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Quantification of angiogenesis as a prognostic marker in human carcinomas: a critical evaluation of histopathological methods for estimation of vascular density

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Abstract

Chalkley counts have been suggested as the primary method for immunohistochemical evaluation of angiogenesis, however, most studies have used microvessel density (MVD). We present paired Chalkley and MVD estimates in carcinomas of the prostate, breast, bladder and lung. The clinical data has previously been reported. In prostate carcinomas, high MVD indicated poor prognosis, whereas high Chalkley counts in breast carcinoma were associated with a poor prognosis. In bladder carcinoma, high estimates using both methods showed good prognosis and were associated with a high degree of inflammation. Neither of the counts revealed prognostic value in lung carcinomas, where the vascular pattern indicated that this cancer was non-angiogenic. We highlight methodological problems with both counting methods. Since angiogenic processes in lung and bladder cancers may be different from those occuring in prostate cancer, we suggest that future analyses also focus on measuring angiogenic factors to obtain more information on the biology of angiogenesis.

Keywords: Angiogenesis; Chalkley; Microvessel density; Breast; Bladder; Prostate; Non-small cell lung carcinoma

1. Introduction

Angiogenesis is the development of new vessels from pre-existing vessels. The prognostic value of estimates of angiogenesis in various types of carcinomas has been investigated since Weidner and colleagues reported that high vascular scores were associated with distant metastases [1]. The principle of the method was based on identifying the most densely vascularised area of the tumour (designated a 'hot spot') under the microscope, and the maximal microvessel density (MVD) in this hot spot was counted and used to characterise the tumour. Slight variants of the method have been presented, but the most frequently used approach has been based on reporting one maximal MVD score for each tumour.

In 1994, Fox and colleagues introduced an alternative principle based on using a Chalkley grid [2,3]. The Chalkley eyepiece graticule is a circle with 25 randomly placed dots, and the circle was projected onto a hot spot and rotated to hit as many vessels as possible in an area of 0.155 mm^2 at $\times 250$ magnification. The average of three Chalkley counts represented the tumour. The Chalkley count has been presented as a surrogate area estimate of the vessels [2]. However, it seems more correct to say that the Chalkley count is related to the number and area of vessels.

In 1996, Vermeulen and colleagues reported a consensus on immunohistochemical evaluation of angiogenesis in human carcinomas, and among other subjects they also reached a ''consensus'' regarding the counting method of choice [4]. In a table on ''proposed standard method'' for assessing angiogenesis, only the Chalkley method was mentioned, and the advantage of the Chalkley method was ''exclusion of the subjective step of identifying individual microvessels in an endothelial cell cluster''. It appears, when looking at the evidence behind this proposed consensus, that data from two papers were used as the basis. The first was by Fox and colleagues, who evaluated 30 breast carcinomas using

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both the Chalkley grid and the previously used MVD method [5]. They reported a Pearson's $r = 0.71$ between counts in the same hot spots using both counting methods on the same occasion, after which only the Chalkley method was used to estimate angiogenesis in a total of 211 breast carcinomas. The second paper was a joint study including 113 breast carcinomas from Italy and 65 breast carcinomas from England [6]. The vascular data in the report consisted of original Chalkley counts in the 65 tumours, whilst MVD counts from the 113 tumours were converted into Chalkley counts using the correlation coefficient = 0.71 from the paper by Fox and colleagues without real counting. To our knowledge, only two other studies have reported vascular counts using both methods; in a paper by Hansen and colleagues, the reproducibility of the two methods was extensively investigated in 40 breast carcinomas but no attempt to correlate the two methods was made [7]. In a correspondence, Fox and colleagues reported Chalkley counts on 169 of the 220 breast tumours Axelsson and colleagues investigated in their report on MVD scores 5 years previously [8,9]. Neither of the counting methods were able to generate scores that could separate the patients into groups of different prognosis. No published studies have reported data directly investigating the prognostic value of estimates of angiogenesis using both the Chalkley grid and the MVD method on the same tumours stained with optimal anti-endothelial antibodies.

The aim of the present analysis was to provide Chalkley counts in prostate and bladder carcinomas, in which MVD has previously been described, and to provide MVD in early breast carcinomas, whose Chalkley counts have previously been reported. Together with MVD and Chalkley data from two additional investigations on prostate and non-small cell lung carcinomas, we present and discuss complete vascular information on MVD and Chalkley counts in five studies including patients diagnosed with carcinoma of the prostate (51 and 221 patients) [10,11] , breast (455 patients) [12], bladder (107 patients) [13], or non-small cell lung carcinoma (NSCLC) (143 patients) [14]. Based on this presentation, we discuss the ability of the MVD and Chalkley methods to generate data for prognostic use. Tumours from these 977 patients have been evaluated in the same laboratory using the same methods for both Chalkley and MVD estimates.

2. Patients and methods

2.1. Immunohistochemical staining

The exact procedures have previously been described in detail for each study [10–14]. Briefly, formalin-fixed and paraffin-embedded tissues were used, and anti-CD34 was the endothelial marker in all but the report on 221 prostate carcinomas, where anti-von Willebrand Factor (vWF) was used [11]. All slides were heated in a microwave oven either in a buffer of 10 mM sodium citrate (pH 6.0) [11,12] or in a buffer of 10 mM Tris and 0.5 M EGTA (Bis-aminoethyl-glycolether-N,N,N′,N′-tetra acid; 3,6 Dioxaoctamethylendirit rilotetra acid) (pH 9.0) [10,13,14].

