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INCIDENCE OF LYMPH NODE METASTASES AFTER PIECEMEAL LASER-SURGICAL AND EN BLOC COLD STEEL RESECTION OF AURICULAR VX2 CARCINOMA. A COMPARATIVE STUDY

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Meinen Eltern in Liebe und Dankbarkeit

Contents

List of abbreviations

CO_2	Carbon dioxide
CSR	Cold steel resection
H&E	Hemalaun and eosin
HNSCC	Head and neck squamous cell carcinoma
ICO:NZW	Iffa Credo New Zealand White rabbits
LN(s)	Lymph node(s)
LSR	Laser-surgical resection
ND	Neck dissection
SCC	Squamous cell carcinoma

1. Introduction

Cancer of the upper aerodigestive tract presents nowadays an important medical problem. This anatomical region is the sixth most often localisation of malignancies. In up to 90% of all these cases the histological type is squamous cell carcinoma (SCC) [9,40,55]. The most pronounced causative agents are the tobacco and alcohol consumption [56,89].

An important characteristic in the natural course of head and neck SCC (HNSCC) is its tendency to disseminate to the regional lymph nodes (LN) and on a later stage to distant organs [84,117,119]. The presence of LN metastases plays a decisive role for the therapeutic approach to the patient and the overall prognosis [14,91,123]. The neoplastic dissemination of HNSCC has a quite predictable direction, determined by the lymph drainage pathways. The lymph from every single region drains along relatively constant collectors to only some particular LNs of the total about 300 LNs in the head and neck [53,119]. As a result of extensive anatomic and clinical studies the LNs in the neck are systematized in several clinico-pathologic groups, which have clearly delimitable anatomic borders and collect the lymph from well defined areas of the mucosa [57,90,117]. In this way to every tumor location in the upper aerodigestive tract correspond certain regional lymph nodes that are most likely to harbour metastases from this primary locations. Apart of this lymphatic spread tumoral cells may further propagate from the primary site and the involved LN with the blood circulation to distant locations (usually the lungs, liver or bones) [27,34]. From this background the SCC of the head and neck is not just a tumor in this region, but a multilevel pathologic process, determined by the spread from this primary locus to its specific region, and in advanced disease stages - to distant foci.

The CO_2 laser surgery has become a widely used clinical treatment in otorhinolaryngology. Its major advantages include precise tissue removal with good haemostatic effects and generally very good functional results [58]. In the surgery for laryngeal cancer the trend in the last decades is clearly from the classical open partial laryngeal resections and laryngectomy towards organ-preservation by endoscopic

CO_2 laser surgery or irradiation with curative intent [28,122]. The rich clinical experience from the last few decades has proven the advantages of the CO_2 laser surgery for early stage glottic [16,41,93], supraglottic [15,28], oral and oropharyngeal [58,118] and hypopharyngeal [24,96,105] squamous cell carcinoma.

However in advanced disease it is often difficult and even impossible to expose well the whole tumor through the surgical laryngoscope. In such cases the tumor is usually divided with the CO_2 laser in several parts, which are excised separately [15,106]. This approach was found useful for estimating the depth of tumoral infiltration under the magnification of the operating microscope [2,95]. It is quite controversial whether this piecemeal resection is oncologically acceptable as it seems opposed to the basic principles of oncologic surgery [4,126]. The proponents of the piecemeal resections refer back to two major types of studies. 1. Studies on the interaction between the tumor and tissue (usually healthy one), showing some ability of the laser to seal the lymphatic vessels on the cutting edge [29,36,120]. 2. Nonrandomized clinical studies, showing similar results with endoscopic CO_2 laser piecemeal resection and classical open surgery and no evidence of increased incidence of metastases after the piecemeal resection [15,41,104,105]. However in this inductive logic chain (from some specific observation to a broader generalization) there is a gap, which has not been addressed by any study in the reviewed literature, namely there are no studies, investigating the actual influence of the transtumoral laser cut (piecemeal resection) on the incidence of lymphatic metastases.

2. Study objective

On this background the aim of the present study was to compare the piecemeal laser surgical complete (R0) tumoral resection with cold steel complete (R0) en bloc tumoral resection in an animal model. For the study the VX2 SCC model in New Zealand White rabbits was chosen. This is an established SCC model, characterised by early predominantly lymphatic metastasing along a constant cascade similar to the one of the human HNSCC. Both surgical approaches had to be compared in uniform conditions on the following major aspects:

- Effectiveness for achieving local control and incidence of local recurrences

- Incidence of regional and distant lymphatic metastases

- Incidence of distant visceral metastases

3. Material and method

3.1. Study protocol

The animal use protocol was approved by the Institutional Animal Care Use Committee (IACUC) of the government of Giessen, Germany, protocol number VI 25.3-19 c 20-15 (1) MR 20/3-Nr. 25/2002. The experiments were performed in accordance with the guidelines of the Declaration of Helsinki and in accordance with the Public Health Service Policy of Humane Care and Use of Laboratory Animals [73].

