgery alone versus chemoradiotherapy followed by surgery for respectable cancer of the oesophagus: a randomised controlled phase III trial. Lancet Oncol. 2005;6:659–668.

(20)Suntharalingam M, Mougham J, Coia LR, (1999) The national practice for patients receiving radiation therapy for carcinoma of the esophagus:

results of the 1996–1999 Patterns of Care Study. Cancer. 1999;85:2499–2505.

(21)Kelsen, D. (2001). "Preoperative chemoradiotherapy for oesophageal cancer." J Clin Oncol 19(2): 283-285.

(22)Kelsen DP, Ginsberg R, Pajak TF (1998) Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. N Engl J Med. 1998;339:1974–1984.

(23)Medical Research Council Oesophageal Cancer Working Group. (2002) Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet. 2002;359:1727–1733

(24)Duhaylongsod, F. G., Gottfried, M. R., Iglehart, J. D., *et al.* (1995). "The significance of c-erb B-2 and p53 immunoreactivity in patients with adenocarcinoma of the esophagus." Ann Surg 221(6): 677-683; discussion 683-674.

(25)Green, R. J., Haller, D. G. (1999). "Neoadjuvant Therapy for Cancer of the Esophagus." Cancer Control 6(1): 43-52.

(26)Lackey, V. L., Reagan, M. T., Smith, R. A., Anderson, W. J. (1989). "Neoadjuvant therapy of squamous cell carcinoma of the esophagus: role of resection and benefit in partial responders." Ann Thorac Surg 48(2): 218-221.

(27)Naunheim, K. S., Petruska, P. J., Roy, T. S., *et al.* (1995). "Multimodality therapy for adenocarcinoma of the esophagus." Ann Thorac Surg 59(5): 1085-1090; discussion 1090-1081.

(28)Bates, B. A., Detterbeck, F. C., Bernard, S. A., *et al.* (1996). "Concurrent radiation therapy and chemotherapy followed by esophagectomy for localized oesophageal carcinoma." J Clin Oncol 14(1): 156-163.

(29)Carey, R. W., Hilgenberg, A. D., Wilkins, E. W., Jr., et al. (1993). "Long-term follow-up of neoadjuvant chemotherapy with 5-fluorouracil and cisplatin with surgical resection and possible postoperative radiotherapy and/or chemotherapy in squamous cell carcinoma of the esophagus." Cancer Invest 11(2): 99-105.

(30)Forastiere, A. A., Orringer, M. B., Perez-Tamayo, C., et al. (1993). "Preoperative chemoradiation followed by transhiatal esophagectomy for carcinoma of the esophagus: final report." J Clin Oncol 11(6): 1118-1123.

(31)Wolfe, W. G., Vaughn, A. L., Seigler, H. F., *et al.* (1993). "Survival of patients with carcinoma of the esophagus treated with combined-modality therapy." J Thorac Cardiovasc Surg 105(4): 749-755; discussion 755-746.

(32)Reynolds JV,* Muldoon C, Hollywood D, Narayanasamy R, Rowley S, O'Byrne K, Kennedy J, Murphy TJ (2007) Long-term Outcomes Following Neoadjuvant Chemoradiotherapy for Esophageal Cancer. Ann Surg. 2007 245: 707–716.

(33)Bosset, J. F., Gignoux, M., Triboulet, J. P., *et al.* (1997). "Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the esophagus." N Engl J Med 337(3): 161-167.

(34)"Occult axillary lymph-node micrometastases in breast cancer." (1990). Lancet 336(8712): 434-435.

(35)Papac, R. J. (1994). "Bone marrow metastases. A review." Cancer 74(9): 2403-2413.

(36)Engell (1955). "Cancer cells in the circulating blood; a clinical study on the occurrence of cancer cells in the peripheral blood and in venous blood draining the tumour area at operation." Acta Chir Scand Suppl 201: 1-70.

(37)Christopherson, W. M. (1965). "Cancer cells in the peripheral blood: a second look." acta Cytol 86: 169-174.

(38)Sloane, J. P., Ormerod, M. G., Neville, A. M. (1980). "Potential pathological application of immunocytochemical methods to the detection of micrometastases." Cancer Res 40(8 Pt 2): 3079-3082.

(39)Ghossein, R. A., Bhattacharya, S., Rosai, J. (1999). "Molecular detection of micrometastases and circulating tumor cells in solid tumors." Clin Cancer Res 5(8): 1950-1960.

(40)Pantel, K., Izbicki, J., Passlick, B., et al. (1996). "Frequency and prognostic significance of isolated tumour cells in bone marrow of patients with non-small-cell lung cancer without overt metastases." Lancet 347(9002): 649-653.

(41)Lindemann, F., Schlimok, G., Dirschedl, P., et al. (1992). "Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients." Lancet 340(8821): 685-689.

(42)Braun, S., Pantel, K., Muller, P., et al. (2000). "Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer." N Engl J Med 342(8): 525-533.

(43)Hermanek, P., Hutter, R. V., Sobin, L. H., Wittekind, C. (1999). "International Union Against Cancer. Classification of isolated tumor cells and micrometastasis." Cancer 86(12): 2668-2673.

(44)Pierga, J. Y., Bonneton, C., Vincent-Salomon, A., et al. (2004). "Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients." Clin Cancer Res 10(4): 1392-1400.

(45)Denis, M. G., Lipart, C., Leborgne, J., et al. (1997). "Detection of disseminated tumor cells in peripheral blood of colorectal cancer patients." Int J Cancer 74(5): 540-544.

(46)Weitz, J., Kienle, P., Lacroix, J., et al. (1998). "Dissemination of tumor cells in patients undergoing surgery for colorectal cancer." Clin Cancer Res 4(2): 343-348.

(47)Pantel, K., Cote, R. J., Fodstad, O. (1999). "Detection and clinical importance of micrometastatic disease." J Natl Cancer Inst 91(13): 1113-1124.

(48)Goetz, A., Egen, C., Mueller, V. (2002). "Enrichment of disseminated breast cancer cells from human peripheral blood by advanced density gradient centrifugation." Proc American Assoc Cancer Res 43: 733 (abst 3637).

(49)Naume, B., Borgen, E., Nesland, J. M., et al. (1998). "Increased sensitivity for detection of micrometastases in bone-marrow/peripheral-

blood stem-cell products from breast-cancer patients by negative immunomagnetic separation." Int J Cancer 78(5): 556-560.

(50)Racila, E., Euhus, D., Weiss, A. J., et al. (1998). "Detection and characterization of carcinoma cells in the blood." Proc Natl Acad Sci U S A 95(8): 4589-4594.

(51)Martin, V. M., Siewert, C., Scharl, A., et al. (1998). "Immunomagnetic enrichment of disseminated epithelial tumor cells from peripheral blood by MACS." Exp Hematol 26(3): 252-264.

(52)Page, D. L., Anderson, T. J., Carter, B. A. (1999). "Minimal solid tumor involvement of regional and distant sites: when is a metastasis not a metastasis?" Cancer 86(12): 2589-2592.

