Correlation of Pretreatment Polarographically Measured Oxygen Pressures with Quantified Contrast-Enhanced Power Doppler Ultrasonography in Spontaneous Canine Tumors and their Impact on Outcome After Radiation Therapy

Carla Rohrer Bley[1]*, Dagmar Laluhova[1], Malgorzata Roos[2], Barbara Kaser-Hotz[1,3], Stefanie Ohlerth[3]*

Purpose: To evaluate the use of noninvasive quantified contrast-enhanced power Doppler ultrasonography as a surrogate in the estimation of tumor hypoxia measured by invasive pO_2 histography in canine tumors.
Material and Methods: Data of pretreatment tumor oxygenation status, tumor vascularity and blood volume, and tumor response after radiation therapy was collected in 48 spontaneous malignant oral tumors (Table 1). Tumor oxygenation status was correlated to vascularity and blood volume, and influences on outcome after treatment were analyzed.
Results: Although vascularity and blood volume correlated moderately with median pO_2 ($r = 0.51$ and 0.61; $p = 0.001$ and < 0.0001) and percentage of pO_2 readings ≤ 2.5, 5, and 10 mmHg ($r = -0.37$ to -0.42; $p < 0.01$–0.03) for all tumors, they did not correlate within the different histology groups ($p = 0.06$–0.9). For all tumors, pretreatment oxygenation status, vascularity and blood volume were not found to be of prognostic value (Tables 2 and 3).
Conclusion: These analyses show that quantified contrast-enhanced power Doppler ultrasonography does not represent a noninvasive indirect method to assess tumor hypoxia measured by invasive pO_2 histography. Both technologies were nonprognostic indicators in spontaneous malignant canine oral tumors.

Key Words: Hypoxia · Polarographic needle electrode · Power Doppler · Microbubble contrast agent · Dog · Tumor

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Korrelation des polarographisch gemessenen Sauerstoffdrucks mit quantifiziertem, kontrastverstärktem Power-Doppler-Ultraschall bei spontanen kaninen Tumoren und deren Einfluss auf das Tumorverhalten nach Strahlentherapie

Ziel: Untersuchung des nichtinvasiven quantifizierten, kontrastverstärkten Power-Doppler-Ultraschalls als alternative Methode bei der Einschätzung der Tumorhypoxie, die in spontanen Tumoren beim Hund mittels invasiver pO_2-Histographie gemessen wurde.
Material und Methodik: Oxygenierungsstatus, Gefäßdichte und Blutvolumen vor der Behandlung sowie das Tumorverhalten nach Strahlentherapie wurden bei 48 Hunden mit spontanen malignen Tumoren der Maulhöhle untersucht (Tabelle 1). Der Oxygenierungsstatus wurde zur Gefäßdichte und zum Blutvolumen korreliert, und Faktoren, die die Tumorantwort nach Strahlentherapie beeinflussen können, wurden analysiert.
Ergebnisse: Obwohl die Gefäßdichte sowie das Blutvolumen moderat mit dem Zentralwert (medianer pO_2 [$r = 0{,}51$ und $0{,}61$; $p = 0{,}001$ und $< 0{,}0001$]) und der prozentualen Verteilung der pO_2-Messwerte $\leq 2{,}5$, 5 und 10 mmHg ($r = -0{,}37$ bis $-0{,}42$; $p < 0{,}01$–$0{,}03$) für alle Tumoren korrelierte, verlor sich diese Korrelation innerhalb der einzelnen histologischen Gruppen ($p = 0{,}06$–$0{,}9$). Über alle Tumortypen gesehen, waren Oxygenierungsstatus, Gefäßdichte und Blutvolumen, gemessen vor der Strahlentherapie, nicht von prognostischem Wert (Tabellen 2 und 3).

*Both authors contributed equally to the study.
[1]Section of Radiooncology, Vetsuisse Faculty, University of Zurich, Switzerland,
[2]Institute for Social and Preventive Medicine, Medical Faculty, University of Zurich, Switzerland,
[3]Section Imaging Diagnostics, Vetsuisse Faculty, University of Zurich, Switzerland.

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Schlussfolgerung: Diese Untersuchung zeigt, dass der kontrastverstärkte Power-Doppler-Ultraschall die invasive pO_2-Histographie nicht ersetzt und beide Methoden keine prognostischen Indikatoren bei spontanen Tumoren der Maulhöhle des Hundes darstellen.

