Microvessel density as a prognostic factor in non-small-cell lung carcinoma: a meta-analysis of individual patient data

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Summary

Background **Angiogenesis is a potential prognostic factor that has been investigated in patients with non-small-cell lung carcinoma. However, published studies of the role of angiogenesis as a prognostic factor are inconclusive. We aimed to collect individual patient data to assess microvessel-density counts (ie, a measure of angiogenesis) as a prognostic factor in non-small-cell lung carcinoma.**

Methods **We obtained published and unpublished datasets and extracted appropriate data, taking particular care to ensure data quality. Detailed information was obtained for the laboratory methods used by every research centre that generated the data. The outcome of interest was overall survival. We did a meta-analysis to estimate the prognostic role of microvessel density by combining separately estimated hazard ratios (HR) from every study, which were adjusted for tumour stage and age. Analyses were done separately for studies that used the Chalkley method or for those that counted all microvessels.**

Findings **17 centres provided data for 3200 patients, 2719 of which were included in the analysis. All but three centres (datasets 9, 10, and 13–367 cases) had already published their findings, and six had updated follow-up information (datasets 1, 2, 3, 6, 7, and 8–1273 cases). For all but three centres (datasets 4, 11, and 13) some data corrections were necessary. For microvessel density counts obtained by the Chalkley method, the HR for death per extra microvessel was 1·05 (95% CI 1·01–1·09, p=0·03) when analysed as a continuous variable. For microvessel density counts obtained by the all vessels method, the HR for death per ten extra microvessels was 1·03 (0.97–1·09, p=0·3) when analysed as a continuous variable.**

Interpretation **Microvessel density does not seem to be a prognostic factor in patients with non-metastatic surgically treated non-small-cell lung carcinoma. This conclusion contradicts the results of a meta-analysis of published data only. Therefore, the methodology used to assess prognostic factors should be assessed carefully.**

Introduction

Non-small-cell lung carcinoma has a very high mortality.[1] Conventional treatment—ie, radical excision without radiotherapy or chemotherapy—is curative for only 40% of patients who are eligible for this treatment.[2]

Several biological factors, including measures of angiogenesis, have been investigated for their prognostic role. Such research aims to expand understanding of the disease process, identify patients at risk, and, improve clinical outcome. Although there is much anecdotal evidence to suggest that several biological factors have a prognostic role in non-small-cell carcinoma, their statistical investigation and clinical implementation are limited by a lack of consensus on appropriate investigation methods.

Consideration of all relevant evidence on a particular factor in a systematic way is desirable.[3] A systematic review identifies relevant studies, extracts relevant data, appraises study methods, and might combine results statistically (ie, meta-analysis). However, application of systematic review principles to prognostic studies poses practical and methodological difficulties.[4,5] First, identification of all published studies for a particular prognostic variable is not easy. Second, most prognostic factors in cancer are continuous variables, but researchers tend to dichotomise them into high and low levels using a cut-off point that is convenient or arbitrary[6] and that differs between studies.[7] Third, small studies are likely to give unreliable results, and those that show a large prognostic effect are more likely to be published than are those that do not; such publication bias is well recognised.[8,9] Evidence of publication bias in prognostic studies is accumulating: it has been shown in studies of Barrett's oesophagus as a risk factor for cancer;[10] has been suspected in other reviews;[11] and a review[12] of the prognostic role of P53 in head and neck cancer showed that published studies had larger prognostic effects than did unpublished studies. Fourth, issues associated with the methods of these studies are compounded by a generally poor standard of reporting. Riley and colleagues[13] reviewed prognostic markers for neuroblastoma and by use of ten different methods of data extraction were able to estimate log HR (with SE) from only 204 (35%) of 575 reports of markers. Furthermore, many researchers might not be able to provide missing data.[12]

Systematic reviewers might use only summary information extracted from published studies, or they might attempt to retrieve individual patient data. Methodological concerns suggest that a systematic review

of prognostic factors, based only on published data might be unreliable.[4,14] Therefore, a multicentre collaborative framework to obtain raw data from as many relevant studies as possible is a desirable approach for investigation of prognostic factors.

Angiogenesis is the process of haphazard new vessel formation required for tumour growth, invasion, and metastasis. Microvessel density is a measure of angiogenesis and a widely studied putative prognostic factor. We aimed to do a study of individual patient data to assess microvessel-density counts as a prognostic factor in non-metastatic surgically treated non-small-cell lung carcinoma, by obtaining data from all identified sources.

Methods

The Prognosis In Lung Cancer project

The Prognosis in Lung Cancer (PILC) project—an international collaborative study group—was set-up to obtain individual patient data for study of potential prognostic factors in non-metastatic, surgically treated non-small-cell lung carcinoma. Microvessel-density count was the first factor we assessed between 1999 and 2003 at the Centre for Statistics in Medicine, Oxford, UK, under the guidance of a steering committee. Details of the conduct of this study have been published previously;[15,16] in particular, the feasibility study[15] has details of methods.

During the recruitment stage, we produced a list of potential participants—ie, individuals and centres worldwide who were researching lung cancer—by doing a thorough literature search and by getting in touch with professional contacts of members of the steering committee to obtain details of unpublished studies. The literature search was carried out by MT, DGA, and FP in Medline without language or date restrictions. The terms used were: "non-small-cell lung carcinoma"; "angiogenesis", "microvessel density"; "MVD"; and "prognos*", and included all alternative spellings and combinations of terms. Details of PILC were presented at the 1st international workshop in lung cancer (Athens, Greece; June 17–19, 1999), and as a result one more centre was recruited (other conference participants had already agreed to the study). Centre selection was unrelated to the findings of any research done at the centres, or to whether the centre had published their findings previously.

