Relationship between vascularity, age and survival in non-small-cell lung cancer

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Summary Lung tumours in the elderly show reduced growth potential; impaired angiogenesis may contribute to this phenomenon. Recent studies have suggested that the angiogenic potential of a tumour may be inferred by the vascularity measured in histological sections. The purpose of this study has been to determine whether vascularity is related to age, survival or other clinical parameters in resected non-small-cell lung cancer (NSCLC). A group of 88 consecutive patients with a follow-up period of at least 5 years was selected. The group exhibited a wide age range (37–78 years) and similar survival characteristics to those of the general NSCLC population. Tumour sections were stained with a pan-endothelial antibody (vWF) and vascularity was quantitated, without knowledge of the clinical details, by three methods: highest microvascular density; average microvascular density; and average microvascular volume. The results were analysed by non-parametric statistical tests. A correlation was found between all three methods of quantitation. Vascularity was not associated with age, sex, tumour type, stage, volume, size (TNM-T) nodal status (TNM-N) or survival. However, survival time was generally longer for patients with higher vascularity, reaching borderline significance ($P = 0.06$) for the average microvascular density values. Higher tumour volume ($P = 0.02$) and stage ($P = 0.05$) were associated with lower survival times. Using multivariate survival analysis, tumour volume was the only factor related to survival. We conclude that vascularity is not associated with age and has no significant prognostic value in NSCLC.

Keywords: lung cancer; vascularity; microvasculature; age; tumour volume; prognosis

The incidence of squamous cell lung cancer increases with age, accounting for approximately 40% of all lung cancers by the age of 80 years (Bryne and Carney, 1995). Clinical studies have indicated that lung carcinomas in the elderly show reduced growth rate and metastatic potential in comparison with those of younger patients (Ershler et al, 1993; Holmes 1989). This is consistent with the finding of an inverse relationship between stage and age in non-small-cell lung cancer (O'Rouke et al, 1987; Teeter et al, 1987).

The formation of new blood vessels, or angiogenesis, has been shown to be essential for tumour growth and metastasis in experimental animal models (Folkman et al, 1990). In such experimental models both angiogenic potential and tumour growth are reduced with age (Yuhas et al, 1974; Kim et al, 1989; Kreisle et al, 1990; Ershler et al, 1993). It is therefore possible that angiogenic suppression may play a part in the reduced growth and metastatic potential observed in lung tumours of elderly patients (Ershler et al, 1993).

Intratumoral microvessels can be easily visualized in histological sections using immunostaining with endothelium-specific antibodies. The stained vessels can then be counted by a variety of methods, the most popular of which is the measurement of the highest microvascular density or 'hotspot' (Weidner 1993). A large number of studies have found the highest microvascular density value to be associated with metastasis and poor survival in various types of tumours (reviewed by Gasparini, 1994; Craft and Harris, 1994). These results suggest, therefore, that the angiogenic potential of a tumour may be inferred by its vascularity. This

hypothesis is widely accepted, so that vascularity measured in histological sections using pan-endothelial antibodies is increasingly referred to as 'angiogenesis'. The validity of this hypothesis, however, is questioned by results of several studies that found either no association between vascularity and prognosis (Hall et al, 1992; van Hoef et al, 1993; Axelsson et al, 1995; Busam et al, 1995; Tahan and Stein, 1995; Morphopoulos et al, 1996) or highest vascularity associated with good prognosis (Kainz et al, 1995; Zatterström et al, 1995).

A small number of reports concerning the association between vascularity, metastasis and survival in non-small-cell lung cancer have been published. The results are also divided along the same lines as described above, i.e. the majority of studies found high vascularity to be associated with poor prognosis (Macchiarini et al, 1992; Yamazaki et al, 1994; Fontanini et al, 1995; Yuan et al, 1995; Giatromanolaki et al, 1996), although one large study (comprising over 200 patients) found that vascularity had no prognostic value (Mattern et al, 1995).

The objectives of our study have been to determine whether vascularity in resected non-small-cell lung cancer is associated with either survival or age. It has previously been suggested that contradictory results in the literature may be explained by variations in vascularity among blocks taken from the same tumour (van Hoef et al, 1993; De Jong et al, 1995) and by the methods used to measure vascularity (Pazouki et al, 1997). As quantitative methods in diagnostic pathology must be reliable and straightforward, we have attempted to clarify this situation by measuring vascularity in a well-defined group of NSCLC patients, using three different methods. The patients exhibited a wide age range and similar survival characteristics to those from a larger series previously reported and from the general NSCLC population (Jefferson et al, 1996).