Schlüsselwörter: Hypoxie · Polarographische Sauerstoffmessung · Power-Doppler · Ultraschallkontrastmittel · Tumor · Hund

Introduction

Tumor hypoxia, measured by invasive oxygen electrodes, has been found prognostic for tumor control and treatment outcome in a variety of human tumors [43]. Similarly, color and power Doppler studies have been performed in human tumors to determine the prognostic value of vascularity and perfusion. Significant associations between high vascularity and tumor aggressiveness, metastatic rate and decreased patient survival have been shown for a variety of tumors [24, 25, 32]. Vascularization and tissue hypoxia are known to be closely related and different noninvasive imaging methods have been evaluated to estimate tumor hypoxia relative to the invasive gold standard method of oxygen pressures measured polarographically. As a result, an estimation of average tissue oxygenation was described to be possible in spite of individual methodological limitations [13, 16, 39, 44].

For this study, the dog was chosen as a large animal tumor model in order to measure oxygen pressures polarographically and perform contrast-enhanced power Doppler ultrasonography prior to radiation therapy (RT).

While often providing partially overlapping data, complementary techniques can be used to validate each other [28]. Therefore, the purpose of the present study was to investigate if pretreatment oxygenation levels measured via invasive polarographic measurements, and vascularity and blood volume as assessed by noninvasive quantified contrast-enhanced power Doppler ultrasonography correlated in different spontaneous canine oral tumors undergoing ionizing radiation. Furthermore, the influence of pretreatment oxygenation levels, vascularity and perfusion on treatment outcome was evaluated in these patients.

Material and Methods

Animals

Dogs with spontaneously originating tumors of the oral cavity scheduled for treatment with RT at the Section of Radiation Oncology of the Vetsuisse Faculty, University of Zurich, Switzerland, were included in the study. All dogs underwent diagnostic work-up as indicated for staging of the disease. Owner consent was obtained for the study which was approved by the Animal Ethics Council of the Canton of Zurich.

Anesthesia

All measurements and sampling procedures as well as subsequent RT were performed under general monitored anesthesia as described [2]. For the study, contrast-enhanced power Doppler ultrasound was performed first, followed by the pO_2 measurements. Finally, dogs underwent RT.

Contrast-Enhanced Power Doppler Ultrasound of Tumors

Imaging was performed by use of a 5- to 12-MHz linear transducer (ATL 5000, Philips AG, Zurich, Switzerland). For contrast-enhanced power Doppler ultrasonography, settings as described in Ohlerth et al. [33] were used. A region of interest (ROI) was drawn around the tumor boundaries and two measurements were computed for each ROI. Fractional area (FA) is calculated as the number of colored pixels in the ROI divided by the total number of pixels in the ROI multiplied by 100; it represents a vascularity index and calculates the percentage area of the tumor occupied by blood vessels. Color-weighted fractional area (CWFA) is used to assess perfusion and determines the mean blood volume within the tissue. FA and CWFA were determined by calculating the median for five images.

pO_2 Measurements and Tumor Volumes

Polarographic tumor oxygen partial pressure measurements were performed as previously described with an Eppendorf or Phönix pO_2 Histograph (Helzel Medical System, Kaltenkirchen, Germany) [1, 2, 14]. The needle was introduced into the tumor under ultrasound guidance in the same image plane in which the ultrasonographic examination was performed [1, 3]. Three different electrode tracks and a minimum of 50 recorded values were acquired for reliable statistical analysis [1, 3, 20]. To assess the biological relevance of tissue oxygenation status, the relative frequency of pO_2 values $\leq$ 2.5, 5, and 10 mmHg, as well as the median pO_2 were calculated. Tumor volume was calculated based on a combination of caliper and ultrasonographic measurements using the rotation ellipsoid formula ($\pi/6 \times$ height $\times$ width $\times$ depth) [40].

Radiation Therapy

RT was delivered with a 6-MV linear accelerator (Dynaray LA20, ABB/VARIAN, Zug, Switzerland) using 6-MV photons or 9- to 16-MeV electrons, as appropriate. Individualized treatment plans were generated using a three-dimensional treatment-planning system (Varian CadPlan® 6.0.8, Varian Medical Systems, Inc., Palo Alto, CA, USA) for photon plans, electron fields were calculated manually. Treatment plans were individually optimized to obtain optimal tumor coverage (100% ± 7.5%). Treatment protocols were assigned either with curative intent (15 × 3.5 Gy [for sarcomas], 17 × 3 Gy [for

squamous cell carcinomas], over 3.5 weeks) or in a coarse fractionated scheme (5 × 6 Gy [sarcomas and malignant melanomas], over 10–14 days). Response was defined as described earlier [36].