(53)AJCC Cancer Staging Manual (2002). New York (YK), Springer-Verlag.

(54)Krag, D., Weaver, D., Ashikaga, T., et al. (1998). "The sentinel node in breast cancer--a multicenter validation study." N Engl J Med 339(14): 941-946.

(55)Moll, R. (1994). "Cytokeratins in the histological diagnosis of malignant tumors." Int J Biol Markers 9(2): 63-69.

(56)Pantel, K., Schlimok, G., Angstwurm, M., et al. (1994). "Methodological analysis of immunocytochemical screening for disseminated epithelial tumor cells in bone marrow." J Hematother 3(3): 165-173.

(57)Braun, S., Muller, M., Hepp, F., et al. (1998). "Re: Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status." J Natl Cancer Inst 90(14): 1099-1101.

(58)Schlimok, G., Funke, I., Holzmann, B., et al. (1987). "Micrometastatic cancer cells in bone marrow: in vitro detection with anti-cytokeratin and

in vivo labeling with anti-17-1A monoclonal antibodies." Proc Natl Acad Sci U S A 84(23): 8672-8676.

(59)Passlick, B., Izbicki, J. R., Kubuschok, B., et al. (1994). "Immunohistochemical assessment of individual tumor cells in lymph nodes of patients with non-small-cell lung cancer." J Clin Oncol 12(9): 1827-1832.

(60)Hitchcock, C. L., Arnold, M. W., Young, D. C., et al. (1996). "TAG-72 expression in lymph nodes and RIGS." Dis Colon Rectum 39(4): 473-475.

(61)Campana, D., Pui, C. H. (1995). "Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance." Blood 85(6): 1416-1434.

(62)Gerhard, M., Juhl, H., Kalthoff, H., et al. (1994). "Specific detection of carcinoembryonic antigen-expressing tumor cells in bone marrow aspirates by polymerase chain reaction." J Clin Oncol 12(4): 725-729.

(63)Lee, M. S., Chang, K. S., Cabanillas, F., et al. (1987). "Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification." Science 237(4811): 175-178.

(64)Brennan, J. A., Mao, L., Hruban, R. H., *et al.* (1995). "Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck." N Engl J Med 332(7): 429-435.

(65)Belinsky, S. A., Nikula, K. J., Palmisano, W. A., *et al.* (1998). "Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis." Proc Natl Acad Sci U S A 95(20): 11891-11896.

(66)Mao, L., Schoenberg, M. P., Scicchitano, M., *et al.* (1996). "Molecular detection of primary bladder cancer by microsatellite analysis." Science 271(5249): 659-662.

(67)Kobayashi, Y., Yoshinouchi, M., Tianqi, G., *et al.* (1998). "Presence of human papilloma virus DNA in pelvic lymph nodes can predict unexpected recurrence of cervical cancer in patients with histologically negative lymph nodes." Clin Cancer Res 4(4): 979-983.

(68)Osborne, M. P., Asina, S., Wong, G. Y., *et al.* (1989). "Immunofluorescent monoclonal antibody detection of breast cancer in bone marrow: sensitivity in a model system." Cancer Res 49(9): 2510-2513.

(69)Naume, B., Borgen, E., Beiske, K., *et al.* (1997). "Immunomagnetic techniques for the enrichment and detection of isolated breast carcinoma cells in bone marrow and peripheral blood." J Hematother 6(2): 103-114.

(70)Pantel, K., von Knebel Doeberitz, M. (2000). "Detection and clinical relevance of micrometastatic cancer cells." Curr Opin Oncol 12(1): 95-101.

(71)Pantel, K., Braun, S., Passlick, B., Schlimok, G. (1996). "Minimal residual epithelial cancer: diagnostic approaches and prognostic relevance." Prog Histochem Cytochem 30(3): 1-60.

(72)Braun, S., Pantel, K. (1998). "Prognostic significance of micrometastatic bone marrow involvement." Breast Cancer Res Treat 52(1-3): 201-216.

(73)Heiss, M. M., Allgayer, H., Gruetzner, K. U., *et al.* (1995). "Individual development and uPA-receptor expression of disseminated tumour cells

in bone marrow: a reference to early systemic disease in solid cancer." Nat Med 1(10): 1035-1039.

(74)Pardoll, D. M. (1993). "Cancer vaccines." Immunol Today 14(6): 310-316.

(75)Pantel, K., Schlimok, G., Braun, S., *et al.* (1993). "Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells." J Natl Cancer Inst 85(17): 1419-1424.

(76)Klein, C. A., Schmidt-Kittler, O., Schardt, J. A., *et al.* (1999). "Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells." Proc Natl Acad Sci U S A 96(8): 4494-4499.

(77)Dietmaier, W., Hartmann, A., Wallinger, S., *et al.* (1999). "Multiple mutation analyses in single tumor cells with improved whole genome amplification." Am J Pathol 154(1): 83-95.

(78)Putz, E., Witter, K., Offner, S., *et al.* (1999). "Phenotypic characteristics of cell lines derived from disseminated cancer cells in bone marrow of patients with solid epithelial tumors: establishment of working models for human micrometastases." Cancer Res 59(1): 241-248.

(79)O'Sullivan G, C., Sheehan, D., Clarke, A., *et al.* (1999). "Micrometastases in esophagogastric cancer: high detection rate in resected rib segments." Gastroenterology 116(3): 543-548.

(80)Wells, C. A., Heryet, A., Brochier, J., *et al.* (1984). "The immunocytochemical detection of axillary micrometastases in breast cancer." Br J Cancer 50(2): 193-197.

(81)Stosiek, P., Gerber, B., Kasper, M. (1996). "[Prognostic significance of micrometastases in axillary lymph nodes in breast carcinoma]." Pathologe 17(6): 433-439.

(82)Jeffers, M. D., O'Dowd, G. M., Mulcahy, H., *et al.* (1994). "The prognostic significance of immunohistochemically detected lymph node micrometastases in colorectal carcinoma." J Pathol 172(2): 183-187.

(83)Maehara, Y., Oshiro, T., Endo, K., *et al.* (1996). "Clinical significance of occult micrometastasis lymph nodes from patients with early gastric cancer who died of recurrence." Surgery 119(4): 397-402.

(84)Freeman, J. A., Esrig, D., Grossfeld, G. D., *et al.* (1995). "Incidence of occult lymph node metastases in pathological stage C (pT3N0) prostate cancer." J Urol 154(2 Pt 1): 474-478.

(85)Cochran, A. J., Wen, D. R., Morton, D. L. (1988). "Occult tumor cells in the lymph nodes of patients with pathological stage I malignant melanoma. An immunohistological study." Am J Surg Pathol 12(8): 612-618.

(86)Mansi, J. L., Gogas, H., Bliss, J. M., *et al.* (1999). "Outcome of primary-breast-cancer patients with micrometastases: a long-term follow-up study." Lancet 354(9174): 197-202.