All authors agreed on a list of potential participants. We sent a speculative letter to potential participants, who we encouraged to forward it to anyone they thought appropriate. Of 38 groups contacted, 28 replied positively; however, one of these groups could not participate because of lack of resources. Ten centres did not reply, and follow-up attempts to make contact were not successful: three were untraceable and seven did not respond to a reminder.

Of the 27 willing participants, 18 had the required data and were able to supply it within project time limits.

***Panel 1:* Centre inclusion criteria**

- All patients in centre's dataset had a diagnosis of non-small-cell lung carcinoma
- For all patients, the cancer diagnosed was a primary tumour and not a result of metastasis or recurrence
- There was at least 2-years of follow-up after surgery for all patients in the dataset
- Patients were not participating in a clinical trial or study where blinding was involved to the extent that the researcher contributing the data was not able to answer questions crucial to this study

***Panel 2:* Patient inclusion criteria**

- Complete anatomical resection of lung carcinoma
- Survival information available, and patient had survived at least 30 days after surgery (otherwise death was regarded as perioperative)
- Microvessel-density count was available
- Tumour stage was less than stage IV, at which stage metastasis has already occurred and surgery has only palliative benefit

Nine centres stated that they had no relevant data, four of which had no separate data of their own, but were in collaboration with other centres that had agreed to participate in PILC. However, all nine centres expressed an interest and willingness to be contacted at a later time if another suitable collaborative project was planned. Of the 18 centres with the required data, 17 fulfilled the basic participation criteria set by the PILC committee (panel 1); we obtained 100% of the data from the 17 centres. We excluded one of the 18 centres because patients were participating in a chemotherapy trial. We sent every centre a follow-up pack that contained the PILC study proposal and a request for individual patient data. We regarded it important to have a formal agreement with the data owners, and we asked the lead collaborator of every participating research group to sign a consent form. Informed consent was not sought from the patients by PILC because the obtained data was anonymised, and at the time of the start of the study consent was not regarded as necessary for this type of study.

We received the following information from contributing centres for every patient: unique identifier; age; sex (available in all but one centre); dead or alive status; survival (in days); microvessel-density counts; and tumour stage, type, grade, and size (tumour size and grade not available from all centres). Data for performance status were not available. We sent participating centres a dataset characteristics form (webappendix), which asked about: whether the study was prospective or retrospective; the method for obtaining microvessel counts; antigen used in immunohistochemistry; microscope magnification; number of hot spots (areas of dense vascularisation); number of pathologists involved in measurement; calculation of survival; and whether the data had been published. The information gathered from

See Online for webappendix

***Panel 3:* Most prominent data problems found in the participating datasets**

- Some patients' disease-free survival was longer than total survival
- Manual calculation of survival, with or without a calendar, resulted in errors
- Incorrect calculation of cancer stage from the tumour, node, and metastasis values
- Inconsistencies between vital status and information on date of death, commonly because cause of death was not related to lung cancer
- Incorrect inclusion of some patients in the database, commonly those that only had a biopsy and not surgery
- On one occasion, while enquiring about a numerical mistake, we found that during an attempt to sort a spreadsheet, only half the columns had been sorted

***Panel 4:* Measuring angiogenesis—available variations**

- There are three main staining antibodies in laboratory tumour preparations—CD31, CD34, and factor VIII
- There are two main methods of measurement—counting of all microvessels or use of the Chalkley camera
- One or more hot spots in one or more fields can be chosen
- One observer or more can count microvessel density either simultaneously or at different times
- Variation exists on how to reach consensus between observer. Options include: add together counts from different fields of view; calculate mean density; or report only the highest value
- Microscope magnification varies

using this form substantially improved our understanding of the nature and structure of the individual datasets, especially the methods used to count microvessel density.

Individual patient data

We obtained data for 3200 patients. Panel 2 shows the inclusion criteria for patients. All cases with a microvessel-density count of zero were excluded from the analysis. Different centres use the number zero in different ways. For instance, in some datasets a microvessel-density count of zero meant a microvessel-density count was missing, whereas in others it meant that no vessels were identified on the slide (a technical impossibility, it can only mean aberrant laboratory procedures). Cases were included once in any PILC analysis. If a patient was present in more than one dataset, the most updated data were used, or the most appropriate data as agreed with data-owners.

Collection of individual patient data and of details about the methods by which these data were obtained gave information that was more detailed than would have been possible if only published studies were reviewed. For 14 datasets, data corrections were necessary—sometimes major; three datasets had not been published; updated follow-up information was available for six datasets; and duplicates were removed after identification of pairs of studies with overlapping patient groups.

We identified many inaccuracies and inconsistencies during data validation. Individual researchers were pivotal for resolution of these issues. Checking, validation, and standardisation of all datasets took nearly 2 years, which is consistent with previous estimates for meta-analysis of individual patient data.[17] Panel 3 highlights some of the most prominent data problems.

After checking and validation of data, we produced a profile for every centre and sent it to the corresponding researcher for checking. The profile contained basic data description and exploratory statistics to ensure that all information supplied had been understood and interpreted correctly, especially data for microvessel-density counts. As a result, some further problems were highlighted and corrected. After identification and exclusion of unsuitable cases according to the criteria (panel 2), there were 2719 unique valid patients.