2.2. Vascular assessments

Two methods were applied: the MVD method and the Chalkley method. Exactly the same MVD method was used in all five studies and represented a slight modification of the original MVD method described by Weidner, since we applied a 10×10 grid on the hot spots and made all vessel counts at $\times 200$ magnification corresponding to an area of 0.25 mm^2. All vessels hitting two of the sides of the grid were included in the count, whereas vessels hitting the other two sides of the grid were excluded from the count, using the principles of Gundersen and colleagues [15]. Counts were typically made in 3-5 hot spots, and the highest MVD was used to characterise the tumour. The Chalkley method used by us was similar to the one originally described by Fox and colleagues [2], except the estimates were done by a single observer, as in the study by Hansen and colleagues [16]. At $\times 200$ magnification under the microscope, corresponding to an area of 0.196 mm^2, the Chalkley grid was projected on a hot spot in the carcinoma and rotated to hit as many vessels as possible. The average of the highest Chalkley count in three separate hot spots was used to characterise the tumour. Originally, Chalkley introduced his point-counting graticule based on projecting the 25 randomly placed dots in the graticule randomly on the test object, which is in contrast to the above mentioned method. However, to avoid confusion with the terminology, we have chosen to designate the estimates of angiogenesis derived using the Chalkley graticule 'Chalkley count', since this has been done in many other similar studies [5,8,16–18]. In the report on 221 prostate carcinomas, the average of two Chalkley counts instead of three was used to characterise each tumour. All stained separate vessels were included in the counts, a lumen was not necessary, although usually present. Counts were always performed in areas of invasive carcinoma.

2.3. Studies

Five studies investigating patients diagnosed with either of four different types of carcinoma were included in the present analysis, and the clinical data on the patients have been described in detail in each report [10–14].

2.3.1. Prostate carcinoma I

One paper reported MVD and Chalkley scores in tumour specimens from 51 consecutive patients suffering from prostatism and diagnosed with invasive prostate

carcinoma [10]. Median follow-up was 29 months, and at analysis 39 patients (76%) had died. Median age was 76 years. Tumours were classified as T1, T2, and T>2 in 3 (6%), 14 (27%) and 34 patients (67%), respectively, and 20 patients (39%) were M0, whereas 18 patients (35%) were M1, and the rest had unknown M-status. Fifteen tumours (29%) were clinically intracapsular. World Health Organization (WHO) grades well, moderate and poor were seen in 5 (10%), 25 (49%) and 21 cases (41%), respectively, and Gleason scores were assigned as 3–6, 7 and 8–10 in 15 (29%), 18 (35%) and 18 tumours (35%), respectively. After transurethral resection of the prostate (TURP), the patients were offered only palliative therapy.

2.3.2. Prostate carcinoma II

Another study reported MVD on 221 consecutive prostate cancer patients followed-up to 15 years [11]. Median follow-up was 3.5 years, and at analysis 98% had died. Median age was 75 years. The tumours were T1, T2, and T>2 in 104 (47%), 27 (12%) and 90 cases (41%), respectively, and 161 patients (73%) were classified as M0. Histopathological grades well, moderate and poor were encountered in 59 (27%), 90 (41%) and 72 cases (33%), respectively. Clinically localised disease was present in 125 patients (57%). The patients were treated with no intent to cure.

2.3.3. Early breast carcinoma

The third investigation included 455 consecutive early breast carcinoma patients (i.e. patients were treated with intent to cure) with a median follow-up of 101 months, and at analysis 168 patients (37%) had died from breast cancer [12]. Median age was 57 years, 321 patients (71%) were postmenopausal, in 246 cases (54%) tumour size was 21–50 mm, whereas in 172 cases (38%) the tumour was smaller, and in 37 cases (8%) the tumour was larger. Lymph node status was N0 and N+ in 217 patients (48%) and 238 patients (52%), respectively. In the 383 ductal carcinomas (84%), the histopathological grades I, II and III were encountered in 88 (19%), 155 (34%) and 140 cases (31%), respectively. One hundred and thirty cases (29%) were oestrogen receptor-negative.

2.3.4. Bladder carcinoma

The fourth analysis provided MVD data on 107 consecutive patients diagnosed with invasive transitional cell carcinoma of the bladder [13]. Median follow-up was 33 months, and at analysis 60 patients (56%) had died from bladder carcinoma. Median age was 64 years, and 91 patients (85%) were male. Tumours were classified as T1, T2 and T>2 in 16 (15%), 39 (36%), and 52 cases (49%), and 61 patients (57%) were N0. Malignancy grade II, III and IV a.m. Bergkvist was seen in one tumour (1%), 89 tumours (83%) and 17 tumours (16%), respectively. Forty patients (37%) received radiotherapy (60 Gy) and/or chemotherapy, and 67 patients (63%) underwent radical cystectomy.