(87)Diel, I. J., Kaufmann, M., Costa, S. D., *et al.* (1996). "Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status." J Natl Cancer Inst 88(22): 1652-1658.

(88)Gebauer, G., Fehm, T., Merkle, E., *et al.* (2001). "Epithelial cells in bone marrow of breast cancer patients at time of primary surgery: clinical outcome during long-term follow-up." J Clin Oncol 19(16): 3669-3674.

(89)Gerber, B., Krause, A., Muller, H., et al. (2001). "Simultaneous immunohistochemical detection of tumor cells in lymph nodes and bone marrow aspirates in breast cancer and its correlation with other prognostic factors." J Clin Oncol 19(4): 960-971.

(90)Corey, E., Corey, M. J. (1998). "Detection of disseminated prostate cells by reverse transcription-polymerase chain reaction (RT-PCR): technical and clinical aspects." Int J Cancer 77(5): 655-673.

(91)Calaluce, R., Miedema, B. W., Yesus, Y. W. (1998). "Micrometastasis in colorectal carcinoma: a review." J Surg Oncol 67(3): 194-202.

(92)Taylor, C. R., Cote, R. J. (1997). "Immunohistochemical markers of prognostic value in surgical pathology." Histol Histopathol 12(4): 1039-1055.

(93)de Mascarel, I., Bonichon, F., Coindre, J. M., Trojani, M. (1992). "Prognostic significance of breast cancer axillary lymph node micrometastases assessed by two special techniques: reevaluation with longer follow-up." Br J Cancer 66(3): 523-527.

(94)Meyer, J. S., Steinberg, L. S. (1969). "Microscopically benign thyroid follicles in cervical lymph nodes. Serial section study of lymph node inclusions and entire thyroid gland in 5 cases." Cancer 24(2): 302-311.

(95)Fisher, C. J., Hill, S., Millis, R. R. (1994). "Benign lymph node inclusions mimicking metastatic carcinoma." J Clin Pathol 47(3): 245-247.

(96)Weeks, D. A., Beckwith, J. B., Mierau, G. W. (1990). "Benign nodal lesions mimicking metastases from pediatric renal neoplasms: a report of the National Wilms' Tumor Study Pathology Center." Hum Pathol 21(12): 1239-1244.

(97)Perrone, T. (1985). "Embolization of benign colonic glands to mesenteric lymph nodes." Am J Surg Pathol 9(7): 538-541.

(98)Buer, J., Probst, M., Ganser, A., Atzpodien, J. (1995). "Response to 13-cis-retinoic acid plus interferon alfa-2a in two patients with therapy-refractory advanced renal cell carcinoma." J Clin Oncol 13(10): 2679-2680.

(99)Izbicki, J. R., Hosch, S. B., Pichlmeier, U., et al. (1997). "Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected oesophageal cancer." N Engl J Med 337(17): 1188-1194.

(100) Moll, R., Franke, W. W., Schiller, D. L., et al. (1982). "The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells." Cell 31(1): 11-24.

(101) Osborne, M. P., Rosen, P. P. (1994). "Detection and management of bone marrow micrometastases in breast cancer." Oncology (Williston Park) 8(8): 25-31; discussion 35-26, 39-42.

(102) Solakoglu, O., Maierhofer, C., Lahr, G., et al. (2002). "Heterogeneous proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors." Proc Natl Acad Sci U S A 99(4): 2246-2251.

(103) Hughes, T., Janssen, J. W., Morgan, G., et al. (1990). "False-positive results with PCR to detect leukaemia-specific transcript." Lancet 335(8696): 1037-1038.

(104) Traweek, S. T., Liu, J., Battifora, H. (1993). "Keratin gene expression in non-epithelial tissues. Detection with polymerase chain reaction." Am J Pathol 142(4): 1111-1118.

(105) Krismann, M., Todt, B., Schroder, J., et al. (1995). "Low specificity of cytokeratin 19 reverse transcriptase-polymerase chain reaction analyses for detection of hematogenous lung cancer dissemination." J Clin Oncol 13(11): 2769-2775.

(106) Zippelius, A., Kufer, P., Honold, G., et al. (1997). "Limitations of reverse-transcriptase polymerase chain reaction analyses for detection of micrometastatic epithelial cancer cells in bone marrow." J Clin Oncol 15(7): 2701-2708.

(107) Bostick, P. J., Chatterjee, S., Chi, D. D., et al. (1998). "Limitations of specific reverse-transcriptase polymerase chain reaction markers in the detection of metastases in the lymph nodes and blood of breast cancer patients." J Clin Oncol 16(8): 2632-2640.

(108) Ruud, P., Fodstad, O., Hovig, E. (1999). "Identification of a novel cytokeratin 19 pseudogene that may interfere with reverse transcriptase-polymerase chain reaction assays used to detect micrometastatic tumor cells." Int J Cancer 80(1): 119-125.

(109) Champelovier, P., Mongelard, F., Seigneurin, D. (1999). "CK20 gene expression: technical limits for the detection of circulating tumor cells." Anticancer Res 19(3A): 2073-2078.

(110) Funaki, N. O., Tanaka, J., Kasamatsu, T., et al. (1996). "Identification of carcinoembryonic antigen mRNA in circulating

peripheral blood of pancreatic carcinoma and gastric carcinoma patients." Life Sci 59(25-26): 2187-2199.

(111) Mori, M., Mimori, K., Ueo, H., et al. (1996). "Molecular detection of circulating solid carcinoma cells in the peripheral blood: the concept of early systemic disease." Int J Cancer 68(6): 739-743.

(112) Liefers, G. J., Cleton-Jansen, A. M., van de Velde, C. J., et al. (1998). "Micrometastases and survival in stage II colorectal cancer." N Engl J Med 339(4): 223-228.

(113) Bostick, P. J., Hoon, D. S., Cote, R. J. (1998). "Detection of carcinoembryonic antigen messenger RNA in lymph nodes from patients with colorectal cancer." N Engl J Med 339(22): 1643-1644.

(114) Wharton, R. Q., Jonas, S. K., Glover, C., *et al.* (1999). "Increased detection of circulating tumor cells in the blood of colorectal carcinoma patients using two reverse transcription-PCR assays and multiple blood samples." Clin Cancer Res 5(12): 4158-4163.

(115) Goessl, C., Heicappell, R., Munker, R., *et al.* (1998). "Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma." Cancer Res 58(20): 4728-4732.

(116) Nawroz, H., Koch, W., Anker, P., *et al.* (1996). "Microsatellite alterations in serum DNA of head and neck cancer patients." Nat Med 2(9): 1035-1037.

(117) Sanchez-Cespedes, M., Monzo, M., Rosell, R., *et al.* (1998). "Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients." Ann Oncol 9(1): 113-116.

(118) Chen, X. Q., Stroun, M., Magnenat, J. L., *et al.* (1996). "Microsatellite alterations in plasma DNA of small cell lung cancer patients." Nat Med 2(9): 1033-1035.