Statistical Analysis

Distribution of data (continuous variables) was analyzed with boxplots, scattergrams and histograms. Although data was normally distributed, nonparametric statistical analyses were performed due to small group sizes. Association between continuous variables (age, body weight, hematocrit, hemoglobin concentration, tumor volume, and Doppler and oxygen measurements) was assessed by the Spearman rank correlation analysis. The relationship between histological classification of the tumors and the continuous variables was analyzed by use of the Kruskal-Wallis test.

Endpoints after RT were recorded as in Rohrer Bley et al. [36]. Progression-free interval (PFI) and overall survival were calculated with respect to tumor histology and patient characteristics by Kaplan-Meier method, log-rank and Breslow-Gehan-Wilcoxon tests, univariate proportional hazards and multiple Cox regression analysis (forward/backward stepwise model). For statistical analysis, StatView® 5.0.1 (SAS Institute Inc., Cary, NC, USA) and SPSS® 10.0 (SPSS Schweiz AG, Zurich, Switzerland) were used. Significance was set at a p-value of < 0.05.

Results

Animals and Tumors

48 dogs with no life-compromising disease other than the known cancer were included. Hematologic parameters were in the reference range for all but two dogs, respectively (data not shown). Distribution of histology was as follows: sarcomas (n = 18), squamous cell carcinomas (n = 17), and malignant melanomas (n = 13). Although sarcomas had higher tumor volumes (median, 21.2 cm^3; range, 2.7–65.4 cm^3) than squamous cell carcinomas (median, 8.2 cm^3; range, 0.3–57.6 cm^3) and melanomas (median, 15.7 cm^3; range, 0.2–29.9 cm^3), the median difference was not significant (p = 0.09). Similarly, dogs with malignant melanoma tended to be older (median, 12 years; range, 6–15 years) than animals with sarcoma or squamous cell carcinoma (median, 8.5 years; range, 4–13 years; and median, 8 years; range, 1–13 years; p = 0.08). Patient characteristics such as age, body weight, hematocrit, hemoglobin concentration and tumor volume did neither significantly correlate with the Doppler nor the oxygen measurements for the different tumor histology groups (p = 0.2–0.9).

Contrast-Enhanced Power Doppler Ultrasound of Tumors

Median vascularity (FA) and blood volume (CWFA) differed significantly between histology groups ($p < 0.0001$). Squamous cell carcinomas were highly vascularized and perfused (median FA, 36.9%; range, 12.9–69.2%; median CWFA, 26.1; range, 6.5–86.1) whereas in malignant melanomas vascularity and blood volume were moderate (median FA, 18.1%; range, 0.2–68.7%; median CWFA, 15.3; range, 0.05–81.4) and in sarcomas they were low (median FA, 5.0%; range, 0.1–38.8%; median CWFA, 3.2; range, 0.02–-19.1). However, there was overlap in the ranges between the different types of tumors.

pO_2 Measurements

Median pO_2 value was lower in sarcoma (median, 8.0 mmHg; range, 0–36.5 mmHg) than in squamous cell carcinoma (median, 22.2 mmHg; range, 6.4–56.7 mmHg) and malignant melanoma (median, 19.3 mmHg; range, 0–95.4 mmHg; p = 0.05). Correspondingly, the numbers of pO_2 readings ≤ 2.5, 5, and 10 mmHg were highest in sarcoma (28.4%, 41.9%, and 54.4%) in comparison to squamous cell carcinoma (0%, 2.7%, and 6.9%) and malignant melanoma (10.4%, 12.1%, and 17.2%; p = 0.04, 0.05, and 0.1, respectively).

For all tumors, vascularity (FA) and blood volume (CWFA) correlated significantly and positively with median pO_2 (r = 0.51 and 0.61; p = 0.001 and < 0.0001) and negatively with the percentage of pO_2 readings ≤ 2.5, 5, and 10 mmHg (r = –0.37 to –0.42; p < 0.01–0.03). However, within the different tumor histology groups, values scattered widely and correlation between power Doppler and pO_2 values was not significant (p = 0.06–0.9).