Adult male specific pathogen free Iffa Credo New Zealand White (ICO:NZW) rabbits weighing 3.0±0.6 kg (range 1.9-4.5 kg) were used. The animals were purchased from Behring (Behring Werke, Marburg, Germany). The study protocol is schematically presented on Fig. 1. After randomisation to the both study arms in 143 male New Zealand White rabbits a VX2 squamous cell carcinoma was induced in a uniform way on day 0. Male animals were preferred, because they have relatively less fatty tissue, which diminishes anaesthesia risks and simplifies the neck dissection [42]. On day 8 a complete (R0) resection of the tumour with curative intent was performed: for the first group - with cold steel resection (CSR) ***en bloc***; for the second group the cancer was transected by the CO_2 laser following which it was removed in two pieces - ***piecemeal*** laser-surgical resection (LSR). On day 51 all animals were sacrificed and subjected to evaluation of the tumoral spread. During the whole study period measurements of the tumoral growth and body mass and temperature were performed on a regular base.

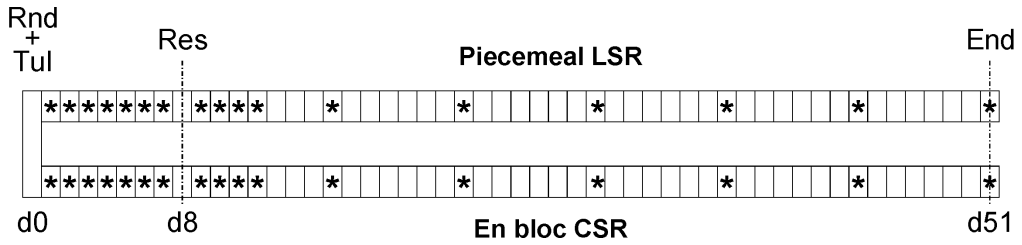

Figure 1. Study protocol: Randomisation (Rnd) and tumor induction (TuI) on day 0, resection (Res) on day 8 and animal sacrifice on day 51. With asterisk the days when the general condition and the signs of the tumoral disease were evaluated.

3.2. Induction of the VX2-carcinoma

3.2.1. VX2 cell suspension preparation

The original VX2 cell line was obtained from Prof. Dr. Robert J.J. van Es (Department of Oral and Maxillofacial Surgery, University Medical Center Utrecht, Utrecht, Niederlande) [111]. The cell line was propagated in-vivo by intramuscular passage in the gluteal musculature of NSW rabbits (Fig. 2a, page 63). Two weeks after implantation the animals were sacrificed. The dissected tumoral mass was processed without delay. During the whole preparation the tumoral mass was cooled in DMEM (Dulbecco's Modified Eagle's Medium, Biochrom AG, Berlin, Deutschland) on dry ice at a temperature of 0-4 C^o. The tumor was cut into several small pieces. Apparently necrotic parts were removed. The viable tumor tissue was further minced with razor blades and stirred through a 300 µm nylon sieve. The mass was again rinsed with cold DMEM. After centrifugation at 3000 r.p.m. for 5 minutes the upper phase was pipetted and removed. The lower phase containing the tumor cell suspension was aliquoted. The number of cells in suspension was counted using a haemocytometer (Improved Neubauer Ruling). Viability of cells was estimated by Trypan blue exclusion after dilution 1:1 (Fig. 2b, page 63). The suspension was standardized at a mean density of 8-10x10^6 cells/ml and filled into 1 cc syringes.

3.2.2. Transplantation

The tumor was transplanted between the central auricular neuro-vascular bundle and the caudal margin on the dorsal side of the auricle (Fig. 2c, page 63). The height of transplantation was chosen at the approximate border between the middle and the distal thirds. The targeted area of the auricle was wetted with warm water. In this way the fur did not impair the visualisation. After introduction of the 0.5 mm needle in a subcutaneous manner a small quantity of air was injected in order to dissect and elevate

the skin from the underlying cartilage and so produce a subcutaneous pocket. In this compartment 0.3 ml of the tumor-cell suspension was injected. Minimal regurgitation of the suspension through the injection point could be observed in some animals, which but did not lead to significant reduction of the injected volume.

3.3. Observation, measurements and documentation

At the beginning of the current study the general condition of the animals was judged by an experienced animal tender. Care was taken to start the experiment only on animals in a good condition, after the initial adaptation to the new milieu. During this period the animals were visited daily and several parameters were noted as recommended by the literature [42]. The body weight was measured with a commercially available scale Multina Plus (Soehnle-Waagen GmbH & Co. KG, Murrhardt, Germany), with „weight-lock“ function that compensates for the movements of the animal and allows precise measurement. The measurements were recorded with an accuracy of 0.01 kg. The rectal temperature was measured with a digital thermometer (TMP Tüshaus Medical Produkte GmbH, Velen-Ramsdorf, Germany) over 4 minutes with an accuracy of 0.1 C^{o}. The measurement of the tumoral mass was done with a calliper with an accuracy of 0.05 mm (Fig. 3a, page 64). The longest diameter and one perpendicular to it were measured. The complete thickness of the tumor and the underlying auricle was measured too. The tumor growth was recorded daily.

