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New method of dynamic color doppler signal quantification in metastatic lymph nodes compared to direct polarographic measurements of tissue oxygenation

Thomas Scholbach[1]*, Jakob Scholbach[2], Gabriele A. Krombach[4], Bernd Gagel[3], Payam Maneschi[5] and Ercole Di Martino[5]

[1]*Kinderklinik am Städtischen Klinikum "St. Georg," Leipzig, Germany*
[2]*Department of Mathematics, École Normale Supérieure, Paris, France*
[3]*Department of Radiology, Aachen University, Aachen, Germany*
[4]*Department of Radiotherapy, Aachen University, Aachen, Germany*
[5]*Department of Otorhinolaryngology and Plastic Head and Neck Surgery, Aachen University, Aachen, Germany*

Tumor growth depends on sufficient blood and oxygen supply. Hypoxia stimulates neovascularization and is a known cause for radio- and chemoresistance. The objective of this study was to investigate the use of a novel ultrasound technique for the dynamic assessment of vascularization and oxygenation in metastatic lymph nodes. Twenty-four patients (age 44–78 years) with cervical lymph node metastases of squamous cell head and neck cancer were investigated by color duplex sonography and 17 (age 46–78 years) were investigated additionally with polarography. Sonography was performed after contrast enhancer infusion under defined conditions. Intranodal perfusion data (color hue, colored area) were measured automatically by a novel software technique. This allows an evaluation of blood flow dynamics by calculating perfusion intensity—velocity, perfused area, as well as the novel parameters tissue resistance index (TRI) and tissue pulsatility index (TPI)—for each point of a complete heart cycle. Tumor tissue pO_2 was measured by means of polarographic needle electrodes placed intranodally. The sonographic and polarographic data were correlated using Pearson's test. Sonography demonstrated a statistically significant inverse correlation between hypoxia and perfusion and significant TPI and TRI changes with different N-stages. The percentage of nodal fraction with less than 10 mmHg oxygen saturation was significantly inversely correlated with lymph node perfusion ($r = -0.551$; $p = 0.021$). Nodes with a perfusion of less than 0.05 cm/sec flow velocity showed significantly larger hypoxic areas ($p = 0.006$). Significant differences of TPI and TRI existed between nodes in stage N_1 and N_2/N_3 ($p = 0.028$ and 0.048, respectively). This new method of dynamic signal quantification allows a noninvasive and quantitative assessment of tumor and metastatic lymph node perfusion by means of commonly available ultrasound equipment.

Key words: lymph node perfusion; signal quantification software; color Doppler sonography; tumor perfusion; tumor oxygenation; neck metastases

Tumor growth and metastatic spread depend on sufficient blood supply.[1–6] The radiosensitivity of tumors is influenced by tissue oxygenation. Tumors produce angiogenetic factors regulating spread of vessels.[3,7–9] These vessels pave the way for invasion of tumor cells into environment. The extent of tumor vasculature and blood flow is a requirement of tumor development and invasiveness.

Hypoxia is one predictive factor for the response to radiotherapy.[4,10] Monitoring of tissue oxygenation can thus be important for scheduling therapeutic strategies. We developed a new technique for sonographic perfusion quantification of tissues and applied this technique to metastatic cervical lymph nodes. The results were compared to direct measurements of tissue oxygenation by polarography.

Material and methods

In a prospective nonrandomized controlled study, 24 patients with neck metastases of histopathologically verified squamous cell head and neck cancer were investigated. All patients were admitted between January 2002 and March 2003.

The cohort consisted of 19 men and 5 women with an average age of 60.5 years (44–78 years), all of whom were studied prior to treatment. The patients underwent their diagnostic and therapeutic procedures in the Departments of Otolaryngology, Radiology and Radiotherapy at Aachen University in Germany. The lymph nodes surveyed were clinically, radiologically and sonographically highly suspicious for a malignancy. Due to the technical requirements entailed in performing a polarography, only nodes > 20 mm were selected. Patients displaying necrotic lymph nodes in the sonographic examination were not admitted. In all patients, hemoglobin was > 10 mg/dl at the time of investigation. Anticoagulated patients were excluded from the study. A written informed consent was obtained from every patient and the study was approved by the local ethics committee.