Treatment Protocols and Response to Treatment

RT was given with curative (n = 26) or palliative (n = 22) intent. The median total doses for curatively treated patients with sarcoma were 52.5 Gy (range, 49–56 Gy) and for palliatively treated sarcomas 30 Gy (range, 30–30 Gy). Squamous cell carcinomas were treated with 51 Gy (range, 45–51 Gy) and malignant melanoma with 24 Gy (range, 18–30 Gy). At disease progression, some dogs received additional treatment (n = 8). Complete response to treatment was observed in 21 dogs (44%), partial response in 18 animals (37%), and stable disease in nine patients (19%). At the time of analysis, 18 dogs were free of progression and seven were still alive. Patients were followed up to death or to the close-out date and none was lost to follow-up. The median follow-up time for all patients was 330 days, and for patients still alive (up to close-out date) 1,321 days. 28 patients died of tumor-related disease, ten for other reasons, and in three cases cause of death could not be assessed.

Progression-Free Interval and Overall Survival

PFI and overall survival times are listed in Tables 1a and 1b.

Univariate Models

In the sarcoma and squamous cell carcinoma group, PFI was neither significantly associated with age, tumor volume, Doppler nor pO_2 measurements (Table 2a). However, in squamous cell carcinomas, older dogs lived significantly longer and larger tumor volumes were significantly associated with longer survival (Table 2b). In palliatively treated melanomas,

larger tumor volumes led to a longer PFI and a lower vascularity index was associated with longer survival. For palliatively treated sarcomas, no significant associations were found between age, tumor volume, Doppler and pO_2 measurements, and PFI or survival (Tables 3a and 3b).

Table 1a. Progression-free interval for curatively and palliatively treated patients: while overall progression-free interval (PFI) of all patients is significantly different for curatively versus palliatively treated patients, no significant differences in PFI are found within the treatment groups. CI: confidence interval; NA: not assessed.

Tabelle 1a. Progressionsfreie Zeit für kurativ und palliativ bestrahlte Patienten: Während sich die kurativen und die palliativen Patientengruppen signifikant unterscheiden, ist für die einzelnen Gruppen innerhalb eines Behandlungsschemas die progressionsfreie Zeit nicht signifikant unterschiedlich. CI: Konfidenzintervall; NA: nicht untersucht.

Histology group	Curatively treated patients Median (days) [95% CI]	Palliatively treated patients Median (days) [95% CI]	Log-rank test p-value
All	504 [0;1,386]	142 [32;362]	0.0005
Squamous cell carcinoma	609 [0;1,261]	NA	
Fibrosarcoma	1,134 [0;2,232]	139 [0;285]	
Malignant melanoma	NA	112 [10;214]	

Table 1b. Overall survival for curatively and palliatively treated patients: while overall survival of all patients is significantly different for curatively versus palliatively treated patients, no significant differences in survival are found within the treatment groups. CI: confidence interval; NA: not assessed.

Tabelle 1b. Überlebenszeit für kurativ und palliativ bestrahlte Patienten: Während sich die kurativen und die palliativen Patientengruppen signifikant unterscheiden, ist für die einzelnen Gruppen innerhalb eines Behandlungsschemas die Überlebenszeit nicht signifikant unterschiedlich. CI: Konfidenzintervall; NA: nicht untersucht.

Histology group	Curatively treated patients Median (days) [95% CI]	Palliatively treated patients Median (days) [95% CI]	Log-rank test p-value
All	711 [0;1,481]	137 [77;197]	0.0008
Squamous cell carcinoma	733 [0;17,559]	NA	
Fibrosarcoma	437 [222;649]	159 [61;257]	
Malignant melanoma	NA	142 [0;290]	

Table 2a. Univariate proportional hazard Cox regression results for influences on progression-free interval: curatively treated patients. CWFA: color-weighted fractional area; FA: fractional area; SE: standard error; TV: tumor volume.

Tabelle 2a. Resultate der univariaten proportionalen Risikoregressionsanalyse für Einflüsse auf die progressionsfreie Zeit: kurativ behandelte Patienten. CWFA: Blutvolumen; FA: vaskularisierte Fläche; SE: Standardfehler; TV: Tumorvolumen.