The macroscopic aspect of the tumor was described with a grading system, based on the presence on tumoral bleeding, necrosis and crust (Fig. 3bc, page 64). For all these characteristics a 4 level grading system was used (Table 1). For the bleeding: 0 - absence of any signs of bleeding; 1 - minimal traces of coagulated blood on the skin; 2 - capillary bleeding, fresh blood cloths on the skin; 3- important bleeding from a larger vessel, important blood traces in the cage from the night. For the necrosis: 0 - no signs of tumoral necrosis; 1 - the tumor is soft, with signs of fluctuation upon palpation, but the covering skin is intact; 2- skin defect with necrotic tissues visible inside or coming out through the defect; 3- important tumoral necrosis with large skin defect.

For the crust: 0 - no crusts; 1- thin crust gluing the fur; 2 - well-developed crust, covering small area of the tumor; 3- thick crust, covering important surface of the tumor. The neck status was clinically estimated by palpation on a daily basis. The findings were recorded in the following way: 0 - no palpable or suspicious neck masses; 1 - suspicious structure, which could not well be differentiated from the normal surrounding structures; 2 - clearly palpable mass. For larger masses approximate dimensions in centimetres were recorded. Notes were taken for the general condition of each animal, or other particular details not included in the above-described grading system. The quantities of water and food eaten and feces and urines were daily closely followed.

Grade	Tumoral bleeding	Tumoral necrosis	Tumoral crust	Neck LN
0	no bleeding	no signs of necrosis	no crusts	no palpable LN
1	minor blood traces	tumor fluctuation under intact skin	thin crust gluing the fur	suspicious neck masses
2	capillary bleeding, fresh blood cloths	small skin defect	crust on a small surface	clearly palpable LN
3	important bleeding	large necrosis	thick, large crust	size in cm

Table 1. Grading system for semiquantitative assessment of the tumoral and neck status

On the day of the surgical resection the weight and body temperature were measured preoperatively and the size and morphology of the tumor and neck status were examined and recorded with the animal already in anaesthesia.

After the resection the weight, body temperature and the presence of enlarged neck LN were evaluated on a daily basis for 5 days in order to detect postoperative complications from the anaesthesia or the intervention. Afterwards this control was done once a week with last measurements on the day of sacrifice. If recurrences appeared at the resection line their size (largest and perpendicular diameter and thickness) was measured weekly.

All the measurements of the tumoral size (with callipers) and the estimation of the size of the LN metastases by palpation were done by one and the same investigator.

3.4. Resection of the tumors

The resection was carried out on the 8^{-th} day after the inoculation of the tumor.

3.4.1. Anaesthesia

The resection of the tumor was performed with the animals anaesthized with 5mg/kg xylazinhydrochlorid (Rompun 2%, Bayer Vital GmbH, Leverkusen, Germany) and 100mg/kg ketaminhydrochlorid (Ketavet, Pharmacia GmbH, Erlangen, Germany) [42]. The dosis of the medicaments was calculated according to the body weight, measured on the preoperative day. The anaesthetised animals were transported from the cages to the operating theatre in carrying boxes. The depth of the anaesthesia was tested by the standard approach of pressing tightly between the fingers of the lower pad. If the animals reacted to this painful stimulus, complementary medication was given. Only after evidence of deep anaesthesia the intervention was started. In the rare cases, when the animals reacted to pain during the intervention it was stopped, complementary medication was given and the work was continued after 5-10 min. After the intervention the animals were transferred back to the cages and observed for 2 to 3 hours till complete restoration of the voluntary mobility occurred.

3.4.2. Scalpel resection

The cold steel resection was performed as ablatio auris (Fig. 4, page 65). The resection line was placed well proximal to the inferior tumor margin (1.5-2.5 cm). At the beginning the fur at this level was shaved and the skin was cleaned with Softasept N (B. Braun Melsungen, Melsungen, Germany). The medial auricle artery was ligated proximal to the resection line. The auricle was cut with a scalpel no. 22. The bleeding from the small skin vessels was controlled with monopolar coagulation. The skin along the resection line was elevated on both sides to form 2-3 mm wide flaps, which were

pulled and sutured together (4/0 Prolene®, curved PS-2 needle, Ethicon, Nordersted) in order to cover well the cartilage. The wound closure was done predominantly with continuous suture and only few single stitches if appropriate. The skin and the fur of the animal were cleaned with water and consecutively with Softasept N. The sutures were removed on the 7-th postoperative day. The same investigator performed all but two resections. The resected specimens were marked with colour threads/pins. After each resection the instrument set was carefully washed and sterilized in a Statim 5000 Cassette Autoclave (SciCan, Toronto, Ontario M3B 3P9 Canada) to avoid bacterial or tumor cell transfer.