Measurement of angiogenesis

Some tumour types need angiogenesis for growth.[18,19] This complex multistep process is associated with invasion of neighbouring areas and ultimately metastasis. Microvessel-density count is a measure of angiogenesis.[18,20] A microvessel-density score can be obtained only after surgical excision of the tumour. A slice of the tumour is treated appropriately in the laboratory, and a pathologist counts the number of vessels showing up in one or more carefully chosen areas of the tumour (ie, hot spots) under the microscope. Dense tumours have a large microvessel-density score and may be more likely to spread to other sites than are those that are less dense. Therefore, angiogenesis is regarded a likely prognostic factor in many types of cancer, including that of the lung.

In 1991, Weidner and colleagues[21] produced a microvessel-density count in an area of intense vascularisation in a tumour. However, the expected range of measurements varies greatly depending on rules and preferences that every research centre uses in its laboratory. Some PILC centres avoided counting single cells and assessed only well defined vessel structures. Thus, their score might be lower than those of other groups. In 1943, Chalkley[22] used a graticule or camera to estimate a relative area of vasculature. On the basis of 25 random point readings, the graticule can be inserted on the microscope while focusing on a hot spot. Rotation of the graticule so that the random points touch as many vessels as possible gives a theoretical maximum of 25 counted vessels per hot spot.[22]

Therefore, two methods of counting microvessel density, and their variations, have been used widely: the density method, in which all vessels in a hot spot (ie, an area of high vessel concentration) are counted; or the Chalkley graticule, which gives an estimate of vasculature relative to the area studied.[22] Although the Chalkley graticule was introduced to simplify counting of vessels, substantial variation exists in measurement procedures

Dataset	Cases sent	Cases used	Antigen	Magnification	Microscopy fields	Hot spots	Observers	Consensus between observers	Mean MVD (SD)	Median MVD (range)
All vessels*										
2	148	34	CD31	×200	3	3	2	Discrepancies resolved by use of conference microscope	16·9 (13·7)	10·5 (5–54)
4	204	163	Factor VIII	×250 and ×400	10	1	3	Mean of the three counts	10·4 (10·7)	7·0 (1–64)
5	119	119	CD34	×250	3	3	1	..	41·5 (23·4)	34·0 (8–132)
6	126	113	CD31	×200	3	3	1	..	20·1 (15·2)	15·3 (3–71)
8	510	407	CD34	×250	3	3	2	Slide reviewed simultaneously if large discrepancies; senior investigator's reading used if no large discrepancies	24·7 (15·5)	20·0 (3–100)
9	111	85	CD31	×200	3	1	1	..	29·1 (13·2)	26·0 (6–64)
10	76	69	CD34	×200	All available under ×100 magnification	2–5	1	1 month after first reading, same observer reassessed all slides and took mean of both readings	85·1 (23·3)	82·7 (35–164)
11	408	404	Factor VIII	×100	10 consecutive fields	1	3	Discrepant scores resolved by consensus of three reviewers	23·7 (18·0)	4·6 (1–179)
12	42	39	Factor VIII	×200	1	3–5	2	Simultaneous reading by use of double-headed microscope	19·8 (7·0)	15·0 (92–50)
13	213	213	CD34	×200	3	1	1	..	107·3 (38·1)	105·0 (35–237)
14	94	33	Factor VIII	×400	5–10 consecutive fields, avoiding necrotic areas	0	2	Simultaneous reading	7·9 (4·7)	6·6 (1–20)
15	115	98	CD31	×400	4	4	1	..	18·9 (8·5)	18·3 (5–64)
16	88	87	CD31	×200	3	3	1	..	132·4 (57·7)	122·5 (46–300)
17	143	136	CD34	×200	Whole section	3–4	1	..	60·8 (29·7)	54·8 (23–242)
Chalkley method†										
1	69	64	CD34	×250	3	3	2	Two independent counts. Mean count if <10% discordance, otherwise consensus by reassessment of slide by use of double-headed microscope	6·9 (1·9)	6·3 (4–13)
3	218	180	CD34	×250	Whole section viewed under low power	3	2	Two observers reviewed every slide on double-headed microscope; agreement on hotspots was reached before counting	6·1 (1·8)	6·0 (3–11)
6	119	105	CD31	×200	3	3	1	..	5·7 (2·7)	5·0 (2–12)
7	515	475	CD31	×200	Not specified	3	1	..	6·2 (1·7)	6·0 (2–12)
13	213	213	CD34	×200	3	1	1	..	5·4 (1·6)	5·0 (2–12)
17	143	136	CD34	×200	Whole section	3	1	..	7·0 (1·8)	7·0 (4–15)

MVD=microvessel density. *Total cases sent=2397; total cases used=2000. †Total cases sent=1277; total cases used=1173. Unique cases after allowance for centres that used both all microvessels and Chalkley method=2719 (2000+64+180+475): datasets 6, 13, and 17 had data for both methods of assessing microvessel density, therefore, to avoid double-counting of cases, only datasets 1 (64 cases), 3 (180 cases), and 7 (475 cases) from centres that used Chalkley method contribute to the count of unique cases.

Table 1: **Description of datasets**

for angiogenesis with no widely agreed best method (panel 4). For six of the 17 datasets, microvessel-density counts were obtained by use of the Chalkley method; 14 datasets measured all vessels (ie, the density method; table 1); three datasets used boths methods. Webfigure 1 shows histograms of microvessel-density counts for every dataset according to whether the centre used the Chalkley method, assessment of all vessels, or both.

When we started the project, we were unaware of the intricate details and substantial differences in the methods used to count microvessel density. We did not anticipate such diversity in methods: although many studies of microvessel density have been published for various cancers, the reporting of methods used has frequently been incomplete.

