Dear Iratxe Puebla,

please find enclosed the revision of my manuscript and a detailed reply to all reviewers in chronological order. Concerning the language revisions, the paper was copyedited by two colleagues who had worked in the UK for several years. Reference 31 that was listed as in press has meanwhile been published. The citation was corrected. Thank you for proceeding my work.

With kind regards,

B. Gagel
1/2

The aim of our study was to find associations between the different methods for quantification of tumor hypoxia (pO2 polarography, FMISO-PET). In addition we wanted to examine one of the main factors influencing tissue oxygenation, the tissue perfusion by the use of different sonographic techniques.

The inhomogeneous distribution of the different measurements is based on logistic problems. In the manuscripts you mentioned we discussed in detail the different new methods, the new parameters and covariants like hemoglobin values and lymph node size that may influence the results.

In the final manuscript all data were pooled to test correlations between the different methods and tissue oxygenation by pO2 polarography on the one hand but also to analyze associations and possible interactions of different factors and even to detect limitations of the different methods by the analysis of outliers. Therefore we used scatter plots and Pearson correlation coefficient without threshold based comparison of hypoxic or normoxic tumors, reflecting a straight (direct) way to show associations. Consequently the current manuscript represents a critical overview of the tested methods.

Discussing pO2 polarography I believe that it is still a standard method for evaluation of therapy relevant tumor hypoxia being validated in numerous clinical studies. This is also stated by J G Eriksen and M R Horsman (Radiat Oncol 2006; 81(2): 119-121) the latter being one co author in the manuscript you cited. In your study being published in 2005 in the Int J Radiat Oncol Biol Phys where you used two different tumor cell lines, you stated that pO2 polarography does not deliver a representative value for the whole tumor volume. In the same year and in the same journal (Int J Radiat Oncol Biol Phys 2005; 61/5: 1493-1502) Humm et al could show a spatial correlation between polarographic pO2 measurements and FMISO. Comparing FAZA and pO2 polarography by the use of a mouse tumor model, Piert et al (J Nucl Med 2005; 46 (1): 106-113) explained missing correlation of PET and pO2 measurements by the fact that exact location of the probe’s tip cannot be monitored. Therefore, measurements from the rim of the tumor are difficult to perform and the results may include pO2 values obtained from necrotic areas
within the tumor tissue. Consequently, a loss of correlation should be explained by inhomogeneous distribution of single measurements.

In our study we realized a CT guided (simultaneous) pO2 measurement for each patient in order to define exact probe movement and to avoid measurements in larger necrotic areas.

“For each patient the needle electrodes were placed and guided CT-controlled in the tumor.” (page 6/7)

In this way we performed 95-400 single pO2 measurements per lymph node [MW=225; SD=61] that should have resulted in a representative distribution of pO2 values.

“In this way we realized 95-400 single pO2 measurements per lymph node (MW = 225; SD = 61), resulting in a representative distribution of pO2 values.” (page 7)

Nevertheless we also have to discuss the problem of measurements in necrotic areas. According to the figures 2 and 3, measurements were performed in lymph node metastasis of the neck, representing superficial tumor locations.

No measurements in regions with different CT signals were performed.
3. “Standard sonographic, CT and PET criteria were used for the diagnosis of metastatic lymph nodes. In 20 patients the diagnosis was confirmed histologically.” (page 4)

In our prior manuscripts, we discussed in detail the new methods, the criteria of malignancy, the new parameters and co variations like hemoglobin values, lymph node size and TNM state. Focusing to the most relevant parameters, we wanted to test the correlations between the parameters of the different methods and to analyze possible interactions between these parameters.

I agree in your point of a more accurate fit of tumor shape and ROI in PET. By the use of rectangular ROIs according to tumor size a slight homogenous underestimation of tumor hypoxia could be expected. Nevertheless it is an accepted technique often used in literature. Because of the homogeneity of underestimation a relevant influence on the results/correlations cannot be expected.

According to comparable data acquisition no computer algorithm for co-registration was used.

“For only measurements in lymph node metastasis (one of each patient) were performed. In order to ensure measurements in the same suspected lymph nodes, sonographically examined lymph nodes were marked on diagnostic CT scans or in the case of lymph node conglomerates, the extension of the scanned node was marked on the skin. PET examinations were realized in most of the patients within two days (maximum time interval four days) using skin markers and positioning lasers for reproducible data acquisition. No immobilization device was used.” (page 4)

4. Some patients were treated by surgery followed by adjuvant radio- or radio-/chemotherapy, others by primary radio-/chemotherapy or chemotherapy alone. Because of the inhomogeneous treatment a clinical analysis of the data was not performed.