3.4.3. Piecemeal laser-surgical resection

For the laser-surgical resection the operating theatre was labelled with the appropriate warning sign on the door and the access of other people was limited. Inside the surgeon and the assistant wore special protecting glasses. For the laser-surgical resection the animals were anaesthetised in the way, described in chapter 3.4.1. The fur was shaved around the tumour. The skin was cleaned with Softasept N (B. Braun Melsungen, Melsungen, Germany). The medial auricle artery was ligated proximal to the resection line. The operating field was covered with wet cotton compresses to protect the animals from incidental laser injuries (Fig. 5, page 66). The CO_2 laser (Lumenis Compact Series C40 - Lumenis GmbH, Dieburg, Germany) was set to continuous wave mode with power settings of 10 watts utilizing the handpiece delivery system with built-in smoke evacuation. The resection was performed in two stages. The first incision line passed through the middle of the tumor, while the second was placed through healthy surrounding tissues some 5 mm away from the visible border of the tumour. The resected specimens were marked with colour threads/pins. The wound was left uncovered, and healing was by secondary intention (Fig. 10, page 70). Only in 5 animals suturing of the part of the wound was necessary (4/0 Prolene®, curved PS-2 needle, Ethicon, Nordersted, Germany) to achieve complete haemostasis. In these cases the sutures were removed on the 7^{-th} postoperative day. The same investigator performed all but four resections.

3.5. Macroscopic and microscopic pathologic evaluation

3.5.1. Animal sacrifice

The animals were sacrificed on the 42^{-th} postoperative day using an intravenous application of 1 ml T-61 (Intervet Deutschland GmbH, Unterschleißheim), after being anaesthetised with 100mg/kg ketaminhydrochlorid (Ketavet, Pharmacia GmbH, Erlangen, Germany).

3.5.2. Dissection

The dissection was performed in a standard way in all animals. All obtained specimens were put into glass vials, marked carefully with animal number, type of specimen, unique identifier of the specimen and date of sacrifice. The specimens were fixed in 4% neutral-buffered formalin. Of the auricle a 1 cm wide tissue band was cut along the whole resection line and marked with colour threads/pins so the anterior, posterior distal and proximal margins and the lateral and medial surfaces could be clearly identified.

For evaluation of the extent of metastatic spread to the regional LNs the ipsilateral neck side was dissected with the animal lying in lateral position. The region was accessed after a hockey-stick skin incision, starting from nuchal and extending along the back and turning right and anteriorly at the level of the scapula. The skin flap was dissected along the superficial fascia and elevated in a rostral direction, so the whole lateral and anterior parts of the neck could be visualised. The jugular vein presented a major landmark as the LN-groups are situated along it (Fig. 6, page 67). The parotideal LNs lie under the parotid fascia along one of its branches - the caudal auricular vein. The caudal mandibular lymph nodes are located between the superficial and the deep layers of the cervical fascia at the bifurcation of the jugular vein between the linguofacial vein and the maxillary veins. The rostral mandibular lymph nodes are located at the

bifurcation of the linguofacial vein close to the posterior pole of the mandibular gland. The dissection was started with opening of the superficial layer of the cervical fascia. First the caudal mandibular LN group was dissected, followed by the rostral one. Last the parotideal LNs were dissected. This sequence was found very useful as bleeding from occasionally opened venous vessels in the parotid region complicates the dissection in the other two regions. On the contrary, bleeding from the perijugular regions does not disturb the work around the parotideal LN group. This approach was found superior to previously described ones [21]. In cases when the LN could not be identified macroscopically, the whole complex of soft tissues around the typical localisations was excised as a single block and placed in 4% neutral-buffered formalin for further serial sectioning. Often after several days in this solution the changes in the tissue consistence allowed macroscopic identification of the LN – the fatty tissue remained white and softer, while the LN became darker and palpable.

In the next step the presence of distant metastases was evaluated with the animal in supine position. The medial skin incision started below the mandible and extended to the pelvis. After preparation of the skin flaps the ribs were cut 1-2 cm laterally to the sternum with sharp scissors, starting from the superior thoracic aperture. After removal of this sternocostal block the larynx was freed from the pharynx and excised. With a gentle pull and sharp dissection the trachea was separated from the oesophagus in a caudal direction. It served as a handle for the extirpation of the attached lungs and heart. The lungs were separated, sliced in 3-4 mm thick slices, examined for macroscopic changes and put into marked vials. The metastases were counted and the diameters of the largest and smallest measured and recorded. The trachea was opened along the posterior wall and examined for metastases. Followed section of the diaphragm and preparation of the liver. It was sliced into 1 cm thick slices and examined for macroscopic changes. The whole thoracic and abdominal cavities were examined for metastases.