Comparison of Chalkley and all-vessels measurements

Even when the same method was used to count microvessel density (ie, Chalkley *vs* all vessels), individual laboratories used very different procedures for measurement of microvessel density. Three of the 17 datasets (6, 13, and 17) used both Chalkley and all vessels methods. We assessed the association between the two methods of angiogenesis measurement.

See Online for webfigure 1

Although the three centres generated similar data types, they differed in the way they obtained them. In centre 6, two pathologists reported the sum of counts for three different hot spots—one pathologist obtained Chalkley counts and the other assessed all vessels. In centre 13, one pathologist measured microvessel density by the Chalkley method and by counting all

vessels, using the same three hot spots for the two methods. In centre 17, one pathologist measured microvessel density count using both methods, but chose different hot spots for each method; for the Chalkley method, the mean of three hot-spot measurements was reported, whereas for measurement of all vessels the maximum of three or four hot-spot counts was reported (the mean was eventually estimated—see measurement standardisation below).

These variations in method are highlighted graphically when mean microvessel density as assessed by all vessels are plotted against mean Chalkley values (webfigure 2). The best correlation (r=0·74) was recorded for dataset 13, where the same pathologist used both counting methods on the same three hot spots. For dataset 6, the correlation is less good, probably because different hot spots were used by two different observers for the two counting methods. For dataset 17, in which one observer assessed different hot spots, there are substantial differences between the counts, which are barely correlated.

See Online for webfigure 2

The method used to count microvessel density is clearly a major differentiating factor of the PILC datasets. Consequently, we did separate analyses according to the method of microvessel-density count. Data for 1173 patients were available by use of the Chalkley method, and data for 2000 patients were available by assessment of all vessels; 454 patients in three centres were included in both analyses.

Measurement standardisation

Throughout the analyses, we used mean microvessel-density count for every patient. Although measurements were given in various forms, the mean was straightforward to obtain for most patients. However, for three datasets (10, 16, and 17), only the maximum measurement from several hot spots was available. We estimated the mean value for patients in these datasets by using information in three other datasets (5, 9, and 13), for which all individual hot-spot measurements (and hence their mean) were available. These data were used to calculate an appropriate correction for estimation of the mean.

We established that in each of datasets 5, 9, and 13 there was a linear relation between the maximum measurement and their mean. For every patient in each of the three datasets separately, we calculated the ratio between the dataset-specific mean and maximum value. Then we calculated the three dataset-specific mean ratios, and finally we obtained the mean value of the three ratios (which was equal to 0·87). We applied this correction to the three datasets for which only the highest values were available (ie, 10, 16, and 17). Further information about the correction method has been published previously.[16]

Other considerations

Survival was defined as the number of days a patient survived from the date of surgery to the date of last follow-up or death. We defined as deceased any patients with a date of death recorded at last follow-up irrespective of cause of death.

After agreement with our collaborators, the datasets are not identified by name but by an identification number. However, several datasets used in this PILC project have been published.[23–36] We asked collaborators during data collection in 1999–2001 to specify which publication appropriately reflects the dataset they supplied to PILC. However, this publication might not be the only version of the dataset. The study was approved by Imperial Cancer Research Fund (now, Cancer Research UK) and complied with all formal issues required at the time of the start of the study (in 1998).

Statistical analyses

Analyses were done separately for centres that used the Chalkley method and for those that assessed all vessels. Furthermore, we noted substantial heterogeneity of methods in studies that counted all vessels (table 1).

Microvessel-density counts, irrespective of the method used to obtain them, are continuous quantities. However, most published studies have assessed angiogenesis as a dichotomous variable.[23,23,28,29] There are no guidelines for choice of cut-off points, and researchers have different approaches for choosing them. This practice is statistically inefficient[37] and might be misleading when cut-off points are chosen by a data-dependent method, such as minimisation of the p value (a method of doing multiple analyses of data by use of different cut-points with the aim of choosing the method that makes the p value as small as possible).[6,7] The most common cut-off point is the dataset's median, and many publications relating to the PILC datasets used this method. Our primary analysis used the recorded microvessel-density counts without grouping, but for comparison of some analyses microvessel-density counts were dichotomised by use of the dataset-specific median as the cut-off point.

Tumour stage is an established prognostic factor. Greatens and co-workers[38] assessed seven putative prognostic factors in non-small-cell lung carcinoma (not microvessel density) and found that none had any prognostic value beyond that provided by pathological stage alone. Microvessel density might have a different prognostic value according to tumour stage, and we investigated stage in our analyses.

We used Cox regression models to obtain the log HR (with SE) for death separately for each dataset; microvessel density, stage, and age were included in the models. We investigated linearity by assessing a quadratic term for microvessel density in every dataset; we found no evidence for non-linearity. We included tumour stage in these models because a useful prognostic factor has to add information to what is already known (ie, to standard prognostic factors for that disease).[2] In univariate analysis, age (as a continuous variable) was significant for some datasets (including two of the three largest) and, therefore, was included as