(119) Hibi, K., Robinson, C. R., Booker, S., *et al.* (1998). "Molecular detection of genetic alterations in the serum of colorectal cancer patients." Cancer Res 58(7): 1405-1407.

(120) Qiu, S. D., Young, C. Y., Bilhartz, D. L., *et al.* (1990). "In situ hybridization of prostate-specific antigen mRNA in human prostate." J Urol 144(6): 1550-1556.

(121) Enzinger, P. C., Mayer, R. J. (2003). "Oesophageal cancer." N Engl J Med 349(23): 2241-2252.

(122) Glickman, J. N., Torres, C., Wang, H. H., *et al.* (1999). "The prognostic significance of lymph node micrometastasis in patients with oesophageal carcinoma." Cancer 85(4): 769-778.

(123) Lugo, T. G., Braun, S., Cote, R. J., *et al.* (2003). "Detection and measurement of occult disease for the prognosis of solid tumors." J Clin Oncol 21(13): 2609-2615.

(124) Gaspar, L. E., Winter, K., Kocha, W. I., *et al.* (2000). "A phase I/II study of external beam radiation, brachytherapy, and concurrent chemotherapy for patients with localized carcinoma of the esophagus (Radiation Therapy Oncology Group Study 9207): final report." Cancer 88(5): 988-995.

(125) Cooper, J. S., Guo, M. D., Herskovic, A., *et al.* (1999). "Chemoradiotherapy of locally advanced oesophageal cancer: long-

term follow-up of a prospective randomized trial (RTOG 85-01). Radiation Therapy Oncology Group." Jama 281(17): 1623-1627.

(126) Wang, K. k. (2002). "Multimodality Therapy for Gastroesophageal Cancers." Journal of Gastrointestinal Surgery 6(4): 527-531.

(127) Nygaard, K., Hagen, S., Hansen, H. S., *et al.* (1992). "Pre-operative radiotherapy prolongs survival in operable oesophageal carcinoma: a randomized, multicenter study of pre-operative radiotherapy and chemotherapy. The second Scandinavian trial in oesophageal cancer." World J Surg 16(6): 1104-1109; discussion 1110.

(128) Le Prise, E., Etienne, P. L., Meunier, B., *et al.* (1994). "A randomized study of chemotherapy, radiation therapy, and surgery versus surgery for localized squamous cell carcinoma of the esophagus." Cancer 73(7): 1779-1784.

(129) Dunne, B., Reynolds, J. V., Mulligan, E., *et al.* (2001). "A pathological study of tumour regression in oesophageal adenocarcinoma treated with preoperative chemoradiotherapy." J Clin Pathol 54(11): 841-845.

(130) Mandard, A. M., Dalibard, F., Mandard, J. C., *et al.* (1994). "Pathologic assessment of tumor regression after preoperative chemoradiotherapy of oesophageal carcinoma. Clinicopathologic correlations." Cancer 73(11): 2680-2686.

(131) Greene, F. L., Page, D. L., Fleming, I. D., al., e. (2002). AJCC Cancer Staging Manual, Sixth Edition. New York, NY., Springer-Verlag.

(132) Hayden, J. A., Cote, P., Bombardier, C. (2006). "Evaluation of the quality of prognosis studies in systematic reviews." Ann Intern Med 144(6): 427-437.

(133) Higgins, J., Green, S., editors "Assessment of study quality. Cochrane Handbook for Systematic Reviews of Interventions 4.2.5 [updated May 2005]; Section 6.5, http://www.cochrane.org/resources/handbook/hbook.htm (accessed 4th January 2006)."

(134) Higgins, J., Green, S. (2005). Cochrance Handbook for Systematic Reviews of Interventions. Chiceser, UK, John Wiley & Sons, Ltd.

(135) Parmar, M. K., Torri, V., Stewart, L. (1998). "Extracting summary statistics to perform meta-analyses of the published literature for survival endpoints." Stat Med 17(24): 2815-2834.

(136) Higgins, J., Green, S., editors "Analysing and presenting results. Cochrane Handbook for Systematic Review of Interventions 4.2.5 [updated May 2005]; Section 8.6.8, http://www.cochrane.org/resources/handbook/hbook.htm (accessed 4th January 2006)."

(137) Higgins, J., Green, S., editors "Analysing and presenting results. Cochrane Handbook for Systematic Review of Interventions 4.2.5 [updated May 2005]; Section 8.7.4, http://www.cochrane.org/resources/handbook/hbook.htm (accessed 4th January 2006)."

(138) Higgins, J., Green, S., editors "Analysing and presenting results. Cochrane Handbook for Systematic Review of Interventions 4.2.5

[updated May 2005]; Section 8.7.2, http://www.cochrane.org/resources/handbook/hbook.htm (accessed 4th January 2006)."

(139) Deeks, J., Altman, D., Bradburn, M. (2001). Statistical methods for examining heterogeneity and combining results from several studies in meta-analysis. Systematic Reviews in Health Care: Meta-analysis in Context. M. Egger, G. D. Smith and D. Altman. London, BMJ Publication Group.

(140) Xiao, X.-W. (2002). "Relationship between lymph node metastases in oesophageal carcinoma and it prognosis." Chinese Journal of Cancer Research 14(4): 297-301.

(141) Natsugoe, S., Mueller, J., Stein, H. J., *et al.* (1998). "Micrometastasis and tumor cell microinvolvement of lymph nodes from oesophageal squamous cell carcinoma: frequency, associated tumor characteristics, and impact on prognosis." Cancer 83(5): 858-866.

(142) Mueller, J. D., Stein, H. J., Oyang, T., *et al.* (2000). "Frequency and clinical impact of lymph node micrometastasis and tumor cell microinvolvement in patients with adenocarcinoma of the esophagogastric junction." Cancer 89(9): 1874-1882.

(143) Heeren, P. A., Kelder, W., Blondeel, I., *et al.* (2005). "Prognostic value of nodal micrometastases in patients with cancer of the gastro-oesophageal junction." Eur J Surg Oncol 31(3): 270-276.

(144) Matsumoto, M., Natsugoe, S., Nakashima, S., *et al.* (2000). "Clinical significance of lymph node micrometastasis of pN0 oesophageal squamous cell carcinoma." Cancer Lett 153(1-2): 189-197.

(145) Sato, F., Shimada, Y., Li, Z., *et al.* (2001). "Lymph node micrometastasis and prognosis in patients with oesophageal squamous cell carcinoma." Br J Surg 88(3): 426-432.

(146) Vazquez-Sequeiros, E., Wang, L., Burgart, L., *et al.* (2002). "Occult lymph node metastases as a predictor of tumor relapse in patients with node-negative oesophageal carcinoma." Gastroenterology 122(7): 1815-1821.

(147) Nakamura, T., Ide, H., Eguchi, R., *et al.* (2002). "Clinical implications of lymph node micrometastasis in patients with histologically node-negative (pN0) oesophageal carcinoma." J Surg Oncol 79(4): 224-229.