MATERIALS AND METHODS

Patients and specimens

A group of 88 patients was selected from a larger series of surgical resections for NSCLC performed at the Cardiothoracic Centre, Liverpool, UK (Jefferson et al, 1996). The patients were chosen from consecutive operative resections by the following criteria: (a) complete survival data were available for a follow-up period of at least 5 years; (b) histological material was available in the form of at least four formalin-fixed, paraffin-embedded blocks; (c) only the most common NSCLC cell types, squamous cell or adenocarcinomas, would be included; and (d) the number of patients had to be large enough so that the group would show survival characteristics representative of the larger series. Although part of the aims of the study was to examine the relationship between age and vascularity, the group chosen by the above criteria had a sufficiently wide age range (37–78 years), so that no other selection criteria were used. All patients underwent surgery in 1988. They were classified by tumour cell type, UICC tumour stage and TNM staging system (T and N) according to published criteria (WHO, 1982; Hermanek and Sobin, 1987; Mountain, 1993).

From each case, a minimum of four blocks were sectioned and reviewed by a pathologist (DMC) in order to confirm the diagnosis and select one block per tumour for the analysis of vascularity. Blocks were selected on the basis of (i) being representative of the tumour, (ii) containing ample tumour areas and (iii) including tumour free margins.

Immunocytochemistry

Lung tumour sections were dewaxed in xylene, rehydrated in ethanol and finally washed in distilled water for 5 min followed by phosphate-buffered saline (PBS) for 10 min. Endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide in PBS for 10 min. Sections were then pretreated with protease XXIV (Sigma) 1 mg ml^{-1} in PBS at 37 °C for 30 min. This was found to be the optimum antigen retrieval method for lung tissue. Goat serum (20%) in PBS was used to block non-specific sites for 20 min, the excess serum removed and the primary antibody applied. Blood vessels were localized by immunostaining 5 mm-thick sections with rabbit anti-human von Willebrand factor (vWF) antibody (Dako. cat. no. A0082) diluted 1:4000 in 20% normal goat serum (NGS). Sections were incubated with the primary antibody at 4°C overnight and then with the secondary antibody (biotinylated polyclonal goat anti-rabbit IgG diluted 1:166 in 20% NGS) for 30 min. The slides were then incubated with the Vectastain ABC-peroxidase reagent from the Elite amplification kit (Vector cat. no. 6100) for 30 min. Next, they were washed in PBS for 10 min and then incubated in a freshly prepared diaminobenzidine substrate (0.08 g in 200 ml PBS/ 200 μl 3% hydrogen peroxide) for 4 min. Sections were rinsed in PBS, counterstained with haematoxylin, dehydrated and mounted. In preliminary experiments, the antibody to CD31 (clone JC/70A from Dako) was compared to the anti-vWF.

Assessment of vascularity

Two non-consecutive sections were immunostained per block. In all cases, both histological features and vascularity were similar in the duplicate sections. Vascularity was assessed by light microscopy using three different methods of quantitation and without prior knowledge of patient clinical parameters. These methods were:

1. Highest microvascular density (h-MVD). Any cell or cell cluster showing antibody staining was considered to be a countable microvessel, irrespective of whether or not a lumen was present. Microvessels in sclerotic or necrotic areas within the tumour or those in adjacent areas of unaffected lung parenchyma were not considered in the vessel count. The area of highest microvascular density was located by scanning the section at 120 × magnification. Microvessels were counted at 200 × magnification using a grid that circumscribed an area of 0.476 mm^2. All vessels contained either completely or partly within the grid were included in the count. Three separate fields were counted and the highest of these three counts was taken as the h-MVD and expressed as the number of vessels per mm^2 (Weidner, 1993).
2. Average microvascular density (a-MVD). Using the same criteria, grid and magnification as above, the number of microvessels were counted in 18 fields selected randomly throughout the section. a-MVD was determined by calculating the mean of the 18 values and expressed as the number of vessels per mm^2 ± standard deviation.
3. Microvascular volume (MVV). The MVV was calculated by the conventional stereological method of point counting using an eyepiece graticule that contained 100 points. Vessels that coincided with the points were counted in 18 fields selected randomly across each section (a total of 1800 points) and the results were expressed as percentage volume, taking the mean ± standard deviation of the 18 values.

All specimens were counted by one observer (LMC) after a period of training and assessment of the reliability of the counting methods. This included counting the same (10–15) specimens on separate occasions (a) in conjunction with two other independent observers, using a two-headed microscope, (b) by the first observer alone and (c) by two observers independently.

Calculation of tumour volume

Measurements of the maximum tumour diameter in three dimensions were available for 51 patients. These three values were multiplied to give the 'actual tumour volume'. As often only one dimension per tumour is available, an 'estimated tumour volume' was calculated by cubing the largest tumour dimension. Both actual and estimated tumour volumes were used in the analyses.