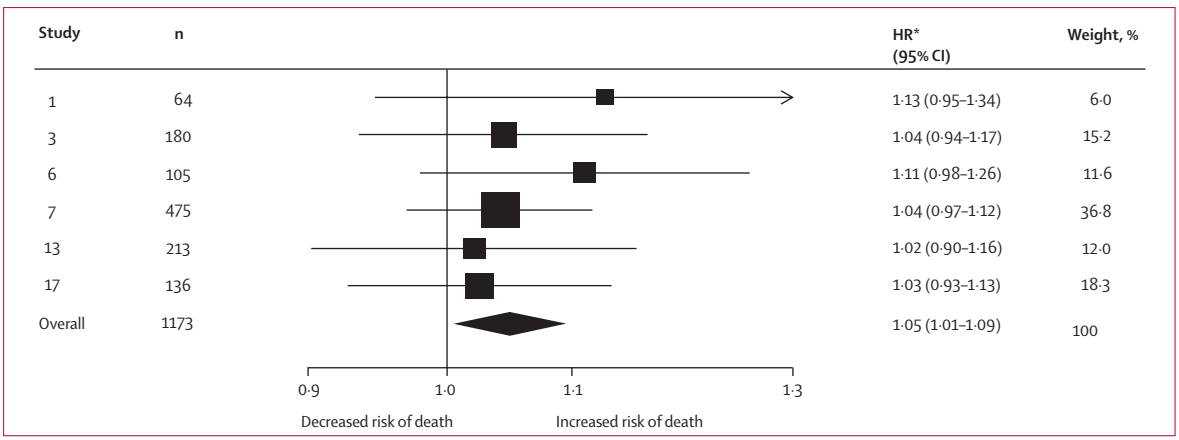

Figure 1: **Association between risk of death and increase of one microvessel count, as measured by Chalkley method**
*Adjusted for age and cancer stage.

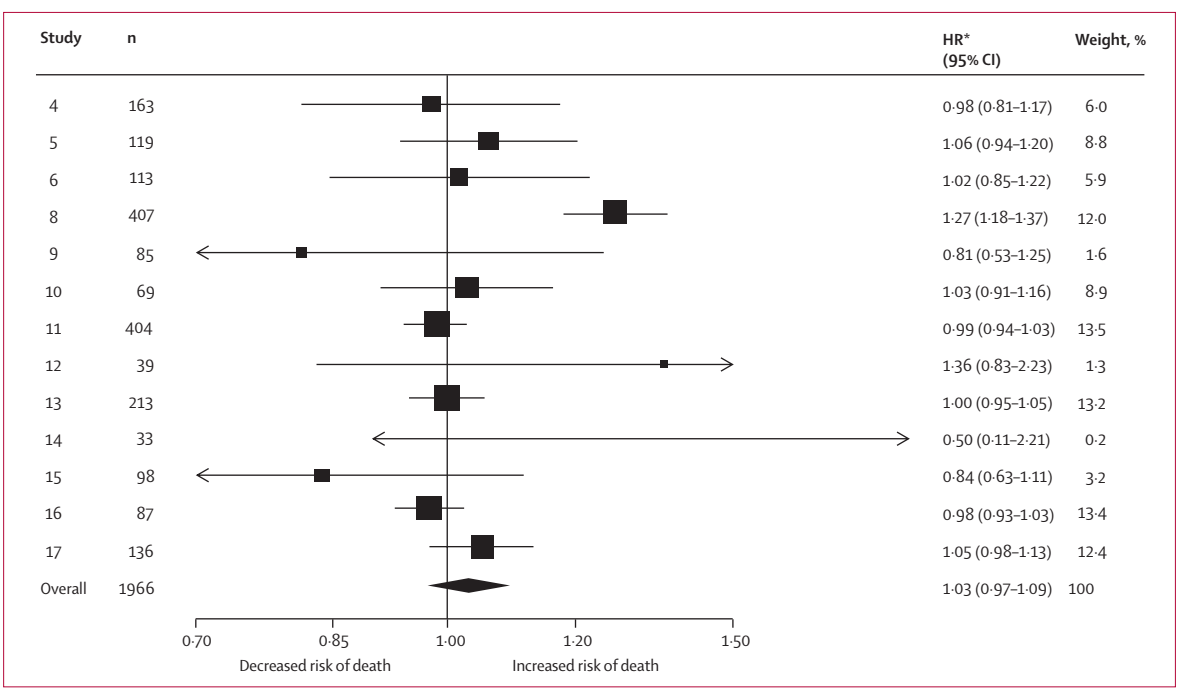

Figure 2: **Association between risk of death and increase of ten microvessel counts, as assessed by measurement of all vessels**
*Adjusted for age and cancer stage.

an adjusting factor. These analyses were done twice: with microvessel density as a continuous variable; and with density categorised as high or low, by use of the dataset-specific median as a cut-off point.

To estimate the prognostic value of microvessel density, individual results from every dataset were pooled by use of random-effects inverse-variance meta-analysis.[39] The pooled effect is expressed as a HR per increase of one vessel for the Chalkley method; per ten vessels for all vessels; or as a comparison of high and low counts for analyses of dichotomised data. All p values, calculated at the 95% level, are two-sided. A dataset was included in meta-analysis only if the number of events (ie, deaths) was at least five. Therefore, dataset 2 was excluded from the meta-analysis. Heterogeneity was quantified by I^2, which describes the percentage of total variation across studies that is due to heterogeneity rather than chance;[40] smaller percentages suggest less observed heterogeneity. Further analysis included investigation of the prognostic value of microvessel density by tumour stage; here, the cox regression model was adjusted for age only.