Diagnoses and tumor staging were as follows (Table I). The new technique employed allows a measurement of classical resistance index (RI) and pulsatility index (PI) not only in a single but also in all vessels of the determined region of interest (ROI). Every single pixel in each vessel is traced through a complete heart cycle. Changes of color hue (representing flow velocity at this point) are measured and RI as well as PI is calculated. By this approach, tissue resistance index (TRI) and tissue pulsatility index (TPI) are calculated that represent mean PI and RI of all vessels inside the ROI.

Sonography

A gray-scale B-mode sonography preceded each color duplex scanning. Extension, size, echogenicity and texture of the suspected area were evaluated. Sonographic criteria for the diagnosis of a metastatic lymph node were as follows: a diameter of more than 10 mm and round in shape, a ratio of longitudinal-to-transverse diameter of < 1.5, or irregular margins and an absence of a clearly defined border between the nodes and the adjacent structures. Inhomogeneity of the internal echo texture and a decline in echogenicity were also presumed to reflect a malignant growth.

In color duplex sonography, characteristics for neoplastic growth were lymph nodes with solitary or conglomerate-type vessels, a loss of radial, unidirectional, or aberrant flow contrary to hilus arterial flow, or tortuous and deformed pictures of vessels and vessel lakes.

The finding of a central anechogenic area and peripheral vascularity, as well as signs of invasion into neighboring blood vessels, was further indications of a malignancy. To visualize as many vessels as possible, a contrast enhancer (Levovist; Schering, Berlin, Germany) was administered. Four grams of Levovist granulate was diluted in 11 ml and injected intravenously: half as bolus injection followed by continuous administration via infusion pump with a rate of 300 ml/hr.

***Correspondence to:** Kinderklinik am Städtischen Klinikum "St. Georg," Delitzscher Strasse 141, D-04229 Leipzig, Germany.
Fax: +49-341-9093615. E-mail: thomas.scholbach@sanktgeorg.de
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TABLE 1 – TUMOR LOCALIZATION AND STAGING IN 24 PATIENTS

	Origin	T	N	M	Oxygen measurement
1	Hypopharynx larynx	4	2	1	Yes
2	Hypopharynx larynx	4	2	0	Yes
3	Hypopharynx larynx	4	3	1	Yes
4	Hypopharynx larynx	4	1	0	Yes
5	Mouth	3	2	0	Yes
6	Mouth	2	3	0	Yes
7	Oropharynx	4	1	0	Yes
8	Oropharynx	4	2	0	Yes
9	Parotid	4	2	0	Yes
10	Pharynx (naso/oro and hypopharynx)	2	2	0	Yes
11	Tongue	3	3	1	Yes
12	Tonsil	4	2	0	Yes
13	Tonsil	1	3	0	Yes
14	Unknown primary tumor	x	3	1	Yes
15	Unknown primary tumor	x	3	1	Yes
16	Unknown primary tumor	x	2	0	Yes
17	Hypopharynx larynx	3	2	0	No
18	Hypopharynx larynx	4	2	0	No
19	Hypopharynx larynx	3	1	0	No
20	Mouth	4	2	X	No
21	Oropharynx	4	2	0	No
22	Schmincke-Regaud tumor	3	1	1	No
23	Tonsil	4	2	0	No
24	Unknown primary tumor	x	2	0	No

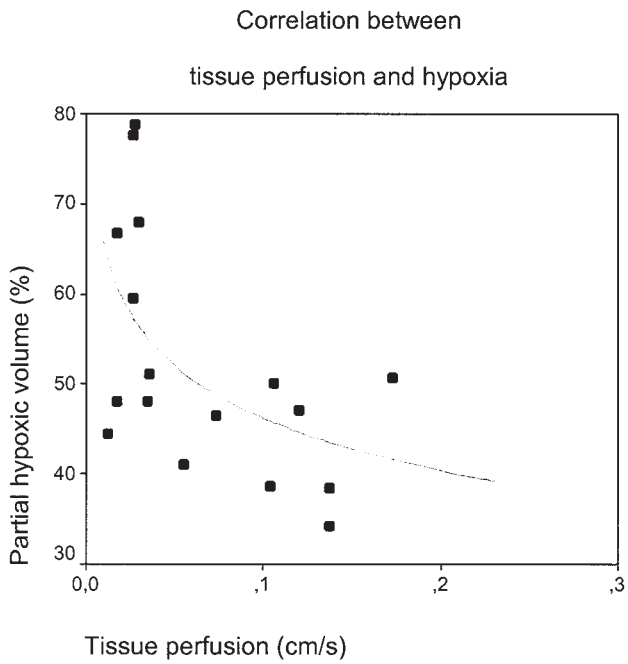

FIGURE 1 – Correlation of hypoxic fraction in metastatic LN in relation to tissue perfusion in 17 patients. Nonlinear curve fit analysis with a logarithmic model (red line).