Variable	Squamous cell carcinoma Coefficient (SE)	p-value	Sarcoma Coefficient (SE)	p-value
Age	0.041 (0.123)	0.736	–0.505 (0.377)	0.180
lnTV	0.420 (0.331)	0.205	–0.133 (0.517)	0.796
FA	0.041 (0.029)	0.150	0.187 (0.098)	0.057
CWFA	0.019 (0.016)	0.235	0.174 (0.096)	0.069
Median pO_2	0.013 (0.030)	0.656	0.051 (0.065)	0.433
% $pO_2 \leq 10$ mmHg	0.019 (0.021)	0.365	–0.023 (0.027)	0.399

Multivariate Models

Multiple Cox regression analyses confirmed the results of the univariate analysis; no other significant prognostic factors were identified for any of the tumor groups (data not shown).

Discussion

The goal of this study was to investigate whether vascularity and blood volume measured via quantified contrast-enhanced power Doppler sonography would allow a noninvasive estimation of tumor tissue oxygenation measured with pO_2 histography in spontaneous canine tumors.

The outcome after RT correlates with prior described responses of oral tumors in dogs [4–6, 9, 10, 34, 41]. Median vascularity and blood volume differed significantly between histology groups: squamous cell carcinomas were highly vascularized and perfused, whereas in malignant melanomas mean vascularity and blood volume were moderate and in sarcomas they were low. These results confirm recently published data from our group [33]. Fibrosarcoma was found to have the lowest tissue oxygen pressure with median values below the prognostically relevant described limit of ≤ 10 mmHg [2, 36], and the high and moderate vascularization and perfusion of squamous cell carcinomas and malignant melanoma, respectively, resulted in only moderate amounts of hypoxic regions. Prognostic significance of parameters possibly reflecting tumor oxygenation status was only described for soft-tissue sarcoma, where increasing microvessel density (MVD) was associated with increasing histological grade. However, no correlation between MVD and survival was found [27]. Accordingly, none of evaluated patient or tumor parameters of this study correlated with outcome after tumor treatment. This finding is in contrast to findings in studies of human tumors, where tumor oxygenation has

been found to be of independent significance in a variety of tumors [7, 8, 12, 18, 19, 21, 29–31, 37].

The parallel use of several different methods including endogenous hypoxia markers was found to be desirable to describe a profile of tumor oxygenation to be used for diagnostic and prognostic purposes [11, 22, 28, 35]. Various techniques leading to partially overlapping data will provide validation of the measurements and result in a more accurate tumor oxygenation profile [13, 23, 28]. The analyzed Doppler parameters correlated significantly with pO_2 measurements for all tumors, but not within the tumor histological groups. This correlation for all tumors may be explained statistically by the fact that both measurements differed within the various groups and values scattered widely. In humans, moderate correlations between the two technologies were found in metastases of head-and-neck cancer [17, 39]. In contrast to these studies, we used contrast-enhanced power Doppler ultrasound which has been shown to be more sensitive than contrast-enhanced color Doppler for detecting low velocities and depicting small vessels [26, 42]. It was hypothesized by our group that using the more sensitive technology, results would correlate to pO_2 measurements to an even higher degree than described in the literature [15, 16]. However, measurement of tumor vascularity and blood volume with contrast-enhanced power Doppler ultrasound and pO_2 histography obviously yielded different biological information in the present study. Nevertheless, according to clinical experience blood flow and tumor oxygenation are important factors which modulate the sensitivity of cancer cells. It has been suggested that measurements of global blood flow and vascularity will be useful in the early assessment of therapeutic response [13, 45].

One important reason for the lack of correlation between the two technologies may have been the fact that vascularity and blood volume may not completely reflect tumor oxygenation status. Approaches used to measure tumor perfusion do not necessarily provide the information on events occurring at the microscopic level [13]. Speed and direction of blood flow in the tumor are unstable; functionality of tumor vessels may be impaired in certain tumors and is likely to lead to the occurrence of fluctuating hypoxia within the tumor microenvironment.

Moreover, results of the present study may be limited because sample

Table 2b. Univariate proportional hazard Cox regression results for influences on survival: curatively treated patients. CWFA: color-weighted fractional area; FA: fractional area; SE: standard error; TV: tumor volume.

Tabelle 2b. Resultate der univariaten proportionalen Risikoregressionsanalyse für Einflüsse auf die Überlebenszeit: kurativ behandelte Patienten. CWFA: Blutvolumen; FA: vaskularisierte Fläche; SE: Standardfehler; TV: Tumorvolumen.