Generally, and therefore in PILC, most patients with non-small-cell lung carcinoma are men, and separate descriptive statistics for men and women are not

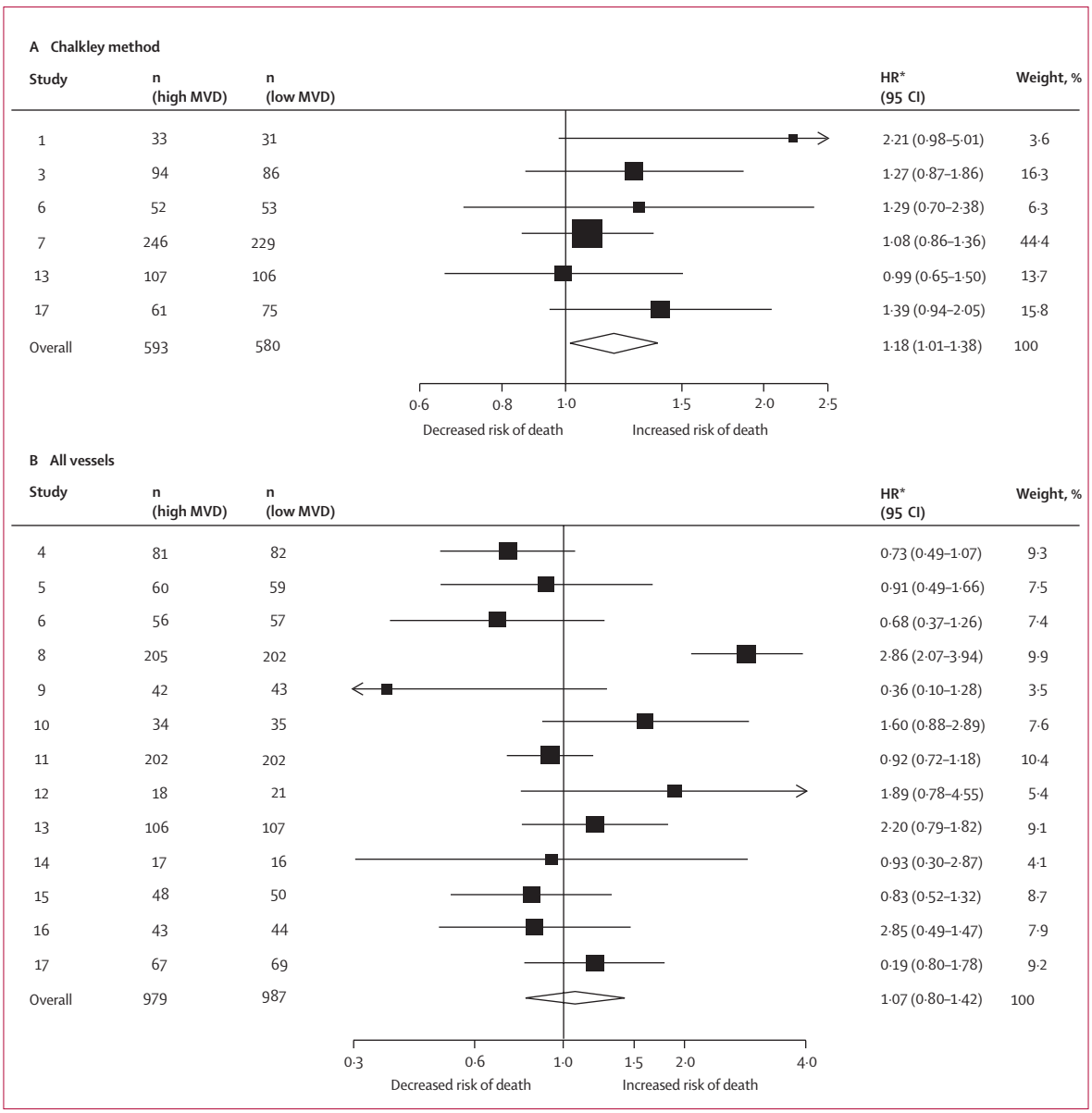

Figure 3: **Association between risk of death and microvessel density classified as binary variables**
(A) Risk of death per increase in one microvessel count, as assessed by Chalkley method. (B) Risk of death per increase in ten microvessel counts, as assessed by measurement of all vessels. Note different scales on horizontal axes. MVD=microvessel density. *Adjusted for age and cancer stage.

presented because of small numbers in some groups. We considered classification by antigen used during immunohistochemical analysis. Of centres that assessed all vessels, four used factor VIII, five CD31, and five CD34. Of centres that used the Chalkley method, no studies used factor VIII, four CD34, and two CD31 (table 1). However, few studies have compared staining performance of the antigens for angiogenesis measurement in lung cancer, and conclusions have been contradictory.[35,41] We did not assess antigens in the analyses. All data analyses was done by use of SPSS (version 11.0) and STATA (version 8.0).

Role of the funding source
The sponsors of the study had no role in the study design; in the collection, analysis, or interpretation of the data; or in the writing of the report. The corresponding author had full access to all data in the study, and had final responsibility to submit for publication.

Results
Four datasets (4, 6, 8, and 10) were obtained prospectively by collection of data as patients were diagnosed; the remaining datasets were obtained retrospectively from hospital historical records. Two datasets (5 and 7) were

generated from some historical records and by collection of prospective data. Sample sizes varied from about 30 patients to nearly 500. 2146 (75%) patients were men (data for sex not available for dataset 10). 1530 (56%) of cases had died; median survival was about 3–4 years. Most patients had stage I or II disease; 421 (15%) of the 2719 PILC patients had stage III disease.

For the Chalkley method, microvessel-density distributions resembled the normal in all six datasets, and means and ranges did not differ substantially between them (webfigure 1). By contrast, distributions for datasets from the all-vessels method varied, and mean values ranged from 10 to 132 (table 1 and webfigure 1).