The described sectioning technique allowed clean preparation of the regions of interest and macroscopic evaluation of the regional and distant spread of the tumoral disease.

3.5.3. Histological preparation and evaluation

After fixation in formalin for at least 48 hours, the specimens were further processed and embedded in paraffin for histological preparation. Of the majority of specimens (main tumor, resection line at sacrifice, lungs and liver one hemalaun and eosin (H&E) stained slide was made per block. Of the complete ND-specimens serial sections were prepared. The slides were cut on a sliding microtome at 100 µm. The slides were examined on conventional light microscope. All specimens were checked for signs of tumoral invasion. For the ND-blocks the total number of studied and metastatically involved LN was noted. In cases of uncertainty complementary slides were prepared from the initially preserved specimen or the paraffin blocks.

3.6. Data processing and analysis

3.6.1. Data processing and analysis

For the initial planning of the experiment timetable MS-Excel was used. For each animal a printed form was prepared and labelled with the appropriate number (Appendix page 75). On this form all parameters from the observation and measurements together with details on the surgical intervention, as well as the findings of the sectioning were recorded in an uniform way. The data from these forms were entered in a specially created databases under Epi Info 6.04d (Centers for Disease Control and Prevention, Epidemiology Programm Office, Division of Public Health Surveillance and informatics, Atlanta, U.S.A.). For the analysis the separate databases were merged with the animal number used as a unique identifier. For the statistical analysis along with Epi Info SPSS for Windows v. 11.5 (SPSS Inc., Chicago, Illinois) was used. Categorical data (frequencies) were analysed using crosstabulation and chi-squared test for independence. In cases of small samples Fisher‘s exact test was used. For comparison of nominal-level variables between groups unpaired t-test was used. For

statistical significance two-tailed test was used. Probability levels less than .05 were considered statistically significant. Relationships were analysed with Pearson product moment correlation coefficient [69].

3.6.2. Exclusion criteria

Four of the 143 animals were not included in further analysis. Three of them were lost intra- or immediately postoperatively because of anaesthesia problems. In the last animal the implanted tumor did gradually regress.

	CSR	LSR
Started animals	73	70
Spontaneous tumor regression	1	0
Intra- perioperative deaths	2	1
Animals included in the main analysis	70	69

Table 2. – Animals excluded from the study

4. Results

From the total of 143 animals at the beginning of the experiment one was excluded because of clinical regression of the tumor and 3 were lost on intra- an early postoperative complications related to the anaesthesia. One hundred thirty-nine animals were included in the final analysis. The mean follow up was 5.85±0.61 weeks (range 2-6). Eleven animals died before the planned timepoint (51^{-st} day). In nine of them the death was related to the main disease – tumor progression on day 36.8±8.2 (23-50) after the tumor induction. The rest two died of pneumonia on days 38 and 42. Of the tumor related deaths 3/9 were in animals with local recurrence, in 9/9 with LN involvement and in 8/9 with distant metastases. There was a high negative correlation between the number of lung metastases and the survival ($r=-0.89$ $p<0.05$). The survival did not correlate with the maximal size of the LN metastases.

4.1. Tumor growth

4.1.1. Success of transplantation

The tumor take rate was 99.3% - a figure that significantly differs from the results of other research groups – 78%-83% [23,111]. Only one animal was excluded from the study because of unsuccessful tumor transplantation. In this animal the tumor had an atypical aspect and its size gradually diminished starting from the 4^{th} day. Under the skin, which regained its normal colour there was still a small hard palpable mass, which resembled scar fibrosis. The histological examination showed resorbtive changes and fibrosis at the site of initial inoculation. Histologically in the centre of the mass 2 regions with vital tumoral cells were found. Despite the presence of potentially proliferating tumor the animal was not included in the further analysis because of the small and atypical primary tumor, which was incomparable to the tumors in the other study animals. In 33/139 animals (23.7%) tumor size reduction in 2 diameters was observed between the second and the third days. The shrinkage in the longitudinal

diameter was 0.73±0.58 (range 0.05-2.5 mm) and in the transversal one 0.52±0.67 mm (range 0.05-3.20 mm). This is to be explained with the drainage of some of the VX2 tumor cell suspension through the injection channel, with partial resorbtion of its liquid fraction and decrease in the traumatic oedema caused by the manipulation. Nevertheless at the moment of tumoral resection in all animals the tumor had viable aspect with all dimensions increased, i.e. apart of the one excluded animal in no other signs of spontaneous tumoral regression were observed.

4.1.2. Tumor growth

For comparison of the tumor growth in both study groups two types of variables were used - absolute dimensions of the tumor and increase of the tumor mass.