Statistical analyses

As vascularity data were not normally distributed, statistical analyses were performed by non-parametric tests. Kaplan–Meier (product estimates) and log-rank tests were used for individual variables and Cox's proportional hazards model was used for multivariate survival analysis. Details of the various tests used are given under the appropriate experimental sections. All analyses were carried out using SPSS software (version 6.0, SPSS. Chicago, IL, USA).
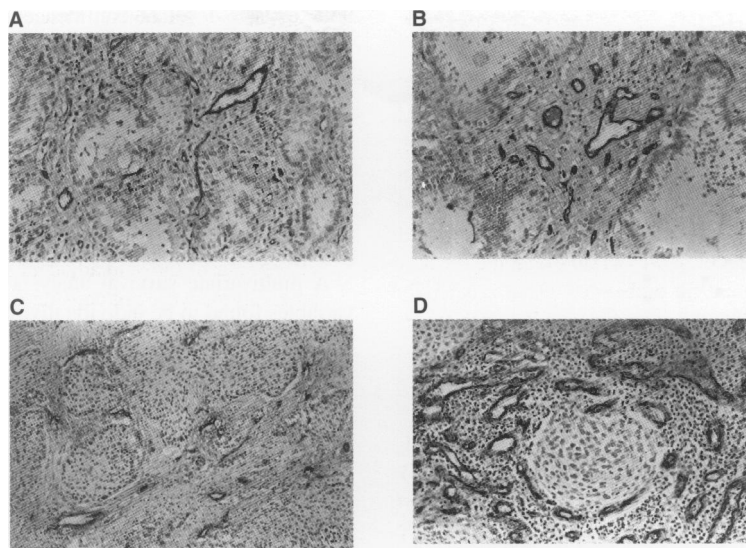
Figure 1 Sections of non-small-cell lung carcinomas stained with vWF antibody. (**A** and **B**) Adenocarcinomas of low and high vascularity respectively. (**C** and **D**) Squamous cell carcinomas of low and high vascularity respectively. Bar = 100 µm for C and 50 µm for A, B and D

RESULTS

Patients

Patient characteristics, shown in Table 1, fell within those of the general population of surgically treated NSCLC (Jefferson et al, 1996). Of the 88 patients, 67% were men, 75% had squamous cell carcinoma, 57% presented with stage I and 63% without bronchial or mediastinal nodal involvement. At the time of the analyses, 53 patients were dead and 35 were alive.

Histological features: microvessel staining and distribution

The protocol adopted to immunostain blood vessels was optimized using nine specimens of NSCLC and two pan-endothelial antibodies: vWF and CD31. Using the optimal antibody concentration and incubation times, we found significant differences in the number of vessels stained depending on the pretreatment of the sections. Under all conditions examined we found vWF preferable to CD31 regarding the intensity of staining and number of vessels detected (results not shown). All subsequent staining was performed with vWF antibody.

Using the optimal protocol (as described in Materials and methods), immunostaining with vWF was satisfactory for all the sections examined and variations in the intensity of the staining were not evident. The distribution and density of the vessels were consistent for the duplicate sections of a given block, but great variation was observed among different tumours. For example, vessels could be either homogeneously or heterogeneously distributed throughout the section, the highest vessel density could be at the periphery of the tumour or towards the centre. These variations were found for both adenocarcinomas and squamous cell carcinomas. Examples of high and low vascularity for both tumour types are shown in Figure 1.

It has been reported previously that the clinical outcome of oral squamous cell carcinoma patients appears to be correlated with different patterns of tumour architecture and blood supply (Lauk et

al, 1989). Therefore, for each tumour we assessed the position of the highest vascularity area (hotspot). In addition, we graded the following features: (i) vascularity throughout the tumour; (ii) vascularity at the edge of the tumour; (iii) lymphocyte infiltration; and (iv) tumour architecture. None of these parameters was related to patient outcome, as determined by the chi-square test (results not shown).

Correlation between three methods of quantitating vascularity

Using the Spearman or Kendall correlations, all three measurements of vascularity were found to be significantly associated ($P<0.0001$) (Figure 2). The highest correlation occurred between the two methods that measure density (h-MVD and a-MVD, $r=0.78$) and the lowest correlation was between the highest density and the volume (h-MVD and MVV, $r = 0.52$).

Relationship between vascularity, age and other clinical parameters

Clinicopathological characteristics of the patients and the vascularity of the tumours are presented in Table 1. Applying the Wilcoxon rank-sum test, tumour vascularity, assessed by any of the three methods used, was not associated with age, sex, tumour type, volume, stage, size (TNM-T), nodal status (TNM-N) or patient outcome. Similarly, age was not associated with any of the parameters examined (Table 1).