(148) Hosch, S., Kraus, J., Scheunemann, P., *et al.* (2000). "Malignant potential and cytogenetic characteristics of occult disseminated tumor cells in oesophageal cancer." Cancer Res 60(24): 6836-6840.

(149) MacGuill, M. J., Barrett, C., Ravi, N., *et al.* (2006). "Isolated tumour cells in Pathological Node-Negative Lymph nodes (pN0) Adversely Affect Prognosis in Cancer of the Oesophagus or Oesophago-gastric Junction." Unpublished.

(150) Bonavina, L., Incarbone, R., Midolo, V., *et al.* (2004). "[Prognostic significance of lymphatic micrometastasis of oesophageal adenocarcinoma]." Chir Ital 56(2): 189-196.

(151) Laso, C. A., Gonzalez, J. J., Fresno, F., *et al.* (2004). "Prognostic value of micrometastases in oesophageal and colorectal carcinoma (a clinical experience)." Hepatogastroenterology 51(58): 964-967.

(152) Komukai, S., Nishimaki, T., Watanabe, H., *et al.* (2000). "Significance of immunohistochemically demonstrated micrometastases

to lymph nodes in oesophageal cancer with histologically negative nodes." Surgery 127(1): 40-46.

(153) Shackney S, M. G., Cuchural G (1978). "Growth rate patterns of solid tumours and their relation to responsiveness to therapy." Annals of Internal Medicine 89: 107-121.

(154) Eloubeidi, M. A., Desmond, R., Arguedas, M. R., *et al.* (2002). "Prognostic factors for the survival of patients with oesophageal carcinoma in the U.S.: the importance of tumor length and lymph node status." Cancer 95(7): 1434-1443.

(155) Bastit, P. C., B. Chevreau, C. et al (2000). "Epirubicin-based chemotherapy in metastatic breast cancer patients: role of dose-intensity and duration of treatment." J Clin Oncol 18(17): 3115-3124.

(156) Budman, D. R., Berry, D. A., Cirrincione, C. T., *et al.* (1998). "Dose and dose intensity as determinants of outcome in the adjuvant treatment of breast cancer. The Cancer and Leukemia Group B." J Natl Cancer Inst 90(16): 1205-1211.

(157) Fisher, B., Anderson, S., Wickerham, D. L., *et al.* (1997). "Increased intensification and total dose of cyclophosphamide in a doxorubicin-cyclophosphamide regimen for the treatment of primary breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-22." J Clin Oncol 15(5): 1858-1869.

(158) Bergh, J., Wiklund, T., Erikstein, B., *et al.* (2000). "Tailored fluorouracil, epirubicin, and cyclophosphamide compared with marrow-supported high-dose chemotherapy as adjuvant treatment for high-risk

breast cancer: a randomised trial. Scandinavian Breast Group 9401 study." Lancet 356(9239): 1384-1391.

(159) Stadtmauer, E. A., O'Neill, A., Goldstein, L. J., *et al.* (2000). "Conventional-dose chemotherapy compared with high-dose chemotherapy plus autologous hematopoietic stem-cell transplantation for metastatic breast cancer. Philadelphia Bone Marrow Transplant Group." N Engl J Med 342(15): 1069-1076.

(160) Thor, A. D., Berry, D. A., Budman, D. R., *et al.* (1998). "erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer." J Natl Cancer Inst 90(18): 1346-1360.

(161) Paik, S., Bryant, J., Park, C., *et al.* (1998). "erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer." J Natl Cancer Inst 90(18): 1361-1370.

(162) Hickman, J. A. (1992). "Apoptosis induced by anticancer drugs." Cancer Metastasis Rev 11(2): 121-139.

(163) Verheij, M., Bartelink, H. (2000). "Radiation-induced apoptosis." Cell Tissue Res 301(1): 133-142.

(164) Raouf, A., Evoy, D., Carton, E., *et al.* (2001). "Spontaneous and inducible apoptosis in oesophageal adenocarcinoma." Br J Cancer 85(11): 1781-1786.

(165) Attardi, L. D., Jacks, T. (1999). "The role of p53 in tumour suppression: lessons from mouse models." Cell Mol Life Sci 55(1): 48-63.

(166) Wallace-Brodeur, R. R., Lowe, S. W. (1999). "Clinical implications of p53 mutations." Cell Mol Life Sci 55(1): 64-75.

(167) Collecchi, P., Baldini, E., Giannessi, P., *et al.* (1998). "Primary chemotherapy in locally advanced breast cancer (LABC): effects on tumour proliferative activity, bcl-2 expression and the relationship between tumour regression and biological markers." Eur J Cancer 34(11): 1701-1704.

(168) Jia, L., Srinivasula, S. M., Liu, F. T., *et al.* (2001). "Apaf-1 protein deficiency confers resistance to cytochrome c-dependent apoptosis in human leukemic cells." Blood 98(2): 414-421.

(169) Johnstone, R. W., Ruefli, A. A., Lowe, S. W. (2002). "Apoptosis: a link between cancer genetics and chemotherapy." Cell 108(2): 153-164.

(170) Brucher, B. L., Weber, W., Bauer, M., *et al.* (2001). "Neoadjuvant therapy of oesophageal squamous cell carcinoma: response evaluation by positron emission tomography." Ann Surg 233(3): 300-309.

(171) Downey, R. J., Akhurst, T., Ilson, D., *et al.* (2003). "Whole body 18FDG-PET and the response of oesophageal cancer to induction therapy: results of a prospective trial." J Clin Oncol 21(3): 428-432.

(172) Flamen, P., Van Cutsem, E., Lerut, A., *et al.* (2002). "Positron emission tomography for assessment of the response to induction radiochemotherapy in locally advanced oesophageal cancer." Ann Oncol 13(3): 361-368.

(173) Cote, R. J., Hawes, D., Chaiwun, B., Beattie, E. J., Jr. (1998). "Detection of occult metastases in lung carcinomas: progress and implications for lung cancer staging." J Surg Oncol 69(4): 265-274.

(174) Cote, R. J., Peterson, H. F., Chaiwun, B., *et al.* (1999). "Role of immunohistochemical detection of lymph-node metastases in

management of breast cancer. International Breast Cancer Study Group." Lancet 354(9182): 896-900.

(175) Edelstein, R. A., Zietman, A. L., de las Morenas, A., *et al.* (1996). "Implications of prostate micrometastases in pelvic lymph nodes: an archival tissue study." Urology 47(3): 370-375.

(176) Muller, P., Weckermann, D., Riethmuller, G., Schlimok, G. (1996). "Detection of genetic alterations in micrometastatic cells in bone marrow of cancer patients by fluorescence in situ hybridization." Cancer Genet Cytogenet 88(1): 8-16.

(177) Pantel, K., Dickmanns, A., Zippelius, A., *et al.* (1995). "Establishment of micrometastatic carcinoma cell lines: a novel source of tumor cell vaccines." J Natl Cancer Inst 87(15): 1162-1168.