Variable	Squamous cell carcinoma Coefficient (SE)	P-value	Coefficient (SE)	Sarcoma p-value
Age	0.328 (0.125)	0.009	0.095 (0.259)	0.713
lnTV	0.610 (0.300)	0.042	–0.528 (0.418)	0.207
FA	–0.014 (0.024)	0.542	–0.008 (0.056)	0.882
CWFA	–0.012 (0.017)	0.490	–0.012 (0.063)	0.843
Median pO_2	–0.006 (0.023)	0.786	–0.020 (0.030)	0.510
% $pO_2 \leq 10$ mmHg	–0.001 (0.017)	0.940	0.002 (0.010)	0.869

Table 3a. Univariate proportional hazard Cox regression results for influences on progression-free interval: palliatively treated patients. CWFA: color-weighted fractional area; FA: fractional area; SE: standard error; TV: tumor volume.

Tabelle 3a. Resultate der univariaten proportionalen Risikoregressionsanalyse für Einflüsse auf die progressionsfreie Zeit: palliativ behandelte Patienten. CWFA: Blutvolumen; FA: vaskularisierte Fläche; SE: Standardfehler; TV: Tumorvolumen.

Variable	Melanoma Coefficient (SE)	P-value	Sarcoma Coefficient (SE)	p-value
Age	–0.027 (0.109)	0.800	–0.448 (0.284)	0.115
lnTV	1.022 (0.490)	0.037	0.389 (0.552)	0.482
FA	–0.029 (0.022)	0.193	0.028 (0.043)	0.510
CWFA	–0.016 (0.018)	0.398	0.025 (0.063)	0.693
Median pO_2	–0.012 (0.009)	0.199	–0.015 (0.032)	0.638
% $pO_2 \leq 10$ mmHg	0.008 (0.010)	0.394	0.002 (0.011)	0.878

Table 3b. Univariate proportional hazard Cox regression results for influences on survival: palliatively treated patients. CWFA: color-weighted fractional area; FA: fractional area; SE: standard error; TV: tumor volume.

Tabelle 3b. Resultate der univariaten proportionalen Risikoregressionsanalyse für Einflüsse auf die Überlebenszeit: palliativ behandelte Patienten. CWFA: Blutvolumen; FA: vaskularisierte Fläche; SE: Standardfehler; TV: Tumorvolumen.

Variable	Melanoma Coefficient (SE)	p-value	Sarcoma Coefficient (SE)	p-value
Age	0.091 (0.106)	0.392	–0.137 (0.218)	0.530
lnTV	0.543 (0.341)	0.111	0.809 (0.596)	0.175
FA	–0.066 (0.029)	0.023	–0.013 (0.035)	0.711
CWFA	–0.046 (0.025)	0.068	–0.025 (0.058)	0.659
Median pO_2	–0.003 (0.009)	0.720	–0.020 (0.030)	0.510
% $pO_2 \leq 10$ mmHg	–0.003 (0.007)	0.678	0.002 (0.010)	0.869

volumes of pO_2 histography and contrast-enhanced power Doppler ultrasound differed. The authors were aware of this problem when planning the study. Therefore, pO_2 measurements were made under ultrasound guidance in the same image plane of the contrast-enhanced power Doppler ultrasound examination, and three needle tracks (at least 50 measurements/track) in different locations were performed. It would have been difficult to obtain more tracks from one tumor, since many tumors were small. However, multiple tracks may still have not reflected the complete ultrasound image or the heterogeneous character of tumors.

In various human tumors, significant associations between high vascularity determined via color Doppler ultrasonography with or without contrast enhancement, and tumor aggressiveness, metastatic rate and decreased patient survival have been shown [24, 25, 32, 38]. However, in the patient population with oral tumors examined in the present study, none of the parameters were of prognostic value.

Conclusion

Although vascularity and blood volume correlated significantly with the descriptors of tissue oxygen tension, significance was lost by investigating the results for the different tumor histology and treatment groups. Neither univariate nor multivariate models were able to show that any of the methods could reliably predict outcome in these patients.

The authors feel that the results will have an impact on further research in tumor oxygenation and perfusion comparing invasive to noninvasive methods, even if the initially proposed hypothesis of correlation and prognostic significance for these tumor and patient populations did not prove true.

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Address for Correspondence
Carla Rohrer Bley
Section of Radiooncology
Vetsuisse Faculty
University of Zurich
Winterthurerstraße 260
8057 Zürich
Switzerland
Phone (+41/44) 635-8487, Fax -8940
e-mail: crohrer@vetclinics.uzh.ch