2.3.5. Non-small cell lung carcinoma

The fifth paper reported both MVD and Chalkley data on 143 consecutive patients diagnosed with NSCLC [14]. At analysis, 109 patients (76%) had died, and the median follow-up of patients who were alive was 110 months. Median age was 62 years, and 104 patients (73%) were male. Tumours were classified as squamous cell, adeno- and large cell carcinomas in 78 (55%), 46 (32%) and 19 cases (13%), respectively, and the majority were T2 (73%). Eighty two patients (57%) had no positive lymph nodes, and 80 (56%), 39 (27%) and 24 patients (17%) had disease stage I, II and III, respectively. No post-operative cytotoxic or radiation therapy was given.

2.4. Statistics

The correlations between the two counting methods were graphically evaluated in scatter plots, and for comparison with other studies we reported Kendall's τ on data stratified by the median or in tertiles depending on the number of patients, since Kendall's τ is especially good for comparison of small sample sizes. Pearson's r on non-ranked data was also listed. Survival plots were drawn using the Kaplan–Meier method, the difference between the curves was evaluated with the Log-Rank test using the test for trend when more than two groups were compared. Cox multivariate analysis was used to evaluate the independent prognostic value of the vascular scores in models including other prognostic parameters depending on the carcinoma type. For a more detailed description of the multivariate analyses, the reader is referred to the original studies.

Table 1
The number of patients (N), anti-endothelial antibody (Ab) and median values stratified by counting method in each study

Study characteristics				Results	
Study [Ref.]	n	Ab	Counting method	Median	(Range)
Prostate I [10]	51	CD34	MVD	67	(32–202)
			Chalkley	5.3	(3.7–8.3)
Prostate II [11]	221	vWF	MVD	43	(16–151)
			Chalkley	5.0	(3.0–10.0)
Breast [12]	455	CD34	MVD	38	(9–181)
			Chalkley	5.0	(2.7–12.0)
Bladder [13]	107	CD34	MVD	71	(21–249)
			Chalkley	6.00	(3.0–13.0)
NSCLC [14]	143	CD34	MVD	63	(27–278)
			Chalkley	7.0	(3.0–15.0)

vWF, von Willebrand Factors; MVD, microvessel density.

3. Results

3.1. Comparison of the counting methods

Table 1 shows the median values of MVD and Chalkley scores from each study. The median MVD scores in the anti-CD34 stained sections were almost alike (63–71), except in early breast cancer, where median MVD was 38, whilst the median MVD in anti-vWF stained sections in the 221 prostate carcinomas was 43. This was comparable with the findings in the report on 51 prostate carcinomas [10], where the median MVD in anti-vWF stained sections was 44. Median Chalkley score in anti-vWF stained sections in both prostate carcinoma studies was 5.0 (range, 3.0–10.0), whereas the median Chalkley scores in the anti-CD34 stained carcinomas varied from 5.0 to 7.0.

Fig. 1 illustrates scatter plots for each of the 5 studies, and also a plot of all counts pooled in one plot. In the study on NSCLC and 221 prostate carcinomas, the correlation was relatively poor, whereas it was moderate in the rest of the studies as judged by the eye and the