(178) Funke, I., Schraut, W. (1998). "Meta-analyses of studies on bone marrow micrometastases: an independent prognostic impact remains to be substantiated." J Clin Oncol 16(2): 557-566.

(179) Bonavina, L., Ferrero, S., Midolo, V., *et al.* (1999). "Lymph node micrometastases in patients with adenocarcinoma of the esophagogastric junction." J Gastrointest Surg 3(5): 468-476.

(180) Hosch, S. B., Stoecklein, N. H., Pichlmeier, U., *et al.* (2001). "Oesophageal cancer: the mode of lymphatic tumor cell spread and its prognostic significance." J Clin Oncol 19(7): 1970-1975.

(181) Komukai, S., Nishimaki, T., Suzuki, T., *et al.* (2002). "Significance of immunohistochemical nodal micrometastasis as a prognostic indicator in potentially curable oesophageal carcinoma." Br J Surg 89(2): 213-219.

(182) Heeren, P. A. M., Kelder, W., Blondeel, I., *et al.* (2005). "Prognostic value of nodal micrometastases in patients with cancer of the gastro-oesophageal junction." European Journal of Surgical Oncology 31(3): 270-276.

(183) Pathology, A. o. D. o. A. a. S. (2001). "Recommendations for processing and reporting of lymph node specimens submitted for evaluation of metastatic disease." Am J Clin Pathol 115(6): 799-801.

(184) Lerut, T., Coosemans, W., De Leyn, P., *et al.* (1999). "Reflections on three field lymphadenectomy in carcinoma of the esophagus and gastroesophageal junction." Hepatogastroenterology 46(26): 717-725.

(185) Ishida, K., Katsuyama, T., Sugiyama, A., Kawasaki, S. (1997). "Immunohistochemical evaluation of lymph node micrometastases from gastric carcinomas." Cancer 79(6): 1069-1076.

(186) Williamson P.R., Tudur Smith C., Hutton J.L and Marson A.G. (2002) Aggregate data meta-analysis with time-to-event outcomes. Statistics in Medicine, 21: 3337-3351.

(187) Donna F. Stroup, Jesse A. Berlin, Sally C. Morton, Ingram Olkin, G. David Williamson, for the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) Group (2000). Meta-analysis of Observational Studies in Epidemiology. A Proposal for Reporting. JAMA. 2000;283:2008-2012.

PUBLICATIONS

1. ANALYSIS OF CLINICOPATHOLOGICAL FACTORS PREDICTING RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY IN OESOPHAGEAL CANCER.

M. MacGuill, E Mulligan, N. Ravi, J.V. Reynolds.

Endoscopy 2004; 36 DOI: 10.1055/s-2004-825002

2. LOW INCIDENCE OF LYMPH NODE MICROMETASTASIS IN CASES OF COMPLETE PATHOLOGICAL RESPONSE TO NEO-ADJUVANT CHEMORADIOTHERAPY IN OESOPHAGEAL CANCER.

MJ MacGuill, JM O'Riordain, C Barrett, G MacDonald, JV Reynolds.

Irish Journal of Medical Science. 2004 Jan-Mar;173(1 Suppl 1): 14-15.

3. ISOLATED TUMOUR CELLS IN PATHOLOGICAL NODE-NEGATIVE LYMPH NODES (PN0) ADVERSELY AFFECT PROGNOSIS IN CANCER OF THE OESOPHAGUS OR OESOPHAGO-GASTRIC JUNCTION.

MacGuill M, Barrett C, Ravi N, Macdonald G, Reynolds JV.

J Clin Pathol. 2007 Oct;60(10):1108-11. Epub 2007 Jan 12

4. CLINICOPATHOLOGIC FACTORS PREDICTING COMPLETE PATHOLOGICAL RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY IN OESOPHAGEAL CANCER.

MacGuill M, Mulligan E, Ravi N, Rowley S, Byrne PJ, Hollywood D, Kennedy J, Keeling PN, Reynolds JV.

Diseases of the Esophagus. 2006;19(4):273-6.

5. THE PROGNOSTIC IMPACT OF OCCULT LYMPH NODE METASTASIS IN CANCER OF THE ESOPHAGUS OR ESOPHAGO-GASTRIC JUNCTION: SYSTEMATIC REVIEW AND META-ANALYSIS

M.J. McGuill, PJ Byrne, N. Ravi, JV Reynolds.

Diseases of the Esophagus (OnlineEarly Articles). doi:10.1111/j.1442-2050.2007.00765.x

APPENDIX 1 SPECIFICATION OF SEARCH STRATEGY.

Database: Ovid MEDLINE(R) In-Process, Other Non-Indexed Citations, Ovid MEDLINE(R)

Search Strategy:

---

1 micrometasta$.mp. [mp=ti, ot, ab, nm, hw] (3118)

2 (OCCULT adj3 (CELL$ or DISEASE? or METASTA$)).mp. [mp=ti, ot, ab, nm, hw] (2550)

3 (MINIMAL$ adj3 (DISEASE? or METASTA$)).mp. [mp=ti, ot, ab, nm, hw] (4555)

4 MICRO?INVOLEMENT.mp. [mp=ti, ot, ab, nm, hw] (0)

5 MICRO?INVOLVEMENT.mp. [mp=ti, ot, ab, nm, hw] (15)

6 MICRO-INVOLVEMENT.mp. [mp=ti, ot, ab, nm, hw] (1)

7 MICROINVOLVEMENT.mp. [mp=ti, ot, ab, nm, hw] (15)

8 MICRO?INVOLVEMENT.mp. [mp=ti, ot, ab, nm, hw] (15)

9 (ISOLATED adj3 (TUMO?R or CELL$)).mp. [mp=ti, ot, ab, nm, hw] (43496)

10 (DISSEMINATED adj3 (DISEASE? or CELL$)).mp. [mp=ti, ot, ab, nm, hw] (4019)

11 (NOD$2 adj3 METASTA$).mp. [mp=ti, ot, ab, nm, hw] (23264)

12 (IMMUNO$ adj3 (METASTA$ or NOD$2)).mp. [mp=ti, ot, ab, nm, hw] (2690)

13 (SUB?CLINICAL adj3 METASTA$).mp. [mp=ti, ot, ab, nm, hw] (162)

14 (micrometasta$ or (OCCULT adj3 (CELL$ or DISEASE? or METASTA$)) or (MINIMAL$ adj3 (DISEASE? or METASTA$)) or MICRO?INVOLVEMENT or (ISOLATED adj3 (TUMO?R or CELL$)) or (DISSEMINATED adj3 (DISEASE? or CELL$)) or (NOD$2 adj3 METASTA$) or (IMMUNO$ adj3 (METASTA$ or NOD$2)) or (SUB?CLINICAL adj3 METASTA$)).ti,ab. (81169)

15 map neoplasms oesophageal.mp. [mp=ti, ot, ab, nm, hw] (0)

16 map neoplasms.mp. [mp=ti, ot, ab, nm, hw] (0)

17 remove duplicates from 7 (15)

20 exp oesophageal neoplasms/ (23489)

21 exp esophagus/ (28349)

22 (oesophag$ or esophag$).ti,ab. (79285)

23 gastro??esophag$.ti,ab. (9546)

24 20 or 21 or 22 or 23 (92475)

25 14 and 23 (1778)

APPENDIX 2 CUSTOMISED DATA SHEET (PAGE 1 OF 3)

Notes:

A = adequate

B = unclear

C = inadequate

METHODS

Study type (cohort or case control):

Were pN1 patients also included in the study?