Methods:
“Because of different treatment modalities (surgery followed by radio- or radio-/chemotherapy and primary radio-/chemotherapy or chemotherapy alone) no clinical analysis was performed.”(page4)

5. As summarized in the title: “New method of dynamic color doppler signal quantification in lymph node metastasis compared to direct polarographic measurements of tissue oxygenation” this manuscript was primary focused on the new technique for sonographic perfusion quantification developed by Scholbach J and Scholbach T. Different parameters of CDS like tissue perfusion (TP), tissue resistance index (TRI) and tissue pulsatility index (TPI) were analyzed and compared to pO2 polarography or N-status/lymph node size. In the present study we only used TP, focusing on the main perfusion parameter influencing tumor oxygenation.
Jan Bussink

General
The detailed numbers of the different examinations were inserted in the methods. The inhomogeneous distribution of the different measurements is based on logistic problems. Based on our experiences and with respect to the limitations of the different methods we believe that it is possible to detect outliers as described in the manuscript.

Minor essential revisions

Abstract (page 2):
From color duplex sonography (CDS) signals two parameters were extracted is correct.

Methods
“CDS was performed in 32 patients.” (page 4)
The detailed numbers of the different examinations were inserted in the methods.

Quantification of perfusion:
“Signal intensity as a measure of tissue perfusion (TP) was quantified as follows: TP = v_mean x A_mean with A = part of the ROI filled with color signals and v = velocity values of all pixels inside the ROI changing due to heart action.” (page 5)

Positron-Emission-Tomography (PET)
“In 24 patients FDG PET as well as FMISO PET examinations could be performed.” (page 5)

“PET examinations were realized in most of the patients within two days (maximum time interval four days) using skin markers and positioning lasers for reproducible data acquisition. No immobilization device was used.” (page 4)
“FMISO PET consisted of one static scan of the relevant region” (page 6)
(One static scan included transmission and emission)

Signal to background ratio

“Three venous blood samples (after 120-, 125- and 130 minutes) were taken at each static scan. After correction according to the half-life period the mean values were calculated.” (page 6)

Discussion page 11:
“It could be shown that the tumor uptake of FMISO was constant between 30 min and 2h and that the tumor to blood and tumor to muscle FMISO uptake ratios were stable 2-4h after injection, suggesting some retention mechanisms of FMISO within the tumor but not within any normal tissue [15].”

Results
When we started our study only CPD calculation was performed. In the course of the study calculation of tumor perfusion could be realized resulting in inhomogeneous distribution of the different measurements
“In the course of the study the commercial software (PixelFlux®, Chameleon-Software Corp., Leipzig, Germany) was available. In 18 patients this software was used for evaluation of blood flow dynamics in the lymph node metastasis until now.” (page 5)

As described in the discussion only the relevant PET parameters were used in table 1. Nevertheless we inserted the result of FMISO<sub>SUVmax/mean</sub>: “No relevant correlation between FMISO<sub>SUVmax/mean</sub> and CPD/TP or the polarographic parameters could be detected.” (page 11)

with an absolute value of the Pearson correlation coefficient ranging between 0.056 and 0.241
Discussion

Page 10:

Using pO2 polarography mean and median pO2 and partly hypoxic fraction ≤ 10mmHg also reflect a mean value for the examined lymph node. In contrast hypoxic fractions ≤ 5mmHg or ≤ 2.5mmHg represent a percentage of values with defined low oxygenation. Normally, patients show homogeneous distributions between the different hypoxic fractions. The mean and median values are influenced by these homogenously distributed parameters and consequently this results in a correlation of hypoxic fractions and mean or median parameters. In patient 1 and patient 2 we see mean and median pO2 values based on a high amount of values < 10mmHg with low percentages of values <5.0mmHg and 2.5mmHg, representing a heterogeneous distribution of values.

Using CDS only a mean value of tumor vascularisation or perfusion could be calculated without any possibility to differentiate between better or worse perfused (=oxygenated) volumes.