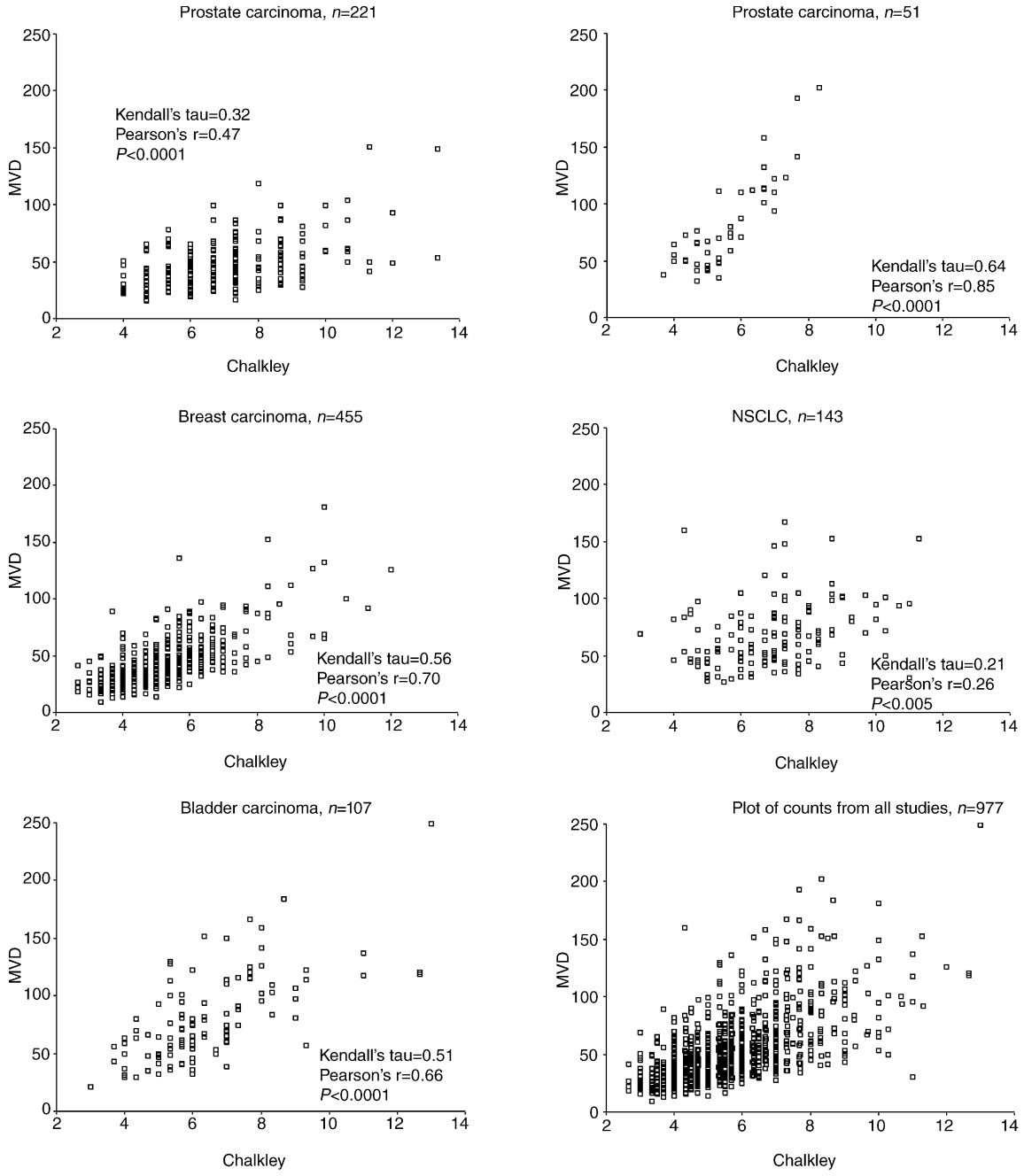

Fig. 1. Scatter plots of paired Chalkley and MVD counts from each study. NSCLC, non-small cell lung carcinoma.

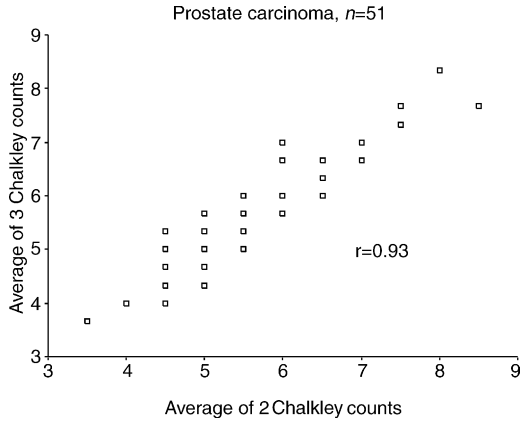

Fig. 2. Plot showing the correlation between Chalkley counts based on two and three separate counts, respectively. In some of the 51 cases, two tumours are represented by the same dot.

association scores. The Chalkley counts made in the study on 221 prostate carcinomas were based on the average of two instead of three separate counts. Fig. 2 is a plot illustrating the association between Chalkley counts based on two and three counts, respectively, from the report on 51 prostate carcinomas, where three Chalkley counts were made. The plot shows a high degree of linearity between the counts, but the average Chalkley based on two counts is higher than the one based on three counts due to less 'dilution' of the score by lower hot spots (data not shown). The almost linear relationship justified the use of Chalkley scores based on the average of two separate counts in the project on 221 prostate carcinomas.

3.2. Angiogenesis estimates and prognostic outcome

Fig. 3 illustrates survival plots of patients whose prostate carcinomas were investigated with both counting methods applied on anti-CD34 and anti-vWF stained tumour sections. It is seen that high vascular scores are associated with poor prognosis. These plots essentially confirm the findings in Fig. 4, where 221 anti-vWF stained tumours were counted and stratified both by the median vascular value and in tertiles. These two figures show that the MVD method is a robust method for evaluating estimates of angiogenesis in prostate carcinoma. Fig. 5 on 455 early breast carcinomas showed that MVD scores were unable to separate the patients, whilst the Chalkley scores revealed a prognostic impact. Both counting methods showed prognostic influence in bladder carcinoma, (Fig. 6), whilst neither of the