In color Doppler sonography (CDS), horizontal and longitudinal scans were performed on every suspected lesion in order to ensure a representative picture of the vascularization. The thickness of these scanned slices was 5 mm each. Lymph node's margins were outlined on the patient's skin. In distances of 5 mm, parallel lines were drawn onto the skin. Parallel feed of the transducer was executed in 5 mm steps according to these skin marks. Depending on the size of the investigated nodes, this resulted in 5–19 images of each lymph node. Color window's size was chosen to encompass the whole node's area. Color Doppler gain was 70 dB and color Doppler frequency was 5.5 MHz. Pulse repetition frequency was adapted to achieve optimal imaging of tumor vessels (range, 551–1,736 MHz, corresponding to a color scale limited to maximum values from 4 to 12 cm/sec). The scanning was performed using a Sonoline Elegra device (Siemens, Erlangen, Germany). A variable transducer (5.2–9.0 MHz) with a linear array was employed. All investigations were performed with a frequency of 7.2 MHz. The transmitting power was 100%; the amplification was 20–28 dB. Focusing depth was set to the appropriate plane of the tumor or the targeted lymph node. To cover and standardize the color sensitivity and frame rate, the color window was always set at the maximum size of the lymph node. For color duplex depiction of intratumoral vessels, the lowest measurable blood flow velocity with the appropriate color gain was individually adjusted while avoiding artifactual color noise and providing maximum visualization quality. Frame rate varied between 6 and 10 images per second depending on the size of the color box (its size varied from 2.4 to 14.1 cm^2), which was adapted to the lymph node's size. All sonographic studies were recorded digitally. The resolution was 576 × 720 pixel in the chosen format. Compression was 1:5 (DV video resolution).

Quantification of vascularization

For evaluation of blood flow dynamics in the metastatic nodes, a commercially available software (Pixelflux, Chameleon-Software, Leipzig, Germany) was employed. Cervical lymph nodes were investigated at the largest diameter to record perfusion signals of intranodal vessels. A color Doppler sonographic video was recorded and perfusion signals of every single image were read out automatically from the ROI. The ROI was defined in advance, encompassing the whole node's transsectional area and sparing out surrounding tissue.

The software extracted information content of color Doppler signals evaluating 2 parameters: color hue, which defined velocity (v), and color area, which defined perfused area (A). Color hue of each pixel inside the ROI is automatically compared by the software with color hues of the color bar of the image. The color bar is calibrated in advance as well as the distances of the image (implemented ruler at the lateral side of the image). After calibration of distances, the software calculates area of ROI and area occupied by colored pixels for further evaluation. A video of the sonographic examination is then automatically analyzed to quantify velocity and area values of each pixel inside ROI. Moreover, all changes of these parameters during the replay of the video are measured. Perfused area (A) is understood as the part of the ROI filled with color signals. In each image of the video, perfused area as well as depicted average of all velocity values of all pixels (v) inside ROI changes due to heart action. Therefore, the software defined a full heart cycle automatically and the mean values of depicted areas and velocities were calculated. At the end, mean flow velocity inside the ROI over one full heartbeat is reckoned and displayed in various graphic and numerical presentations. TRI and TPI are calculated automatically from the raw data according to the commonly used equations for RI and PI. In contrast to conventional RI and PI (which incorporate flow velocity changes of a single vessel only), flow velocities of each pixel inside the tissue region covered by the ROI are incorporated in the calculation. We coined therefore the terms TRI and TPI to characterize this fundamental difference to conventional RI and PI.