Analysis of survival

Assessment of the association between vascularity, age and other clinical parameters with overall survival was investigated by the Kaplan–Meier estimates of survival function, with comparisons made using the log-rank tests (Table 2). The median values were calculated for all three measures of vascularity (4.4 for MVV, 140.8 for h-MVD, 67.2 for a-MVD), as well as for age (65 years),
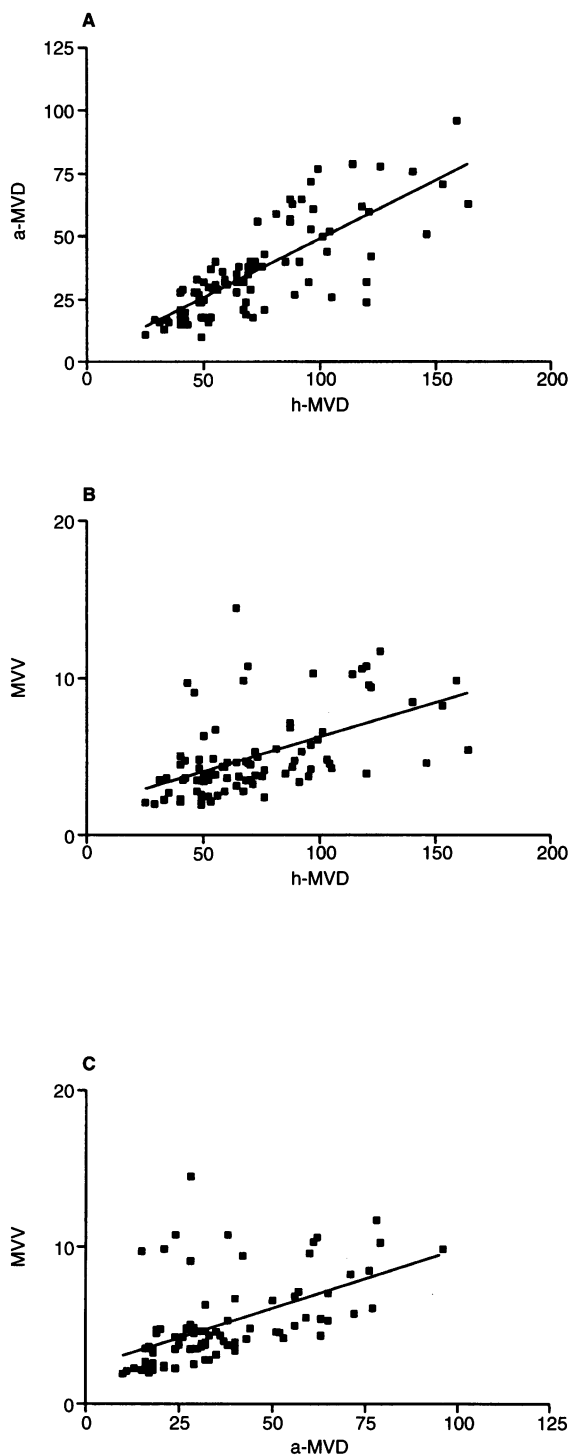
Figure 2 Correlation between different methods of quantitating vascularity. The methods compared were: microvascular volume (MVV), highest microvascular density (h-MVD) and average microvascular density (a-MVD). Correlation coefficients (r) were as follows: (**A**) a-MVD v h-MVD $r = 0.78$; (**B**) MVV v h-MVD $r = 0.52$; (**C**) MVV v a-MVD $r = 0.58$. $P<0.001$ for all groups

'actual' tumour volume (36 cm³) and 'estimated' tumour volume (64 cm³). These values were used as cut-off points for the analyses. Survival time was significantly associated with tumour stage ($P = 0.05$) and tumour volume (both actual, $P = 0.02$ and estimated, $P = 0.003$). Two other parameters were associated with survival at a

93% or 94% level of confidence; these were nodal status ($P = 0.07$) and vascularity measured by the a-MVD method ($P = 0.06$). Survival was not associated with any of the other parameters examined, including age and vascularity measured by either h-MVD or MVV (Table 2). Survival curves according to median values of the three vascularity measurements are shown in Figure 3. It should be noted that the association between a-MVD and survival indicated that high vascularity was associated with good prognosis (Table 2 and Figure 4C).

A multivariate survival analysis was performed with the two variables found to be individually related to survival time (tumour volume and stage). In this model, tumour stage ceased to be predictive of survival; tumour volume was the only measured factor related to survival, with a 58% (95% CI 52–65%) relative increase in hazard for tumours larger than 64 cm³ ($P=0.005$). Applying the Kruskal–Wallis test, higher tumour volumes were significantly associated with greater levels of nodal status (TNM-N) ($P<0.05$) Survival curves according to tumour volume defined by median groupings are shown in Figure 4.

DISCUSSION

This study was designed to determine whether vascularity in resected NSCLC was associated with patient survival or age. Accordingly, the patients were selected from a larger series previously examined (Jefferson et al, 1996) so that their outcome was representative of that of the larger series, a prolonged follow-up period was available and a wide range of ages was included. As with the general population of surgically treated NSCLC, the 88 patients that we examined fell into two distinct groups with either high or low median survival. We found that vascularity was not significantly associated with age, survival or any other clinical parameter, including tumour type, volume, stage, size (TNM-T) or nodal status (TNM-N).