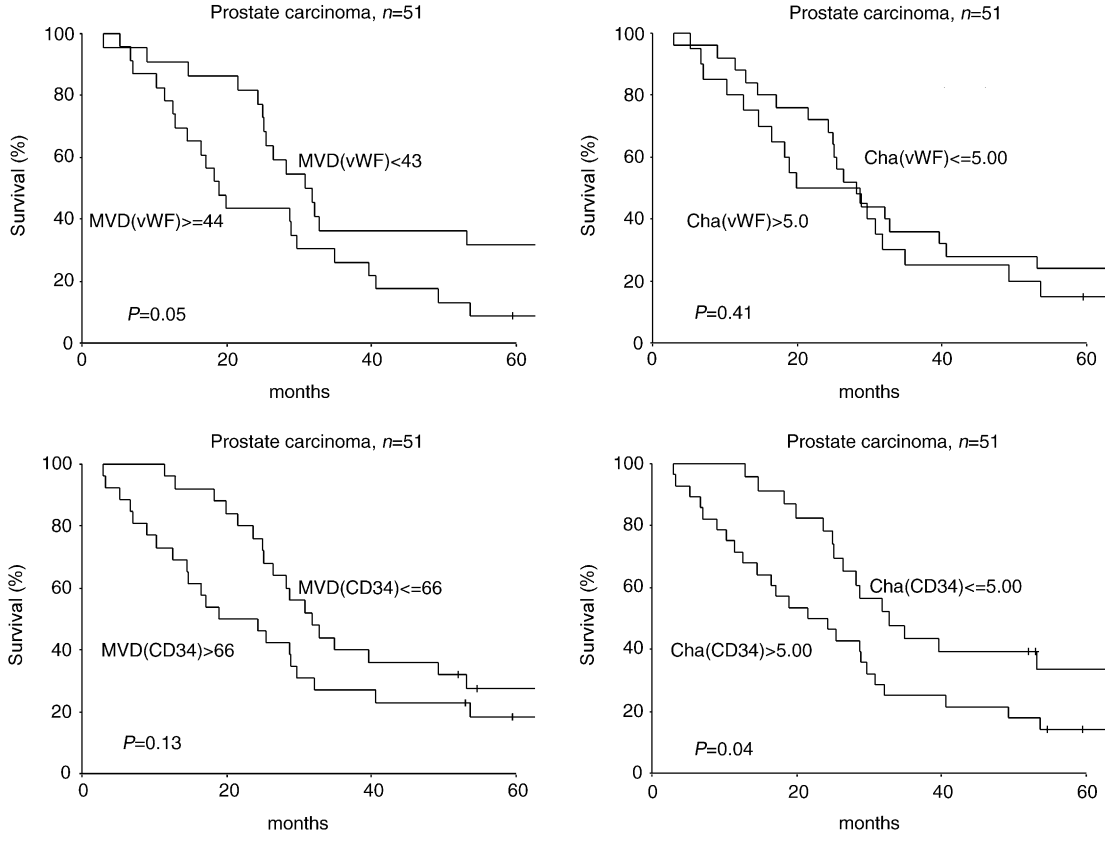

Fig. 3. Survival stratified by median microvessel density (MVD) or Chalkley counts made in either anti-von Willebrand Factor (VWF) or anti-CD34 stained tumours from 51 patients diagnosed with prostate carcinoma [10] (with permission from *APMIS*).

counting methods had a prognostic impact in NSCLC (Fig. 7).

Table 2 highlights the findings in multivariate analyses from each study. In prostate carcinoma, there was a powerful prognostic value of the MVD scores compared with the Chalkley estimates, the relative risk indicating an increased risk of 1% of dying from prostate cancer for each increase in MVD. Using overall or disease-specific survival as the endpoint, the multivariate analyses showed no independent prognostic impact of the vascular scores in the investigated breast, bladder, and NSCLC.