Were the pathologists blinded to patient outcome (A,B or C):

Were pN0 patients who received adjuvant therapies included?

The total number of pN0 patients investigated:

The total number of pN0 patients followed up:

Was there any analysis of the impact of confounding factors on micrometastasis in pN0 patients (eg multivariate analysis or chi-squared testing of pathological variables):

If so which confounding factors were examined (only list those with a scientific rationale eg neo-adjuvant therapy, mean number of nodes examined per case, tumour grade, T-stage versus age or sex):

Mean age of patients in trial:

Mean age of pN0 patients:

Male/female ratio in trial:

Male/female ratio in pN0 patients:

Geographic region (Europe / North America / Japan):

Number of sections examined immunochemically (per block or per node?):

Mean number of nodes examined per case:
Mean number of nodes examined per pN0 case:

Definition of a positive case:

IMMUNOCHEMISTRY

Immunohistochemistry? (if other please specify eg immunocytochemistry):

Antibody used: (eg AE1/AE3):

Type of tissue examined (frozen, paraffin embedded etc):

Positive controls:

Negativc controls:

OUTCOME MEASURES AND RESULTS

Was outcome data on disease-free survival in pN0 patients available?:

Correlation co-efficient for cox proportional hazards model analysis of impact of occult metastasis on disease free survival (eg hazard ratio or relative risk or risk ratio):

Confidence interval:

p-value:

other (eg standard error):

p-value associated with logrank comparison of impact of occult metastasis on disease free survival:

total number of pN0 patients:

total number of pN0 patients in survival analysis:

number of occult metastasis positive pN0 patients:

number of occult metastasis negative pN0 patients:

number of occult metastasis positive pN0 patients in survival analysis:

number of occult metastasis negative pN0 patients in survival analysis:

total number of (relapses + disease related deaths) in pN0 group in survival analysis:

range of follow-up (months):

APPENDIX 3 QUALITY ASSESSMENT SHEET (PAGE 1 OF 2)

Study ID
Date
Study Participation

Is the study population (pN0 patients) adequately described for the key characteristics?

- Were they post oesophagectomy / gastro-oesophagectomy?
- Histological type (adenocarcinoma / squamus cell carcinoma)
- Adjuvant therapies
- T-staging
- Tumour grade
- Age
- Sex
- Tumour location

Was a defined sampling time described?

Were all available cases examined?
(eg were they consecutive cases)

Were there acceptable exclusion criteria
(peri-operative deaths, neo-adjuvant therapies,
distant metastasis, residual tumour)

Study Attrition

What proportion of pN0 patients whose lymph nodes were investigated who went on to be included in survival analysis? Was this proportion adequate?

If incomplete are reasons for loss to follow-up provided and are attempts to locate those lost to follow-up are described?

If follow-up is incomplete is occult lymph node metastasis state of patients lost to follow-up is described?

Prognostic Factor Measurement

Is a clear and suitable description of the staining technique provided? Were suitable positive controls used on every run?

Is it stated in the text or can it be reasonably inferred that lymph nodes which should have been classified as pN1 on standard post-operative examination were reclassified as pN1 for the analysis?

APPENDIX 4 COX CALCULATIONS PAGE 1 OF 1

Paper	UppCI	ln(uppCI)	LowCI	ln(lowCI)	Correlation Coefficient	ln(HR)	(lnUppCI-lnLowCI)
Vazquez-Sequeiros	5,8	1,7579	0,8	-0,2231	2,2	0,7885	1,981
Sato	8,14	2,0968	0,12	-2,1203	0,93	-0,0726	4,2171
Komukai 2000	36,48	3,5968	1,14	0,131	6,45	1,8641	3,4657
MacGuill 2006	4,1884	1,4323	1,0784	0,0755	2,2428	0,8077	1,3568

Paper	2F-1(1-ai/2)	SE [ln(HR)]	ln (HR)
Vazquez-Sequeiros	3,92	0,5054	0,7885
Sato	3,92	1,0758	-0,0726
Komukai 2000	3,92	0,8841	1,8641
MacGuill 2006	3,92	0,3461	0,8077

Appendix 5.1 Log Rank Calculations (Page 1 of 6)

Paper	ln[Hri]	var[ln(Hri)]	Oi	Rri	Rci	Pi	P/2
Vazquez-Sequeiros	0,982558696	0,399376623	25	14	110	0,12	0,06
Matsumoto	1,509655609	0,238653503	17	33	26	0,002	0,001
Glickman adeno	0,413923083	0,313856209	15	15	34	0,46	0,23
Glickman scc	0,135810426	1,168055556	6	5	24	0,9	0,45
Sato	0,609972775	0,462962963	9	20	30	0,37	0,185
Nakamura 2002	0,999898199	0,571632072	9	14	39	0,186	0,093
Hosch	1,87509885	0,414848485	11	25	12	0,0036	0,0018
Izbicki 1997	2,310740241	0,804761905	5	14	12	0,01	0,005
Hosch 2001	1,87509885	0,414848485	11	25	12	0,0036	0,0018
Hosch 2000	1,716721052	0,374025974	11	28	20	0,005	0,0025
Komukai 2000 Surgery	2,12396087	0,472394755	9	14	23	0,002	0,001
Mueller	1,357704027	0,458703156	13	13	48	0,045	0,0225
Laso	1,166347216	0,6890625	8	16	5	0,16	0,08

Appendix 5.2 Log Rank Calculations (Page 2 of 6)

Paper	1-p/2	F-1(1-pi/2)	OiRriRci	OiRriRci^-2	OiRriRci^-22
Vazquez-Sequeiros	0,94	1,55	38500	196,21	196,21
Matsumoto	1,00	3,09	14586	120,77	120,77
Glickman adeno	0,77	0,74	7650	87,46	87,46
Glickman scc	0,55	0,13	720	26,83	26,83
Sato	0,82	0,90	5400	73,48	73,48
Nakamura 2002	0,91	1,32	4914	70,10	70,10
Hosch	1,00	2,91	3300	57,45	57,45
Izbicki 1997	1,00	2,58	840	28,98	28,98
Hosch 2001	1,00	2,91	3300	57,45	57,45
Hosch 2000	1,00	2,81	6160	78,49	78,49
Komukai 2000 Surgery	1,00	3,09	2898	53,83	53,83
Mueller	0,98	2,00	8112	90,07	90,07
Laso	0,92	1,41	640	25,30	25,30