Signal intensity as a measure of tissue perfusion (TP) was quantified during one full heart cycle as follows: $TP = v_{mean} \times A_{mean}$. Mean flow velocity as depicted in Figures 1 and 2 is a product of this signal evaluation and describes flow intensity as if the whole ROI would be perfused with this velocity. Mean flow velocity may be quite low, depending on the ratio of perfused and

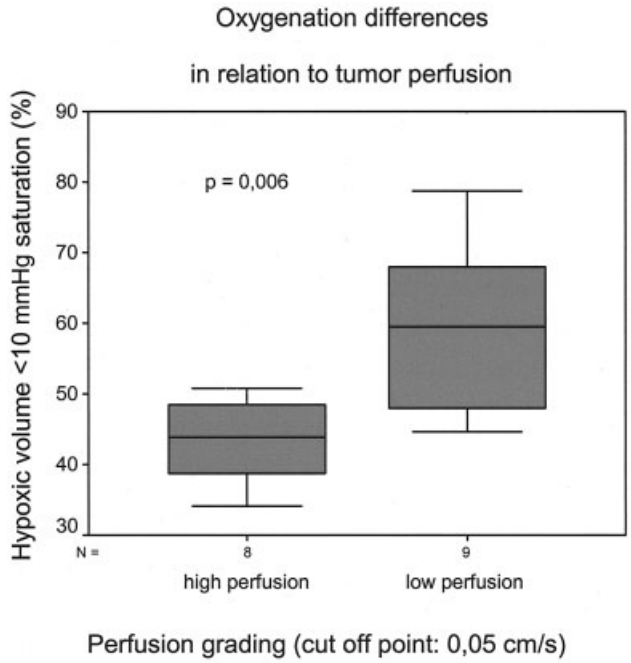

FIGURE 2 – Comparison of hypoxic volume in metastatic LN in relation to level of perfusion.

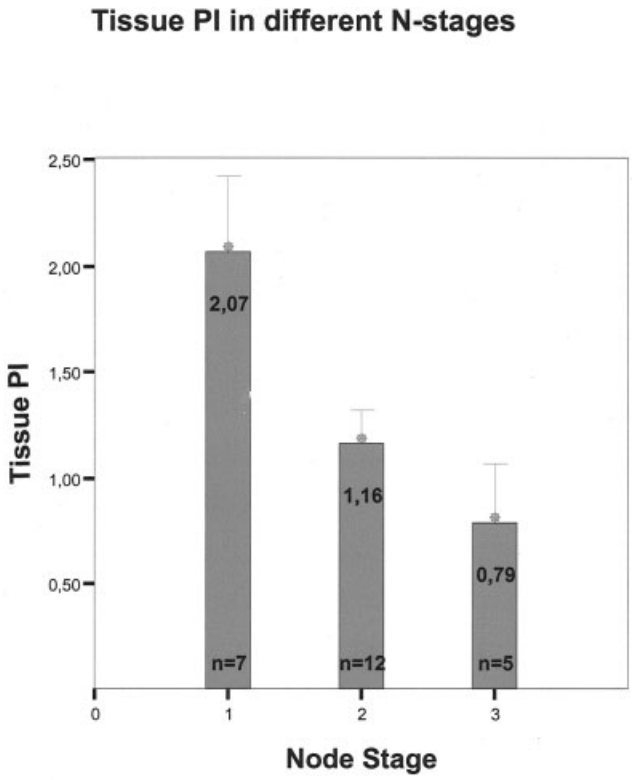

FIGURE 3 – TPI in different N-stages from 24 patients: mean values and standard error of the mean. Significant difference between N_1 and N_2 ($p = 0.028$) and between N_1 and N_3 ($p = 0.030$).

TABLE II – MEAN VALUES AND STANDARD DEVIATION OF PERCENTAGE OF HYPOXIC TISSUE VOLUME (LESS THAN 10 mmHg OXYGEN SATURATION) IN NODES WITH HIGH AND LOW PERFUSION

Perfusion grading (limit 0.05 cm/sec)	Percentage of hypoxic node volume	*n*	SD
High perfusion	43.33	8	6.0
Low perfusion	60.21	9	13.1
Σ	52.26	17	13.3

Cutoff 0.05 cm/sec mean perfusion velocity of the whole ROI.

nonperfused area inside the ROI, and depending also on the actual flow velocity in perfused parts.

pO_2 histography

Tumor tissue pO_2 was measured by means of polarographic needle electrodes using a pO_2 histograph type 6650 (Eppendorf-Netheler-Hinz, Hamburg, Germany). Sterile polarographic needle electrodes with stainless steel shafts were used. The probes had a mean shaft diameter of 300 μm. The needle electrodes were placed, controlled by computed tomography, in the tumor tissue without general or local anesthesia.