“Comparing the parameters of pO2 polarography and CPD there were two patients (patient 1 and 2) with low mean and median pO2 values based on inhomogeneous distribution of the different hypoxic fractions. There was a high percentage of readings ≤ 10 mmHg and a low percentage of readings ≤ 2.5 or ≤ 5.0 mmHg. CPD or TP only deliver a mean value of tumor vascularisation or perfusion. The method is therefore unable to reproduce any heterogeneity of tumor oxygenation resulting in decreased correlation between CPD or TP and the hypoxic fractions ≤ 2.5 and ≤ 5.0 mmHg.” (page 10/11)

Page 11:

In literature, FDG uptake was used for the detection of tissue oxygenation (caused by HIF-1α) as well as correlated with tissue perfusion (partly explained by p53 oncogene).

Therefore we believe that the discussion of p53 oncogene is very important in order to reflect “that tumor hypoxia is caused by innumerable, multifactorial, partly contradictory interacting causes and effects complicating detection of therapy relevant hypoxia by the use of clinical examinations.”(page 11)
Tables 1:
N = number of correlated measurements was inserted in the legend. In addition, the numbers of performed measurements of the different methods were inserted in the method part.

Figure 1: Upon consultation with S Stanzel (Institute of Medical Statistics) we favored scatter plots without depiction of linear best fit and $r^2$.

Figure 2: The value reflects parameter variability, so we decided not to discuss this special value.

Figure 3: In figure 2, two different patients were compared. In figure 3 different images of one patient were presented. In order to underline this difference, different layouts were used.
Mathias Bruehlmeier

The Pearson correlation coefficient displays the degree of linear association of two different parameters.

Results
Page 8: “Correlations are listed in Table 1. To emphasize relevant correlations (r > 0.4) bold numbers were used. Only a slight association between FMISO_{T/B} and the hypoxic fraction ≤ 2.5 mmHg could be detected.”

In our conclusions, we combined a short summary of the work in the first paragraph and possible consequences in the second paragraph.

We used the numbers only to discriminate the different outliers in the text. There was no association between the numbers and listed values!

Page 12: “It reflects that tumor hypoxia is caused by innumerable, multifactorial, partly contradictory interacting causes and effects complicating detection of therapy relevant hypoxia by the use of clinical examinations.”
Lymph node metastasis of 38 patients with histologically verified head and neck malignancies (36 patients with squamous cell cancer, one patient with lympho-epithelial cancer and one patient with Hodgkin’s lymphoma) were enrolled in a prospective clinical evaluation between October 2002 and January 2005.

Measurements were performed in one lymph node metastasis in each patient. In order to ensure measurements in the same suspected lymph nodes, sonographically examined lymph nodes were marked on diagnostic CT scans or in the case of lymph node conglomerates, the extension of the scanned node was marked on the skin. PET examinations were realized in most of the patients within two days (maximum time interval four days) using skin markers and positioning lasers for reproducible data acquisition. No immobilization device was used.

In our prior manuscripts we discussed in detail the new methods, the new parameters and covariants like hemoglobin values, lymph node size and TNM state. In the final manuscript all data were pooled. Focusing on the most relevant parameters we wanted test correlations between the parameters of the different methods and to analyze possible interactions.

Standard sonographic, CT and PET criteria were used for the diagnosis of lymph node metastasis. In 20 patients the diagnosis was confirmed histologically.

Because we used three diagnostic methods no exact cut-off value was defined for FDG PET.

All patients fasted for at least 6 h before examination, verified by determining blood glucose level (mean = 90.8 mg/dl; SD = 16.0 mg/dl). None of the patients showed a higher concentration than 120 mg/dl, so treatment with insulin prior to examination was not necessary.
The detailed numbers of the performed measurements were inserted in the methods.

5. Page 6: “Three venous blood samples (after 120-, 125- and 130 minutes) were taken at each static scan. After correction according to the half-life period the mean values were calculated.” (page 6)

6. Page 4: “Patients underwent the following measurements within one week: contrast-enhanced color duplex sonography (CDS), FDG PET, FMISO PET and polarographic pO₂ measurement (last measurement).” “PET examinations were realized in most of the patients within two days (maximum time interval four days)”

7. Correction was done

8. First paragraph of the discussion was deleted.

In order to discuss our results we believe that it is important to present a few factors of the multifactorial, partly contradictory interacting causes and effects of tumor hypoxia.

Page 12: “It reflects that tumor hypoxia is caused by innumerable, multifactorial, partly contradictory interacting causes and effects complicating detection of therapy relevant hypoxia by the use of clinical examinations. Nevertheless those examinations may enable the transfer of simplified information from cellular micro cosmos into clinical practice.”