Appendix 5.3 Log Rank Calculations (Page 3 of 6)

Paper	Rri+Rci	(OiRriRci^-2)/(Rri+Rci)	(OiRriRci^-2)/(Rri+Rci)2
Vazquez-Sequeiros	124	1,58	1,58
Matsumoto	59	2,05	2,05
Glickman adeno	49	1,78	1,78
Glickman scc	29	0,93	0,93
Sato	50	1,47	1,47
Nakamura 2002	53	1,32	1,32
Hosch	37	1,55	1,55
Izbicki 1997	26	1,11	1,11
Hosch 2001	37	1,55	1,55
Hosch 2000	48	1,64	1,64
Komukai 2000 Surgery	37	1,45	1,45
Mueller	61	1,48	1,48
Laso	21	1,20	1,20

Appendix 5.4 Log Rank Calculations (Page 4 of 6)

Paper	[(OiRriRci^-2)/(Rri+Rci)]*2F-1(1-Pi/2)	[(OiRriRci^-2)/(Rri+Rci)]*2F-1(1-Pi/2)2
Vazquez-Sequeiros	2,46	2,46
Matsumoto	6,33	6,33
Glickman adeno	1,32	1,32
Glickman scc	0,12	0,12
Sato	1,32	1,32
Nakamura 2002	1,75	1,75
Hosch	4,52	4,52
Izbicki 1997	2,87	2,87
Hosch 2001	4,52	4,52
Hosch 2000	4,59	4,59
Komukai 2000 Surgery	4,50	4,50
Mueller	2,96	2,96
Laso	1,69	1,69

Appendix 5.5 Log Rank Calculations (Page 5 of 6)

Paper	OiRriRci2	(Rri + Rci)^2	(OiRriRci2)/(Rri + Rci)^2
Vazquez-Sequeiros	38500,00	15376,00	2,50
Matsumoto	14586,00	3481,00	4,19
Glickman adeno	7650,00	2401,00	3,19
Glickman scc	720,00	841,00	0,86
Sato	5400,00	2500,00	2,16
Nakamura 2002	4914,00	2809,00	1,75
Hosch	3300,00	1369,00	2,41
Izbicki 1997	840,00	676,00	1,24
Hosch 2001	3300,00	1369,00	2,41
Hosch 2000	6160,00	2304,00	2,67
Komukai 2000 Surgery	2898,00	1369,00	2,12
Mueller	8112,00	3721,00	2,18
Laso	640,00	441,00	1,45

Appendix 5.6 Log Rank Calculations (Page 6 of 6)

Paper	(OiRriRci2)/(Rri + Rci)^22	Ori-Eri	Ori-Eri2	Vri	Vri2	(Ori - Eri)/Vri	ln(HRi)	var(lnHRi)
Vazquez-Sequeiros	2,50	2,46	2,46	2,50	2,50	0,98	0,98	0,40
Matsumoto	4,19	6,33	6,33	4,19	4,19	1,51	1,51	0,24
Glickman adeno	3,19	1,32	1,32	3,19	3,19	0,41	0,41	0,31
Glickman scc	0,86	0,12	0,12	0,86	0,86	0,14	0,14	1,17
Sato	2,16	1,32	1,32	2,16	2,16	0,61	0,61	0,46
Nakamura 2002	1,75	1,75	1,75	1,75	1,75	1,00	1,00	0,57
Hosch	2,41	4,52	4,52	2,41	2,41	1,88	1,88	0,41
Izbicki 1997	1,24	2,87	2,87	1,24	1,24	2,31	2,31	0,80
Hosch 2001	2,41	4,52	4,52	2,41	2,41	1,88	1,88	0,41
Hosch 2000	2,67	4,59	4,59	2,67	2,67	1,72	1,72	0,37
Komukai 2000	2,12	4,50	4,50	2,12	2,12	2,12	2,12	0,47
Mueller	2,18	2,96	2,96	2,18	2,18	1,36	1,36	0,46
Laso	1,45	1,69	1,69	1,45	1,45	1,17	1,17	0,69

Appendix 6.1 example of calculation of lnHr from printed survival curve (Bonavina (130))

T	Start tci	End tci	Fmax	Fmin	Rci(ts)	Rci(t)
1	0	5	60	4	23	23
2	5	15	60	4	23	20,9090909
6	15	20	60	4	20,9090909	19,7474747
7	20	25	60	4	19,7474747	18,5132576
8	25	30	60	4	17,7727273	16,5032468
9	30	40	60	4	15,4717938	12,8931615
10	40	50	60	4	12,0336174	9,02521307

T						
1	Sci(ts)	Sci(te)	Dci(t)	Cci(t)	Cci(t)	Ts2
2	100	100	0	0,20535714	0	4
6	100	100	0	2,09090909	2,09090909	5
7	100	100	0	1,16161616	1,16161616	15
8	100	96	0,7405303	1,23421717	1,23421717	20
9	96	90	1,03145292	1,26948052	1,26948052	25
10	90	84	0,8595441	2,57863231	2,57863231	30
T	84	77	0,75210109	3,00840436	3,00840436	40

T						
1	T	Start tri	End tri	Fmax	Fmin	Rri(ts)
2	1	0	5	60	4	8
6	2	5	15	60	4	8
7	3	15	20	60	4	6,4
8	5	20	25	60	4	5,15151515

9	6	25	30	60	4	4,05681818
10	7	30	40	60	4	3,76704545

Appendix 6.2 example of calculation of lnHr from printed survival curve (Bonavina (130))

T						
1	Rri(t)	Sri(Ts)	Sri(Te)	Dri(t)	Cri(t)	Cri(t)
2	8	100	100	0	0,07142857	0
6	7,272727273	100	88	0,87272727	0,72727273	0,727272727
7	6,044444444	88	75	0,89292929	0,35555556	0,355555556
8	4,829545455	75	63	0,77272727	0,3219697	0,321969697
9	3,767045455	63	63	0	0,28977273	0,289772727
10	3,139204545	63	42	1,04640152	0,62784091	0,627840909

T						
1	Ts2	Te2	Dci(t)	Dri(t)	ln{HRi(t)}	Var [ln{HRi(t)}]
2	4	5	0,000001	0,000001	1,05605267	1999999,832
6	5	15	0,000001	0,87272727	14,7354311	1000000,961
7	15	20	0,000001	0,89292929	14,8861487	1000000,904
8	20	25	0,7405303	0,77272727	1,38629436	2,38342711
9	25	30	1,03145292	0,000001	-12,369213	1000000,643
10	30	40	0,8595441	1,04640152	1,60943791	1,722951013

T					
1	ln(HRi(t))/var(ln(HRi(t))	I/var	ln(Hri)	Var(ln(HRi)	SE(ln(Hri)
2	5,28026E-07	5E-07	1,51581076	1,00003154	1,00001577

6	1,47354E-05	1E-06
7	1,48861E-05	1E-06
8	0,581639084	0,41956391
9	-1,23692E-05	1E-06
10	0,934117047	0,58039955