We measured oxygenation with 95–400 single measurements per single lymph node. From these data, percentage of respective pO_2 fraction was calculated. The distance between the measurement points was 0.7 mm. This was due to an automatic probe movement of 1.0 mm forward and 0.3 mm backward in order to minimize compression effects caused by the forward motion of needle electrode. The directions and overall length of needle penetration were determined by CT scans, thus avoiding necrotic areas. Calibration was performed in sterile 0.9% saline solution immediately before and after pO_2 was measured. To assess the biologic and clinical relevance of oxygenation measurement, the relative frequency of pO_2 readings with values below 2.5, 5.0 and 10.0 mm Hg, as well as mean and median, was stated.

Statistics

Statistical analysis was performed using the SPSS software for Windows 11.05 (SPSS, Chicago, IL). To assess statistical relevance of the results, we calculated correlations according to Pearson and differences between groups with the Mann-Whitney U-test.

Results

TP was correlated with oxygen saturation of the individual lymph node (LN). A significantly inverse correlation of TP with the partial hypoxic nodal volume exhibiting oxygen saturation less than 10 mmHg ($r = -0.551$; $p = 0.021$; Fig. 1) was found. Best curve fit was calculated with a logarithmic model according to following equation: $HV = 26.91 - 8.37 \ln (TP)$, where HV denotes hypoxic volume (less than 10 mmHg oxygen saturation).

Statistically significant differences in hypoxia between metastatic LN by assorting nodes according to their perfusion level were demonstrated. Nodes with a tissue perfusion less than 0.05 cm/sec had a significantly higher degree of hypoxia (60% *vs*. 43%; $p = 0.006$; Table II, Fig. 2). Significant differences of TPI between nodes in stages N_1 and N_2 ($TPI_{N1} = 1.15$; $TPI_{N2} = 2.07$; $p = 0.028$) as well as N_1 and N_3 ($p = 0.030$; Fig. 3, Table III) and of TRI between nodes in stage N_1 and N_3 ($TRI_{N2} = 0.79$; $TRI_{N3} = 0.47$; $p = 0.048$; Fig. 4, Table IV) could be found. There was no significant difference of TRI between N_1 and N_2 ($p < 0.056$) and between N_2 and N_3 ($p = 0.506$). The TPI differences between N_2 and N_3 failed to demonstrate statistic significance ($p = 0.383$).

Discussion

Tumor progression depends on a sufficient vascular network to meet the metabolic needs of growing tumor tissue. Changes in tumor perfusion are likely to correlate with tumor growth and could eventually characterize tumor behavior: central necrosis,

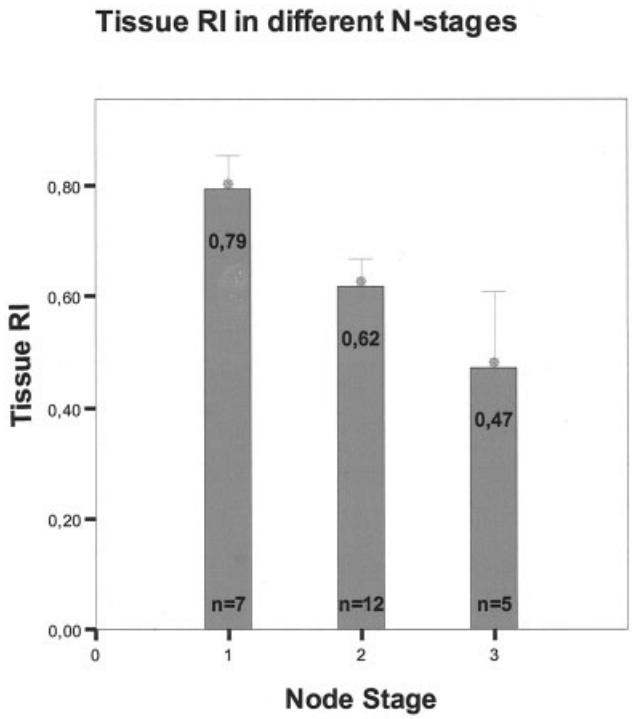

FIGURE 4 – TRI in different N-stages from 24 patients: mean values and standard error of the mean. Significant difference between N_1 and N_3 ($p = 0.048$).