The initial tumor dimension were slightly bigger in the LSR group compared to the CSR group for the longitudinal diameter with 8.3% (mean 0.8 mm), for the perpendicular diameter with 18.9% (mean 1.4 mm) and for the thickness with 24.2% (mean 0.8 mm). These differences appeared statistically significant.

	CSR	**LSR**
Tumor length [mm]	9.6±1.9	10.4±1.9
Tumor width [mm]	7.4±1.8	8.8±1.5
Tumor thickness [mm]	3.3±0.6	4.1±0.9

Table 3. Initial dimensions of the tumor for both the therapeutic groups.

At the time of resection the maximal dimensions of the tumors in the LSR group showed to be bigger than those of the CSR for the longitudinal diameter with 12.7% (mean 2.3 mm), for the perpendicular diameter with 8.3% (mean 1.1 mm) and for the thickness with 17.2% (mean 1.2 mm). These differences appeared statistically significant.

	CSR	LSR
Tumor length [mm]	18.1±4.3	20.4±4.3
Tumor width [mm]	13.2±3.1	14.3±2.4
Tumor thickness [mm]	7.0±2.5	8.3±2.1

Table 4. Maximal dimensions of the tumor for the both therapeutic groups.

The reported differences should be attributed mainly to the technique of tumor induction. Though in all animals 0.3 cc standardized on the cell count suspension was injected there are other important mechanical factors that influence the procedure. A relatively long subcutaneous needle tunnel is indispensable for the successful injection, because it provides tight sealing around the needle for the pneumatic dissection. After the needle withdrawal this tunnel allows some regurgitation of cell suspension and so gets contaminated, which results in complementary tumor growth. The cell suspension itself though standardized for the cell count could differ in its viscosity and so influences the dissection in the subcutaneous plane and the regurgitation. The above differences could be induced or influenced by these mechanical factors.

The tumor growth, which is a better measure of the tumoral viability and aggressiveness, than the absolute dimensions, was estimated by the percentage increase of the tumoral dimensions.

	CSR	LSR
Tumor length increase [%]	192.3±53.8	199.1±46.0
Tumor width increase [%]	184.7±56.4	164.8±29.7
Tumor thickness increase [%]	217.8±80.7	204.8±46.4

Table 5. Percentage increase of the dimensions of the tumor for the both therapeutic groups.

The percentage increase of the tumor dimensions was compared for the both study groups and the results showed to be homogenous. The increase in the longitudinal

diameter was bigger in the LSR group than in the CSR with 3.5% without being statistically significant. On the contrary the increase of the perpendicular diameter and the thickness were bigger in the CSR group with 12.1% ($p<0.05$) and 6.3% (n.s.) respectively.