It is interesting that survival time was generally longer for patients with higher vascularity values, but this association only reached borderline significance for a-MVD, one of the three vascularity measurements used (Table 2 and Figure 3). Several studies have reported that high microvascular density is a good prognostic indicator in carcinoma of the cervix (Siracka et al, 1988; Revesz et al, 1989; Kainz et al, 1995), and head and neck tumours (Zatterström et al, 1995). However, the majority of studies have found the opposite, i.e. that high vascularity is an indicator of poor prognosis (see Introduction).

To date, all studies measuring vascularity in NSCLC have counted the vessels in the most vascularized area or 'hotspot' either as density (h-MVD) (Macchiarini et al, 1992; Fontanini et al, 1995; Yuan et al, 1995) or as point counting a total of 75 points (Giatromanolaki et al, 1996). A good correlation has been found between different methods to measure vascularity, including subjective eye appraisal (Giatromanolaki et al, 1996). However, we have previously reported that different results can be obtained when vascularity is estimated by the microvascular volume, rather than density (Pazouki et al, 1997). Therefore, in this study we have assessed vascularity by three different methods: two that measure density, either randomly (a-MVD) or in the 'hotspot' (h-MVD), and one that estimates volume by stereological point counting throughout the section (MVV). Apart from the near-significant association with survival discussed above, the three measurements of vascularity showed the same lack of association with age, survival and clinicopathological parameters (Tables 1 and 2 and Figure 3).

Table 1 Vascularity, age and other patient characteristics

Variable	Number of cases	MVV median (IQR)	h-MVD median (IQR)	a-MVD median (IQR)	Age median (IQR)
All	88	4.4 (3.3–5.5)	140.8 (103–192)	67.2 (47.3–98.7)	65 (61–68)
Age					
<65 years	46	4.1 (3.3–5.8)	143 (103–183)	67.2 (56.7–84)	–
>65 years	42	4.3 (3.5–5.4)	139 (103–212)	65.1 (44.1–105)	–
Sex					
Male	59	4.2 (3.5–5.4)	135 (99–181)	65.1 (44.1–98.8)	66 (61–69)
Female	29	4.3 (3.4–5.8)	145 (116–202)	67.2 (54.6–88.2)	63 (60–68)
Tumour type					
Squamous cell	66	4.3 (3.4–6.3)	144 (103–202)	67.2 (50.4–105)	65 (61–68)
Adenocarcinoma	22	3.7 (2.8–4.7)	125 (105–160)	67.2 (44.1–84)	64 (63–69)
Actual tumour volume					
<36 cm³	27	4.3 (3.7–5.5)	147 (116–190)	75.6 (58.8–96.6)	63 (57–67)
>36 cm³	24	4.6 (3.2–5.9)	149 (88–234)	67.2 (44.1–120.8)	66 (62–70)
Estimated tumour volume					
<64 cm³	30	4.3 (3.8–5.8)	152 (116–200)	77.7 (60.9–111.3)	63 (58–70)
>64 cm³	21	4.6 (2.7–5.5)	135 (86–216)	67.2 (37.8–107.1)	67 (62–69)
Tumour stage (UICC)					
I	50	4.3 (3.2–5.3)	139 (103–185)	67.2 (44.1–90.3)	64 (61–70)
II	14	3.5 (2.4–4.3)	126 (111–183)	60.9 (37.8–111.3)	66 (59–68)
IIIa	24	4.6 (3.6–6.5)	145 (105–206)	66.2 (53.6–98.7)	64 (63–68)
TNM-T					
T1	32	4.2 (2.8–5)	145 (103–160)	68.2 (44.1–100.8)	63 (57–68)
T2	46	4.4 (3.2–5.5)	135 (101–193)	67.2 (50.4–84)	65 (62–68)
T3	10	4.3 (3.7–6.5)	191 (110–215)	67.2 (50.4–107.1)	66 (61–71)
TNM-N					
N0	55	4.3 (3.5–5.3)	143 (103–193)	67.2 (47.3–99.8)	64 (61–71)
N1	17	3.8 (2.5–5.9)	117 (108–181)	63 (37.8–94.5)	66 (59–68)
N2	16	4.6 (3.7–5.5)	147 (105–204)	65.1 (56.7–92.4)	64 (63–67)
Outcome					
Dead	53	3.9 (3.3–5.8)	135 (99–191)	60.9 (44.1–84)	64 (61–69)
Alive	35	4.3 (3.6–5.2)	147 (112–197)	77.7 (55.7–112.4)	65 (60–68)

Vascularity measurements were: microvascular volume (MVV, as %); highest microvascular density (h-MVD, as vessels mm^{-2}); and average microvascular density (a-MVD, as vessels mm^{-2}). Age is given in years. IQR = inter-quartile range.