TABLE III – MEAN VALUES AND STANDARD DEVIATION OF TPI (MEAN PI OF ALL VESSELS OF THE WHOLE ROI) ACCORDING TO THE N-STAGE

N-stage	Mean	*n*	SD
1	2.07	7	0.952
2	1.16	12	0.551
3	0.79	5	0.626

TABLE IV – MEAN VALUES AND STANDARD DEVIATION OF TRI (MEAN RI OF ALL VESSELS OF THE WHOLE ROI) ACCORDING TO THE N-STAGE

N-stage	Mean	*n*	SD
1	0.79	7	0.153
2	0.62	12	0.174
3	0.47	5	0.303

proliferation potential, metastatic activity.[11,12] Alterations in local tissue perfusion lead to an alteration of local oxygenation. Tissue oxygenation in tumors is reported to be of predictive value for patient survival. Hypoxia contributes to tumor resistance against radio- and chemotherapy and seems to predispose to more aggressive and metastatic behavior.[13–17] Therefore, it is useful to calculate perfusion of tumor tissue to monitor the state of tumor development. Measurement of tissue oxygenation is possible via direct placement of oxygen probes at various sites of the tumor (oxygen histogram).[15,17,18] Recently, the potential of magnetic resonance imaging (MRI) perfusion evaluation has been demonstrated in cervical carcinoma. Lyng *et al*.[19] found significant correlation of perfusion and tissue oxygenation in dynamic gadolinium DTPA-enhanced MRI. No correlation existed between vascular density and pO_2. Since MRI is a valuable but costly procedure that requires the administration of a contrast enhancer with the risk of allergic reaction, we looked for an alternative.

Color Doppler sonography is easy to apply, noninvasive, cost-effective and capable of depicting flow signals. Modern sonography devices allow the visualization of flow even in tiny vessels with a diameter of 0.1–0.2 mm in lymph nodes.[20,21]

Early studies report on the use of single-vessel RI and PI measurements.[22] First attempts made to quantify flow signals by software-based techniques in lymph nodes[23,24] could demonstrate with computer-assisted single image analysis in 25 patients that lower color pixel density came along with significantly longer survival, whereas mean flow velocity had no meaning. In contrast to these findings, we could combine both velocity and pixel area measurements in videos. This may contribute to a more subtle evaluation not to be achieved with earlier still-image analysis. Steinkamp *et al*.[25] demonstrated flow differences in reactive lymph node enlargement and metastases and applied these parameters for diagnosis of a malignancy. Other investigators questioned these findings.[26–28] With the technical means employed by these groups, the results remained unequivocal.

A critical appraisal of advantages and limitations of ultrasound is always necessary, especially when entering a new field opened up by new tools. Aliasing, ratio of heart rate and imaging frame rate, preponderance of vessel orientation inside the ROI, biased distribution of vessel diameters inside ROI and data loss during image signal processing and transfer may serve as examples of influences on the measurements, which cannot be ruled out in certain situations. Other limitations include interobserver variability and inadequate settings of ultrasound equipment such as wall filter, ratio of color box size to ROI size and pulse repetition frequency.[29] Moreover, one should always keep in mind that sonography deals with (imaging) planes and approaches to 3-dimensional sonographic perfusion measurement of tissues still await exploration. Therefore, misinterpretation of local phenomena as being significant for perfusion of the whole tumor volume may be relevant, too.[30] In this context, a correlation of physically different techniques to evaluate tumor perfusion is important. Our results demonstrate a significant correlation of tissue oximetry (a technique based on measurements in the whole volume of a tumor) with the novel dynamic Doppler signal perfusion measurement. It stresses the potential of this software-based method despite the above-mentioned objective and subjective sources of error.

In a recent study, we were able to show a correlation between the extent of vascularization and tissue oxygenation by sonographic means.[31] Dynamic color Doppler signal quantification of tissue perfusion is another advancement toward a more comprehensive measurement of relevant perfusion data.[32–34]

In this technique, color Doppler signals are read out automatically by a recently available software (Pixelflux).[32–34] Each pixel's individual velocity and area value are read out. Velocity values are not angle-corrected. This implies real perfusion velocity is higher than depicted and measured. It is therefore appropriate to use the term "signal intensity" rather than "flow intensity." Nevertheless, such measurements are valuable if the influence of flow direction can be neglected. This is true when vessel distribution inside ROI is chaotic or symmetric and tissue samples under investigation do not differ significantly in this respect. The proof of this is a comparison with an established direct measurement of perfusion. Tissue oxygenation level is a function of perfusion intensity as well as intrinsic properties of oxygen binding to hemoglobin and local influences on transfer of oxygen from hemoglobin to surrounding tissue. Perfusion intensity is an important variable of tissue oxygenation and the principal modulator of substrate supply and tumor metabolism.[35,36]