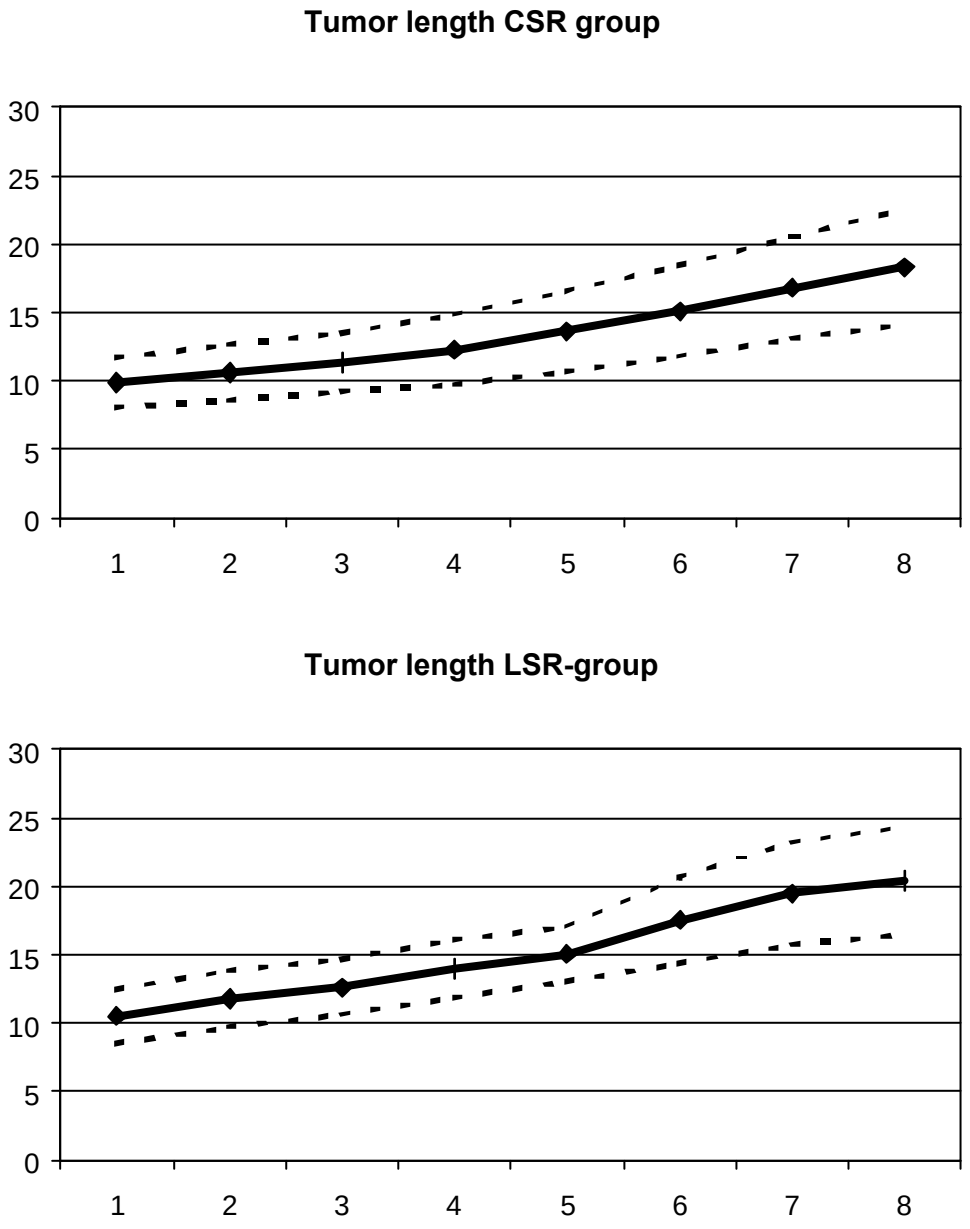

Figure 7a. .Tumor growth over the 8 days after tumor implantation, measured by the longitudinal diameter of the tumor in mm for both therapy groups (mean ±SD)