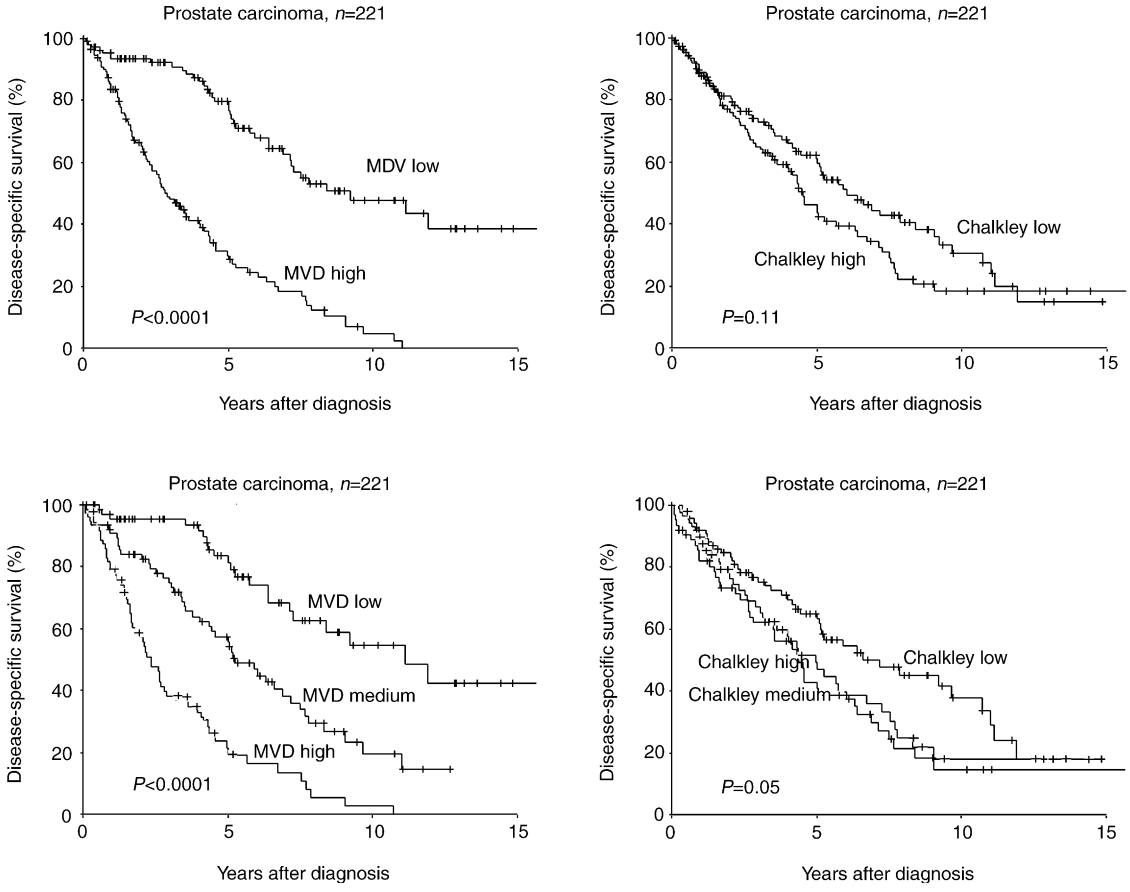

Fig. 4. Disease-specific survival stratified in two or three groups according to microvessel density (MVD) or Chalkley counts made in anti-von Willebrand Factor (VWF)-stained tumours from 221 patients diagnosed with prostate carcinoma [11] (with permission from the *British Journal of Cancer*).

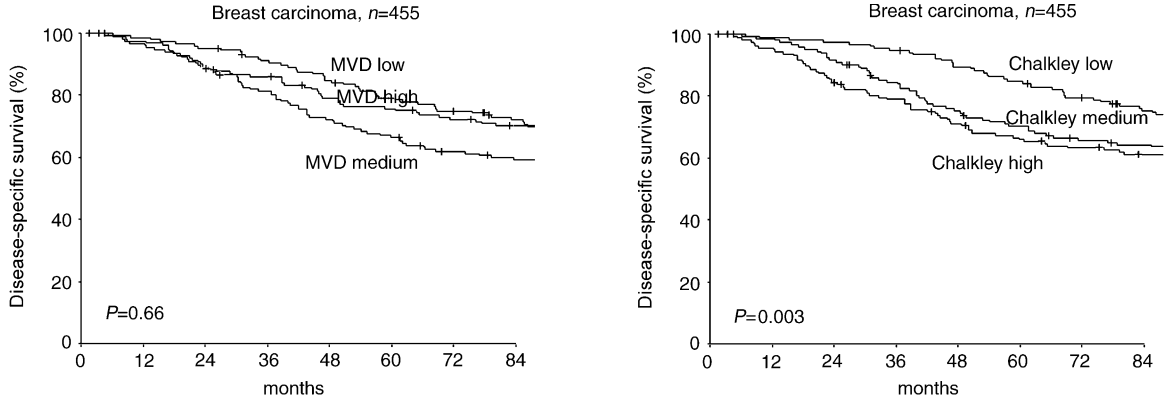

Fig. 5. Disease-specific survival stratified in tertiles according to microvessel density (MVD) or Chalkley counts made in anti-CD34 stained tumours from 455 patients diagnosed with early breast carcinoma [12] (with permission from *Acta Oncologica*).

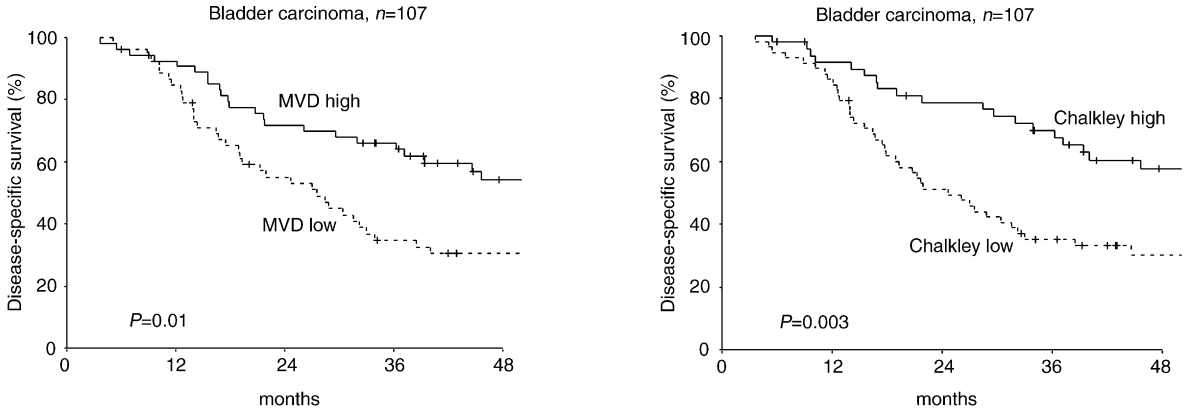

Fig. 6. Disease-specific survival stratified by median MVD or Chalkley counts made in anti-CD34 stained tumours from 107 patients diagnosed with bladder carcinoma [13] (with permission from the *British Journal of Cancer*).