Figure 1 shows a forest plot of the six studies that used the Chalkley counting method, including the pooled estimated HR for death per additional microvessel from a random effects model (p=0·03). The results suggest that higher microvessel density counts were associated with worse survival. There was no noted heterogeneity between studies (I^2=0%).

Figure 2 shows a meta-analysis for studies that counted all vessels, including the pooled HR for death per ten vessels (p=0·3). These data suggest that microvessel density is not associated with survival. Heterogeneity (I^2=73·7%) was due to dataset 8. After exclusion of this dataset, there was no observed heterogeneity (I^2=0%) and the HR was 1·00 (95% CI 0·97–1·02, p=0·84; data not shown).

Figure 3A shows findings from analyses of Chalkley microvessel density as a binary variable, including the pooled estimated HR for death per additional microvessel (p=0·03). There was no heterogeneity between studies (I^2=0%). These findings suggest that high Chalkley counts analysed as a binary variable are associated with a poor prognosis for patients with non-small-cell lung carcinoma. There is no evidence to suggest that microvessel density as assessed by the all-vessel method and analysed as a binary variable has a prognostic effect (figure 3B; p=0·66). Dataset 8 accounted for most of the variability (I^2=77%). After exclusion of this dataset, heterogeneity was reduced (I^2=18%) and the pooled HR was 0·96 (0·82–1·12, p=0·66; data not shown).

The prognostic importance of angiogenesis did not differ by tumour stage, irrespective of microvessel-counting method: all such meta-analyses, with counts analysed as continuous variables (table 2) and as binary variables (data not shown) gave non-significant results. No heterogeneity was noted between studies.

	Disease stage	Number of patients*	Number of datasets	HR (95% CI)†
Chalkley method	Stage I	968	6	1·03 (0·98–1·09)
	Stage II	118	3	1·06 (0·94–1·19)
	Stage III	64	2	1·08 (0·94–1·24)
All microvessels‡	Stage I	1298	12	1·03 (0·96–1·10)
	Stage II	293	8	1·09 (0·96–1·22)
	Stage III	290	8	1·04 (0·94–1·16)

*Total number of patients differs from that in table 1 because data for disease stage were not available for all patients. †Adjusted for age. ‡Dataset 2 excluded from meta-analysis.

Table 2: **Results from meta-analysis by disease stage**

Discussion

Overall, our analyses give only weak evidence to suggest that microvessel density is a prognostic factor for survival in patients with non-small-cell lung carcinoma when analysed as a continuous variable and measured by use of the Chalkley method. No prognostic effect was observed by measurement of all vessels.

Laboratory developments have helped identify many potential cancer prognostic factors. However, there is no consensus on how to design and analyse prognostic studies, despite several published studies.[14,42–46] Studies of prognostic factors are characterised by diversity in methods and poor reporting of those methods, commonly leading to contradictory results.[14,42,47] Systematic reviews done appropriately and perhaps a comprehensive summary of the current evidence of prognostic factors for a specific cancer with regular updates would offer informed opinions.

Despite practical difficulties in the application of systematic-review principles to the area of prognosis,[4] this approach is the best available for efficient use of published information. Drawbacks of systematic review of published data only are well known.[3,4,47–49] The alternative approach of meta-analysis of individual patient data has advantages. However, important considerations for this approach are resources and difficulties in obtaining relevant datasets.[17,50–53] Advantages of individual patient data are reduced effect of publication bias, better understanding of the methods used, and the ability to analyse data consistently.[50–52,54]

Few researchers have investigated cancer prognostic factors using individual patient data.[54–56] To our knowledge, the PILC project is the first attempt to assess the role of an immunohistochemical tumour marker using individual patient data, while attempting to acquire all known data with no geographical restrictions.

Most prognostic studies are too small to identify a modest association with outcome.[14,57] Dichotomisation of the potential prognostic factor is a standard procedure, but has many problems including loss of power; such an approach is not recommended.[37] Furthermore, data-driven dichotomisation to achieve a significant result may lead to bias.[7] On analysis of individual patient data, actual measurements can be used. Publication bias might be leaving many data invisible because non-significant results might not be reported.[12,58,59] A systematic review will be misleading if the sample of primary studies is biased.[9,12] Improved quality of primary studies and reporting would improve this situation.[47,60]

Although as many data sets as possible were collected for PILC, this technique is not essential: a study of

individual patient data that restricted the included studies would be valuable. Sample selection according to geographical origin, homogeneity of laboratory techniques, and sample size (ie, focusing only on large studies) may be possible. Identification of unpublished data may be a problem in all circumstances, but not doing so would leave the review open to bias.

Individual patient data undoubtedly gives the best-quality information in the long term,[3] but a meta-analysis that uses individual patient data cannot overcome either deficiencies of individual studies or heterogeneity of approaches. It does, however, allow such variations to be identified and investigated, and enables a consistent approach to data analysis. Moreover, the inclusion of unpublished data reduces the risk of publication bias. The over-riding disadvantage of individual patient data is that their use is very time-consuming. Whether such an exercise is cost-effective in a given situation or whether resources would be used better on a new high-quality, large, prospective, and possibly multicentre study is debatable. In the future, the best option might be to use data derived from tissue banks.[3,61]

Microvessel-density counts, as a measure of angiogenesis, have been investigated widely as a cancer prognostic factor.[36,62–70] For this factor to yield a useful measure of prognosis, variation between methods used to measure it should be kept to a minimum. Potential sources of variability include choice of antigen, microscope magnification, number of hot spots, number of observers, method of consensus used, and, most importantly, choice between Chalkley method or measurement of all vessels.[18,20,71–76] These two methods produce incompatible measurements, and data obtained from each method should be analysed separately.