Our results disagree with other studies reporting that h-MVD in NSCLC was significantly associated with histological type, stage of disease (Yuan et al, 1995), nodal status (Fontanini et al, 1995; Yuan et al, 1995; Giatromanolaki et al, 1996) distant metastasis (Macchiarini et al, 1992; Fontanini et al, 1995; Yuan et al, 1995; Giatromanolaki et al, 1996) relapse free and overall survival (Yamazaki et al, 1994; Fontanini et al, 1995; Giatromanolaki et al, 1996). In contrast, our results agree with those of Mattern et al (1995), who found that h-MVD was not a prognostic indicator for metastasis or survival in NSCLC; similarly, Yamazaki et al (1994) found that vascularity was not associated with lymph node metastasis in adenocarcinomas.

Established prognostic indicators in NSCLC include nodal involvement, disease stage and tumour grade (Shimosato, 1995). Recently, a clinical study reviewing over 600 patients found that tumour volume was associated with survival (Jefferson et al, 1996). In spite of the small number of tumours examined in our study, we found that larger tumour volumes were associated with shorter survival times ($P<0.02$), as well as with nodal status (TNM-N) ($P<0.05$). It is interesting to note that both 'actual' and 'estimated' tumour volumes correlated with survival, allowing the possibility of using estimated tumour volumes in calculations of survival when only the largest tumour dimension is available. Our study also confirms that tumour stage is significantly associated with survival, whereas the association between nodal involvement and survival only reached borderline significance.

In our study, age was not associated with vascularity or with any other of the parameters tested, including survival. In contrast, an association between age and vascularity has been found in NSCLC (Giatromanolaki et al, 1996) and other types of tumours (Davidson et al, 1994; Miliaras et al, 1995; Leon et al, 1996). When reported, tumours from younger patients had higher vascularity values than those from older patients. However, several studies have been unable to confirm the statistical significance of this association (van Hoef et al, 1993; Axelsson et al, 1995; Bossi et al, 1995; Tahan and Stein, 1995). A lack of association between age and other prognostic indicators has previously been reported for NSCLC (Pendleton et al, 1996), oral carcinoma (Williams et al, 1994) and cutaneous melanoma (Busam et al, 1995).

The estimated vascularity in tissue sections can be significantly affected by variations in the methodology, including antibody used and pretreatment of the sections (Busam et al, 1995; our

Table 2 The association between vascularity, age and other clinical parameters with survival

Variable group (number of cases)	Median survival in days (95% CI)	Probability of survival (%) between 2 and 5 years		Significance of relationship to survival time
All patients (88)	994 (142–1845)	48.9	40.9	–
Age				
<65 years (46)	535 (0–1967)	50	40	$P = 0.79$
>65 years (42)	994 (0–2756)	47.6	45.2	
Sex				
Male (59)	751 (252–1250)	50.9	37.3	$P = 0.47$
Female (29)	2145 (597–3692)	58.6	48.3	
Tumour type				
Squamous cell (66)	1005 (0–2334)	50	42.4	$P = 0.88$
Adenocarcinoma (22)	592 (0–1522)	45.5	36.4	
MVV				
<4.4 (46)	810 (0–2344)	52.1	41.3	$P = 0.74$
>4.4 (42)	1224 (274–2173)	52.4	40.5	
h-MVD				
<140.8 (45)	751 (324–1178)	51.1	35.6	$P = 0.35$
>140.8 (43)	1498 (*,*)	58.1	46.5	
a-MVD				
<67.2 (48)	592 (163–1021)	45.8	33.3	$P = 0.06$
>67.2 (40)	2145 (*,*)	65	50	
Actual tumour volume				
<36 cm³ (27)	2613 (*,*)	66.7	55.6	$P = 0.02$
>36 cm³ (24)	355 (104–606)	25	25	
Estimated tumour volume				
<64 cm³ (30)	2651 (*,*)	66.7	56.7	$P = 0.003$
>64 cm³ (21)	348 (298–412)	19.1	19.1	
Tumour stage (UICC)				
I (50)	2230 (*,*)	60	52	$P = 0.05$
II (14)	810 (0–1861)	57.1	35.7	
IIIa (24)	565 (227–904)	33.3	20.8	
TNM-T				
T1 (32)	2230 (*,*)	65.6	53.1	$P = 0.3$
T2 (46)	565 (22–1108)	40	35.6	
T3 (10)	643 (12–1274)	36.4	27.3	
TNM-N				
N0 (55)	2145 (*,*)	58.2	49	$P = 0.07$
N1 (17)	614 (0–1377)	31.3	31.3	
N2 (16)	205 (163–967)	35.3	23.5	

Medians were used as cut-off values for vascularity measurements (MVV, h-MVD, a-MVD), age and tumour volumes. 95% CI represents the 95% confidence intervals; when they are not calculable *,* is entered. The significance of association with survival is from the log-rank test.

unpublished data). Even after careful optimization of the methodology, heterogeneity among blocks taken from the same tumour can significantly affect the microvascular density attributed to a given tumour (van Hoef et al, 1993; De Jong et al, 1995; Schor et al, manuscript in preparation). We suggest, therefore, that variations in methodology and tumour heterogeneity could account for the contradictory reports regarding the value of vascularity as a tumour prognostic indicator. In a large tumour, such as most resected NSCLC, it may be necessary to examine multiple blocks in order to determine the overall vascularity of the tumour.