It is interesting therefore to correlate investigations of tissue oxygenation with perfusion measurements. Significant differences could be demonstrated in this study between LNs with high and low perfusion. Low perfusion was defined as mean tissue perfusion with a velocity less than 0.05 cm/sec; nodes with a higher perfusion were assorted to the high-perfusion group (Fig. 2). These results indicate that the new method of perfusion measurement in tissues is easily applicable and reflects oxygenation reliably. We were also able to demonstrate a significant inverse correlation of

tissue oxygenation and perfusion. Infusion of contrast enhancer is a useful tool to detect more color signals. In some cases, contrast enhancer may blur flow signals (so-called blooming effect).[37–39] This may have had an impact on the results of this study and negatively affected the statistical correlation found. Our method is novel in its measurement of intrinsic properties of flow signals (numerical description of color hue and pixel density per ROI). Therefore, it is more susceptible to interferences such as blooming and movement artifacts that are not important in other experimental settings (*e.g.*, semiquantitative evaluation of flow from single images). Future studies should be designed to minimize such interferences. The ongoing development of more advanced ultrasound equipment and/or the application of new B-flow techniques[40] could make a valuable contribution to the sonographic assessment of dynamic blood flow parameters.

The technique used for this study allows characterization of tissue perfusion under various aspects. By this approach, conventional RI and PI measurements can now be extended from a single vessel to a whole ROI. Thus, a larger area is characterized with respect to peripheral flow resistance. Our results demonstrated a significant decline of TRI and TPI from lower to higher N-stages. This might reflect differences in vascular spread, branching, caliber tapering, or interstitial pressure. Pressure detection and quantification of such changes of vascularity may have a future potential in the monitoring of antiangiogenetic therapies. Our results are in line with observations of other groups, who found in the single-vessel techniques significantly lower RIs in higher-graded lymph node metastases of endometrial carcinoma[41–43] found a single-vessel RI less than 0.4 predictive for metastases in endometrial carcinoma. In this series of head and neck tumor metastases, N_3 nodes had a mean TRI of 0.47 compared to N_1–N_1 nodes with 0.79. This demonstrates a decrease in peripheral vessel resistance with tumor progression. Further studies are necessary to obtain more details.

The same authors[44] developed this approach further and counted colored pixels in ROI. They calculated a so-called vascularity index (VI) by means of a quantitative image-processing system. This index correlated better with tumor stage, tumor size and pathologic findings, including depth of stromal invasion, lymphovascular emboli and pelvic lymph node metastases than intratumoral RI.

Hypoxia is a promoter of radioresistance. pO_2 polarography is the standard for tissue oxygenation measurements. Despite its reliability, this procedure has various drawbacks. In some cases, bleeding may occur as well as the leaking of necrotic tissue because a needle has to be inserted.

In addition to the invasive nature of this procedure, the necessity of a computed tomography is a further drawback. This imaging procedure is required in order to locate the probe exactly into the suspected tissue. The length and the necessary insertion of the probe mean that the size of the lymph node, which can be investigated, is limited to a minimum of 20 mm. Since bleeding may be a problem, patients with coagulation problems are not eligible for this procedure. Moreover, the influence of the measurement procedure itself on tissue oxygenation state seems possible.[45] These restrictions can be avoided by application of sonography. A number of reports point to a correlation of sonographically detected perfusion changes in tumors and their growth.[46,47] Quantitative analysis of vascularity by means of Doppler techniques can help to classify tumors according to their aggressiveness.[48–50] Conventional sonographic approaches to perfusion evaluation include simple and subjective estimation of vessel density and pattern and calculation of RI and PI in single vessels.[51,52]

The present study of a novel dynamic color Doppler signal quantification technique offers new tools to evaluate tumors. The new parameters TRI and TPI have the potential to differentiate metastases according to their N-stages and specific tumor tissue perfusion allows correlation of oxygenation and perfusion. The results in a limited number of patients suggest that a more comprehensive and differentiated appraisal of tumors could become possible. Further studies are necessary to confirm these findings.

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