Hansen and co-workers[71] compared the reproducibility of microvessel-density counting and observer variability. They found that use of a Chalkley camera produces little variation between observers, whereas measurement of all vessels gave substantial variation between observers. These findings are consistent with those of Offersen and co-workers,[72] who assessed histopathological methods for estimation of microvessel density. These findings were confirmed by analysis from available PILC data. The Chalkley method is a standardised, consistent approach for counting of microvessel density. By comparison, assessment of all vessels has no standard protocol, and the method of counting varies widely. Therefore, there are clear grounds for preference of the Chalkley method. However, there are major variations that can arise in the conduct of both methods (panel 4).

Although several studies have assessed angiogenesis as a potential prognostic factor in non-small-cell lung carcinoma, evidence to date is contradictory.[24,28–31] The large variations in methods might account for this disparity of conclusions. Reliability of measurement techniques for microvessel density, choice of cut-off points, and the sample size in relation to the number of events affect study quality. Published studies might be more likely than unpublished studies to show a strong association, leading to publication bias.

Choice of antibody for investigation of angiogenesis is related to their potential to stain vessels, and thus might affect the number of microvessels available for counting. Fox and colleagues[75] lend support to the use of CD31 for immunohistochemical analysis because of its sensitivity for microvessels compared with other antigens. In the same study,[75] however, the researchers mention the comparison of staining outcomes between CD31 and factor VIII, and found no difference in the prognostic information provided. Other researchers have compared staining performance between antibodies, but conclusions are commonly contradictory.[35,41,76–78] If two different laboratories use the same antibody, staining quality might differ because of variability between methods. Therefore, direct comparisons between antibodies might be unreliable unless they were done under the same laboratory conditions. A recent[18] international consensus statement on the methods and criteria for assessment of angiogenesis suggests—but crucially does not prove—that CD34 should be the antigen of choice. Consequently, we do not present on analysis according to the antigen used.

Another meta-analysis of angiogenesis was published while our study was under way.[79] Meert and colleagues[79] identified 21 published studies, 14 of which were eligible for inclusion in their meta-analysis. Data from ten of these studies are included in PILC, allowing an interesting comparison between the two approaches and results. Overall, Meert and colleagues[79] found that angiogenesis is a prognostic factor—ie, that high microvessel density leads to poor prognosis; this finding contrasts with PILC results.

Meert and colleagues[79] grouped studies by antigen, but did not differentiate between studies that used the Chalkley method and those that assessed all vessels. Cut-off points used in the studies varied: where possible, the authors used the observed median, otherwise the value from the original studies was accepted—frequently an arbitrary cut-off. Furthermore, the researchers used for preference unadjusted HR (rather than the ideally homogeneously adjusted HR across studies). When these data were not given they used any data available to estimate them, introducing inconsistency and making the findings subject to the usual data-extraction errors.[80–83] These choices might have affected the overall result of their systematic review.[77]

By contrast, we obtained individual patient data from all contributing centres, and we extensively checked and standardised data. Therefore, there are differences from the data used by Meert and colleagues[77] because of additional follow-up information, correction of errors, and specific case-selection criteria. We adopted a uniform cut-off point in addition to analysing the data as continuous variables. We included unpublished data, and we had access to further detail about study methods than was

given in published studies. We adjusted all analyses for age and disease stage. Moreover, we separated all analyses by the most contributory factor of variation—ie, the method of counting microvessel density: Chalkley method *vs* assessment of all vessels. Therefore, the HR from the two studies are not comparable, especially for analysis of microvessel density as a continuous variable. However, the effect of the study methodology (ie, individual patient data *vs* published data only) on the results is an issue that can be studied further in future work.

Tumour angiogenesis is a unique therapeutic target shared by many types of tumour.[59] However, we conclude that the methods for obtaining the measured data are so diverse that they mask a confident answer. Our study gives only weak evidence to suggest that high counts of microvessel density in patients with non-metastatic, surgically treated non-small-cell lung carcinoma is a marker of poor survival when analysed as a continuous variable and measured with the Chalkley method. Importantly, the association is fragile and is not consistent with studies that measured all vessels. Therefore, despite Chalkley's apparent superiority, angiogenesis should not be regarded a prognostic indicator of survival in patients with non-small-cell lung carcinoma. With improved and standardised measurement techniques, a measure of angiogenesis might provide useful prognostic information for patients with non-small-cell lung carcinoma in the future.

One variable is unlikely to be adequate to predict survival from any disease.[84] However, systematic reviews of one factor (as in our study) can give sufficient evidence for the usefulness of the factor for future reference in multifactor studies. At present, that microvessel density can play a valuable prognostic part in non-small-cell lung carcinoma seems unlikely, mainly due to diverse laboratory methods for measurement of microvessel density.

Rather than further study of existing data, whether published or individual patient data, better answers to the assessment of prognostic factors will come from prospective studies. The formation of collaborative groups has been recommended to achieve several high-quality individual studies, with the long-term aim of pooling individual patient data from every study in a prospective meta-analysis.[3] Tissue banks can help to realise the objective of high-quality data for assessment of prognostic markers.

Contributors

All authors jointly planned the study, developed the study proposal, and screened datasets for inclusion. MT administered data collection and did data analysis with guidance from DA. MT and DA drafted the paper, and all authors approved the manuscript. FP provided expert advice in pathology throughout the project.

Conflict of interest

The authors declared no conflicts of interest.

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There were 17 centres, but more than one collaborator per centre; only one consenting signature per centre was required.

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