Vascularity, estimated using pan-endothelial antibodies such as vWF or CD31, is often referred to as angiogenesis; however, these antibodies do not distinguish between newly formed (angiogenic) and pre-existing vessels. Furthermore, as microvascular density in

normal bronchial tissue is similar (h-MVD) or higher (a-MVD) than that of lung tumours (Schor et al, manuscript in preparation), there is no evidence that vascularity measured in these tumours by staining the vessels with a pan-endothelial marker represents angiogenesis. Specific anti-angiogenic vessel antibodies for paraffin-embedded tissues are not yet available.

Taken together, our results indicate that vascularity, measured by either density or volume of vessels stained with pan-endothelial markers, is (a) not a prognostic indicator in NSCLC and (b) not related to the age of the patients. Our results do not exclude the possibility that angiogenesis may be important in lung tumours, but suggest that, if this is the case, vascularity measured by current methods is not an accurate index of angiogenesis in these tumours.
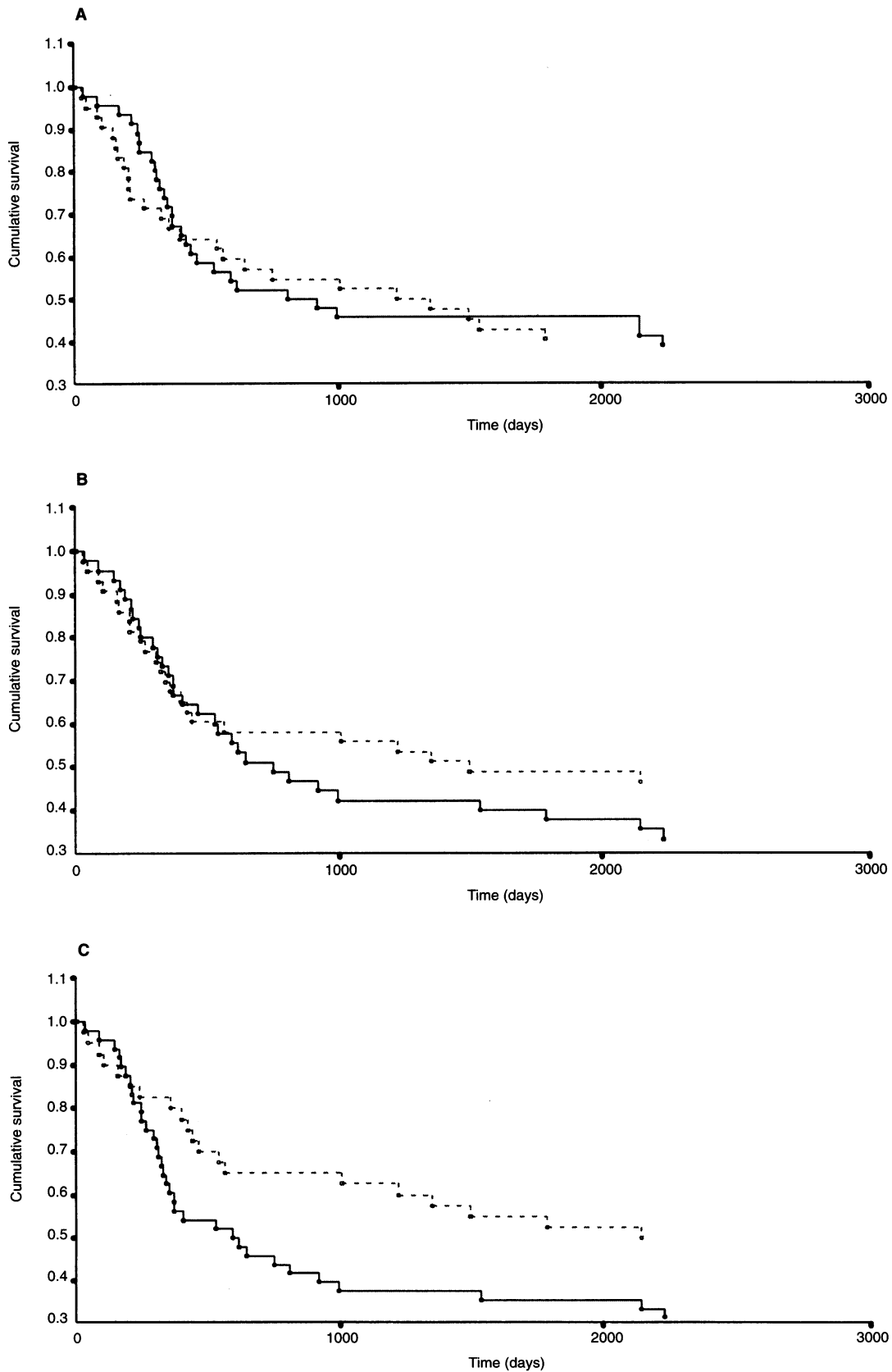
Figure 3 Survival curves according to median vascularity values. Vascularity measurements were: (**A**) microvascular volume (MVV, as %) <4.4, ——; >4.4, ------; (**B**) highest microvascular density (h-MVD, as vessels mm^{-2}) <140.8, ——; >140.8, ------; (**C**) average microvascular density (a-MVD, as vessels mm^{-2}) <67.2, ——; >67.2, ------.
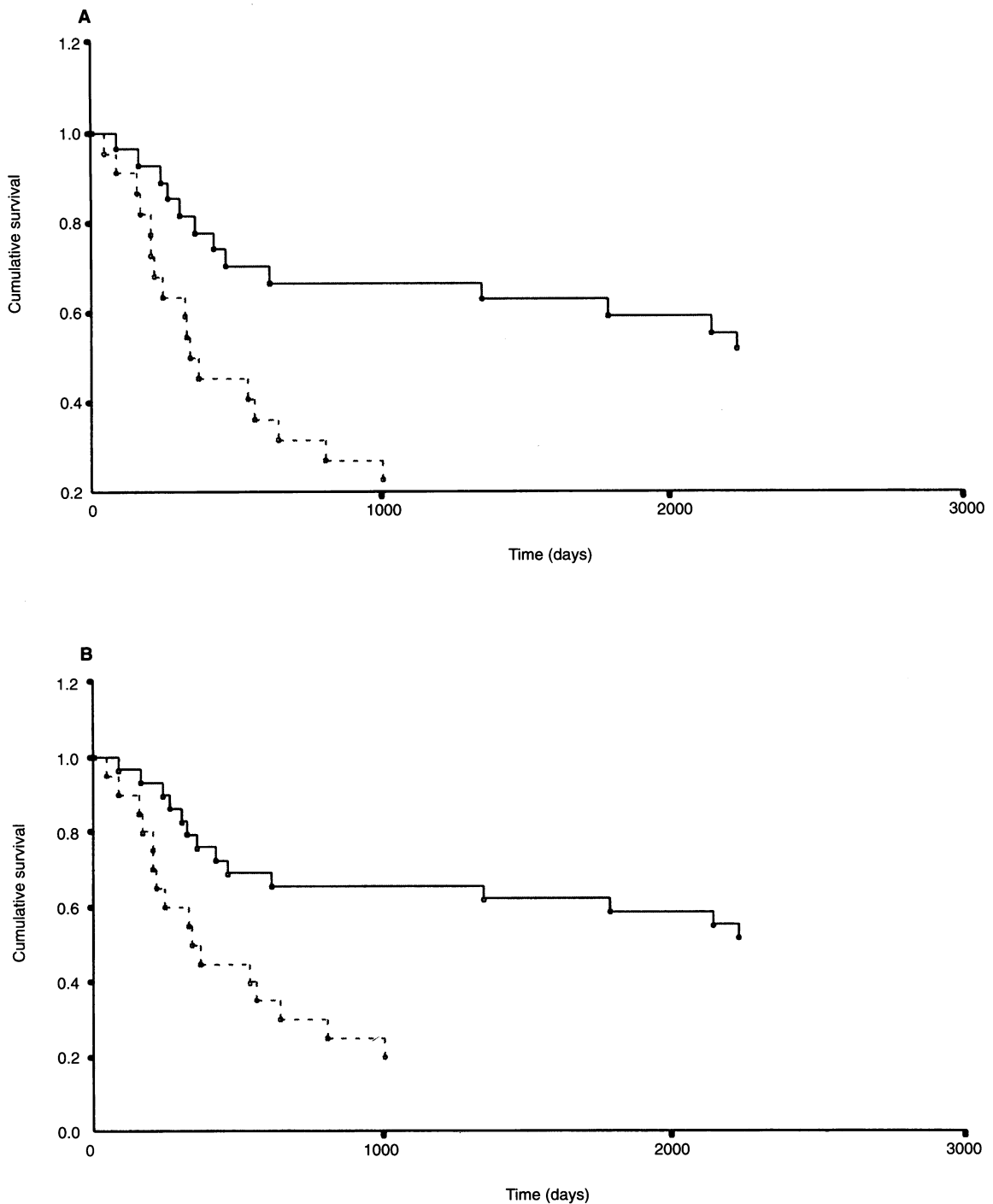
Figure 4 Survival curves according to median tumour volumes. Tumour volume measurements were: **(A)** actual tumour volume divided into those <36 cm³ (———), >36 cm³ (-------); **(B)** estimated tumour volume divided into <64 cm³ (———), >64 cm³ -------

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