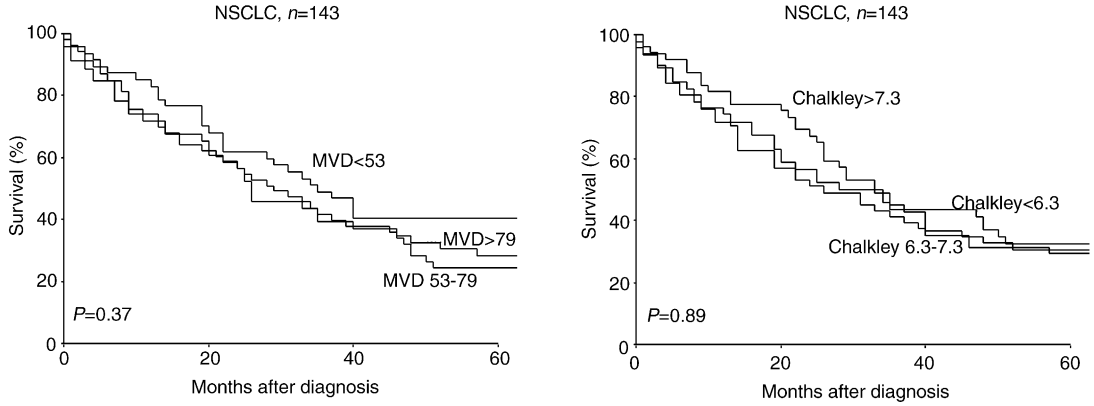

Fig. 7. Survival stratified in tertiles according to microvessel density (MVD) or Chalkley counts made in anti-CD34 stained tumours from 143 patients diagnosed with non-small cell lung carcinoma (NSCLC) [13] (with permission from *Cancer*).

Table 2
Results from univariate and multivariate analyses with relative risks (RR) and 95% confidence intervals (CI) using overall or disease-specific survival as the endpoint

Study		Univariate		Multivariate		
		Dichotomised	Continuous	*P* value	RR	95% CI
Prostate, $n=51$, anti-CD34	MVD	0.1[b]	0.04		ND	
	Chalkley	0.04	0.03		ND	
Prostate, $n=221$	MVD	<0.0001[c]	<0.0001	0.0004 (cont)	1.01	1.01–1.02
	Chalkley	0.11	0.32	NS	–	–
Breast, $n=455$	MVD	0.45[c]	0.81	NS	–	–
	Chalkley	0.02	0.02	NS	–	–
Bladder,[a] $n=107$	MVD	0.01[b]	<0.0001	NS	–	–
	Chalkley	0.003	0.02	NS	–	–
NSCLC, $n=143$	MVD	NS[b]	NS	NS	–	–
	Chalkley	NS	NS	NS	–	–

NS, non significant. ND, not determined due to few events; 95% CI, 95% Confidence Interval.

[a] In bladder carcinoma, the *P* values indicate worse prognostic outcome of a low vascular score, whereas high vascular scores in the other cancer types were associated with worse prognosis.

[b] Overall survival.

[c] Disease-specific survival.

4. Discussion

We present paired MVD and Chalkley estimates of angiogenesis in 977 carcinomas with the aim to discuss the ability of the methods to generate prognostic information. Correlation plots of the data, in general, revealed a moderate correlation between the two methods, however, the prognostic impact of the methods varied with the type of carcinoma. Only in prostate carcinoma was the MVD method superior to the Chalkley method in separating the patients. Importantly, the MVD scores were also strong independent prognostic markers in the multivariate analysis, indicating that the vascular scores in prostate cancer contributed additional knowledge at diagnosis.

In the 455 breast carcinomas, the Chalkley method was superior to the MVD method in univariate analysis; however, in multivariate analysis the Chalkley estimates were only independent markers in node-positive patients. In tumours from node-negative patients, the Chalkley scores did not provide data which could classify the patients into high- and low-risk groups.

In bladder carcinoma, the Chalkley scores showed good agreement with the already reported MVD scores, and in univariate analysis, high Chalkley scores indicated a good disease-specific survival in a similar manner to the MVD scores. In these bladder carcinomas, we have previously identified a close association between inflammation and angiogenesis, and the same association was seen between inflammation and the Chalkley scores [13]. In the previous paper on bladder carcinoma, we decided to report only the MVD scores, since the Chalkley scores, in theory, could be influenced by vasodilatation caused by the inflammatory cells. However, by comparison of the paired MVD and Chalkley counts in the bladder carcinomas to the other carcinoma types in Fig. 1, it appears that the vascular scores in bladder carcinomas have a fairly high agreement between the scores, especially the range of the Chalkley scores is similar to the range in the other carcinoma types. Thus, the impression during the Chalkley measurements that the vessels in areas of inflammation were not dilated, was indirectly confirmed in this way.

Fig. 1 shows that NSCLC has the poorest association between the two counts of the cancers studied. Pearson $r^2 = 0.07$, thus only 7% of the variation of the Chalkley counts can be explained by the variation of the MVD counts, the remaining 93% of the variation is biological and methodological variation. In addition, Fig. 7 highlights that the MVD and the Chalkley scores had no prognostic impact at all in the NSCLC group. While measuring the vascular scores in NSCLC, we identified an alveolar vascular pattern entirely or partly in the carcinomas in 24% of the 143 tumours, and based on weak vascular endothelial growth factor (VEGF) staining and intensity evaluation in parallel sections, this alveolar pattern was characterised as non-angiogenic [14]. Thus, based on the previous study and by comparison to the other carcinoma types in the present report, we show that estimates of angiogenesis are irrelevant in NSCLC since angiogenic processes are apparently not fundamental to tumour growth in this cancer type.

In 1996, a consensus by Vermeulen and colleagues recommending the Chalkley method was published, however, only relatively few studies have used the method [4]. When including studies on carcinomas evaluated with the Chalkley grid and investigating the prognostic influence of Chalkley counts, it appears that five studies have reported on breast carcinomas, four studies have reported on NSCLC, and two studies have reported on bladder carcinomas. A total of 1443 breast cancer patients have been investigated, and four of the studies have been performed by the same group [5,8,17,18] and the last from another group [16]. Only two of the studies identified high Chalkley scores as independent markers of poor prognosis in multivariate analyses [5,16], whereas the other three were non-significant [8,17,18]. The studies on NSCLC included 500 patients in all, where two of the studies identified high Chalkley scores associated with a poor prognosis [19,20], whilst the other two found no association between vascular scores and prognosis [14,21]. Both studies on a total of 133 bladder carcinomas identified high Chalkley scores as an indicator of poor prognosis [22,23]. Thus, the general picture of the prognostic impact of estimates of angiogenesis using the Chalkley method is not clear based on the literature. Although only a few additional studies using the Chalkley method have been published since the first consensus, a ''Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours'' has recently been published by Vermeulen and colleagues [24]. Despite that the additional studies since the first consensus have been conflicting, the second consensus is based on two studies [5,16] and recommends that the Chalkley method should be standard for estimating tumour angiogenesis. The new consensus states that the Chalkley method can be used to predict overall and disease-free survival in node-negative/node-positive breast cancer since ''convincing data suggest that the parameter and method might be implemented in the near future—confirmatory (multicentre) study is needed''. We believe the justification for this new consensus is dubious.

The investigations on 455 early breast carcinomas, reported from our group, also illustrated problems estimating tumour angiogenesis, since the Chalkley and MVD counts showed good agreement, but only the Chalkley scores were able to separate the patients into relevant prognostic groups (Figs. 1 and 5). A similar finding has been described in 330 breast carcinomas in the PhD thesis by Hansen, who found high Chalkley

counts significantly associated with poor prognosis, whilst the MVD scores showed no prognostic impact [25]. In the prostate project on 221 patients, the MVD method was superior to the Chalkley method. The reason why only one of the methods held prognostic information irrespective of a fair correlation between the MVD and Chalkley counts is obscure, especially it is hard to explain why only Chalkley counts, a surrogate estimate of vessel area, are prognostic in breast carcinoma, while only MVD, a measure of vascular density, is prognostic in prostate carcinoma. In our opinion, methodological problems must be part of the explanation, and this may indicate that estimating angiogenesis, using these methods, is not reliable enough for prognostic purposes. If indeed estimating angiogenesis was a powerful method, both counting methods should show poor prognosis associated with high vascular scores, since the scores were associated with each other.

In the study by Fox and colleagues, 30 breast carcinomas were counted using both the Chalkley and the MVD method, and they found a Pearson correlation coefficient of 0.71 when counting in the same hot spot on the same occasion [5]. In the present report in the breast carcinomas, we counted the sections on two separate occasions and still found an almost identical coefficient of 0.70. However, a MVD score of 50 corresponded to a wide range of Chalkley scores (3–10 in the plot of pooled data in Fig. 1), and emphasises that the two counts characterise two different modalities: a density and an area estimate.

Using the vessel density as an estimate of angiogenesis is an indirect measure of the net result of all the angiogenic factors that have been in play. In our analysis of angiogenesis in NSCLC, the vascular pattern, as it appeared under the microscope, together with weak VEGF intensity staining in parallel sections, indicated that invasive tumour growth took place without inducing new vessel formation [14]. The presence of the alveolar vascular pattern was significantly associated with a favourable prognosis. In our investigations on bladder carcinoma, inflammatory cells had a major impact on angiogenesis [13]. Estimates of angiogenesis increased significantly with increasing degree of inflammation, and intense VEGF staining of both inflammatory and carcinoma cells was identified in parallel sections. High estimates of angiogenesis indicated a good prognosis. Importantly, these two reports illustrated that the appearance of the vascular bed in the tumours, as seen under the microscope, is caused by processes of which we know only very little. In this context, part of the explanation as to why high estimates of tumour angiogenesis do not consistently indicate poor prognosis in numerous studies could be that the biology of angiogenesis is different in different tissues. Therefore, investigations on VEGF [26], Hypoxia Inducible Factor 1α [27] and other angiogenic factors (for example) are warranted to provide a more direct picture of the biology of angiogenesis.

This analysis presents paired MVD and Chalkley data in 977 tumours. All vascular scores in four of the studies were made by the same investigator [10,12–14], and another investigator performed both counts in the last study [11]. The conclusion, drawn from these studies, is that the two methods yield counts that are correlated, but the prognostic information from the counts is not consistently associated with one of the methods. We propose that this to some extent reflects methodologically problems that must be addressed in the future, before considering implementation of the methods in the daily pathological setting. More studies using both counting methods are needed to test the proposed cut-off points from already published studies, and inter-observer variation should also be investigated. However, since the biology of tumour angiogenesis is complex and may vary among different tissue types, investigations on different angiogenic factors should also be made in addition to vascular counts. Prospective studies, using well-defined cut-off points, is the ultimate test of the strength of estimates of angiogenesis, but these should await a higher degree of consensus between published retrospective studies.

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