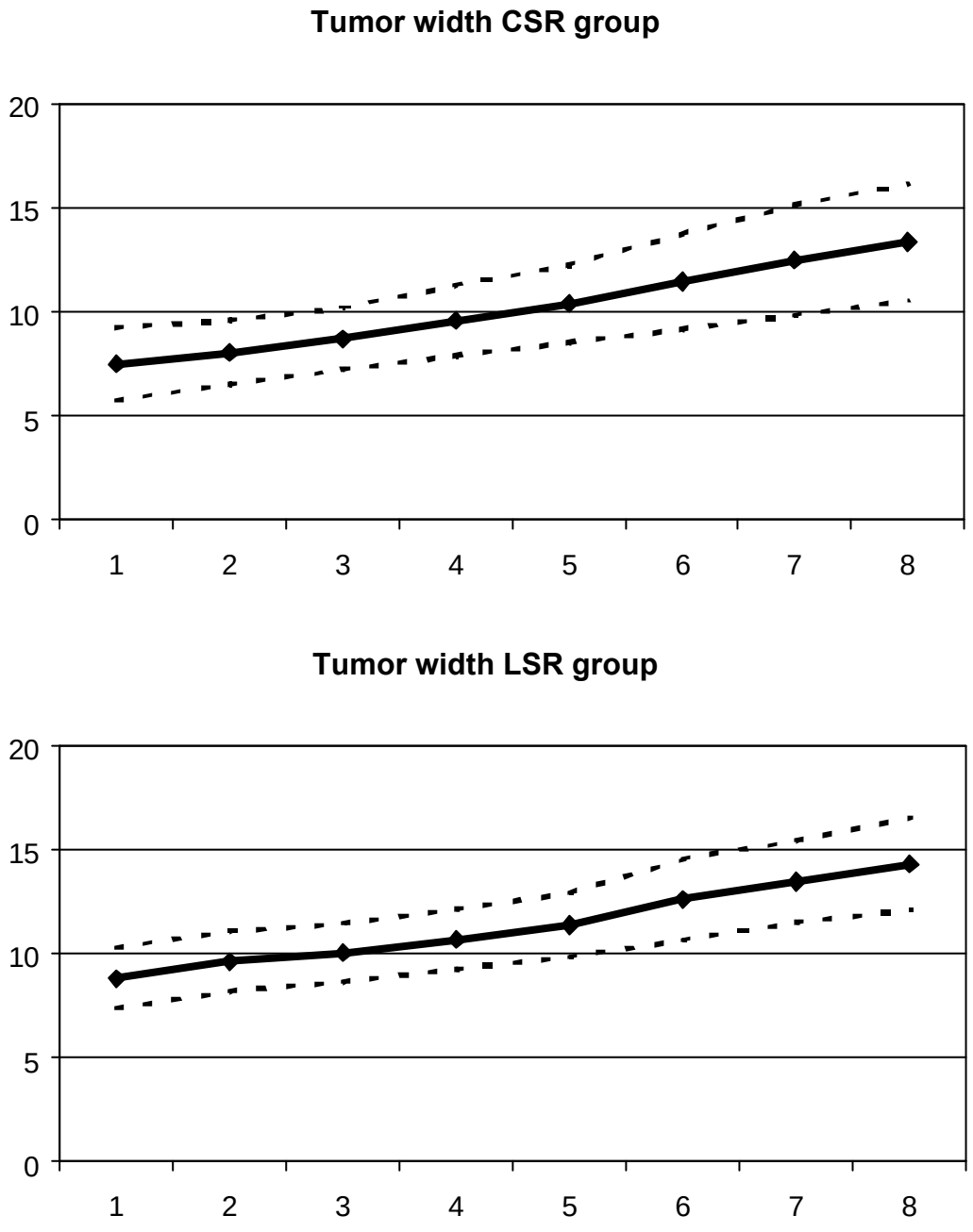

Figure 7b. Tumor growth over the 8 days after tumor implantation, measured by the transversal diameter of the tumor in mm for both therapy groups (mean ±SD)

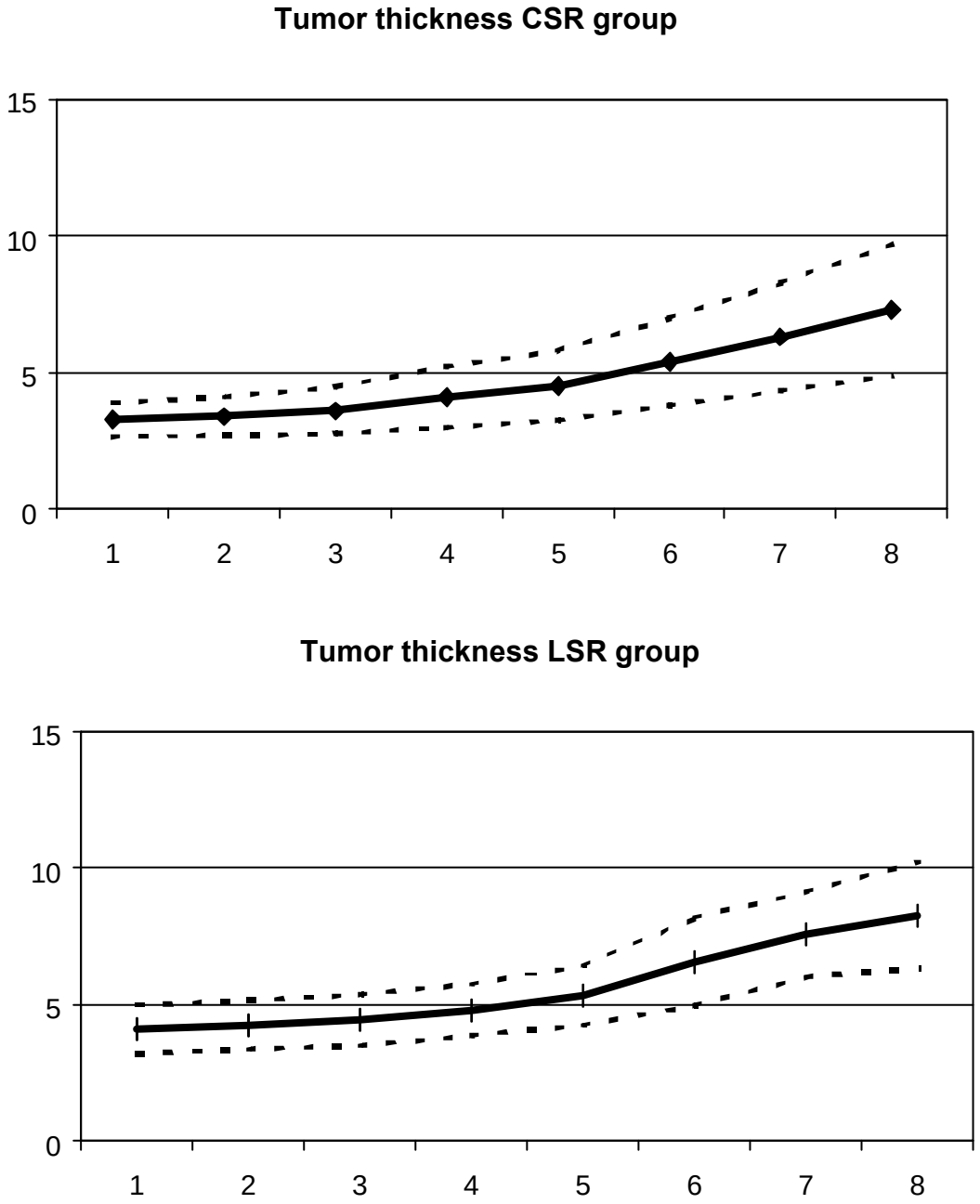

Figure 7c. Tumor growth over the 8 days after tumor implantation, measured by the tumor thickness in mm for both therapy groups (mean ±SD)

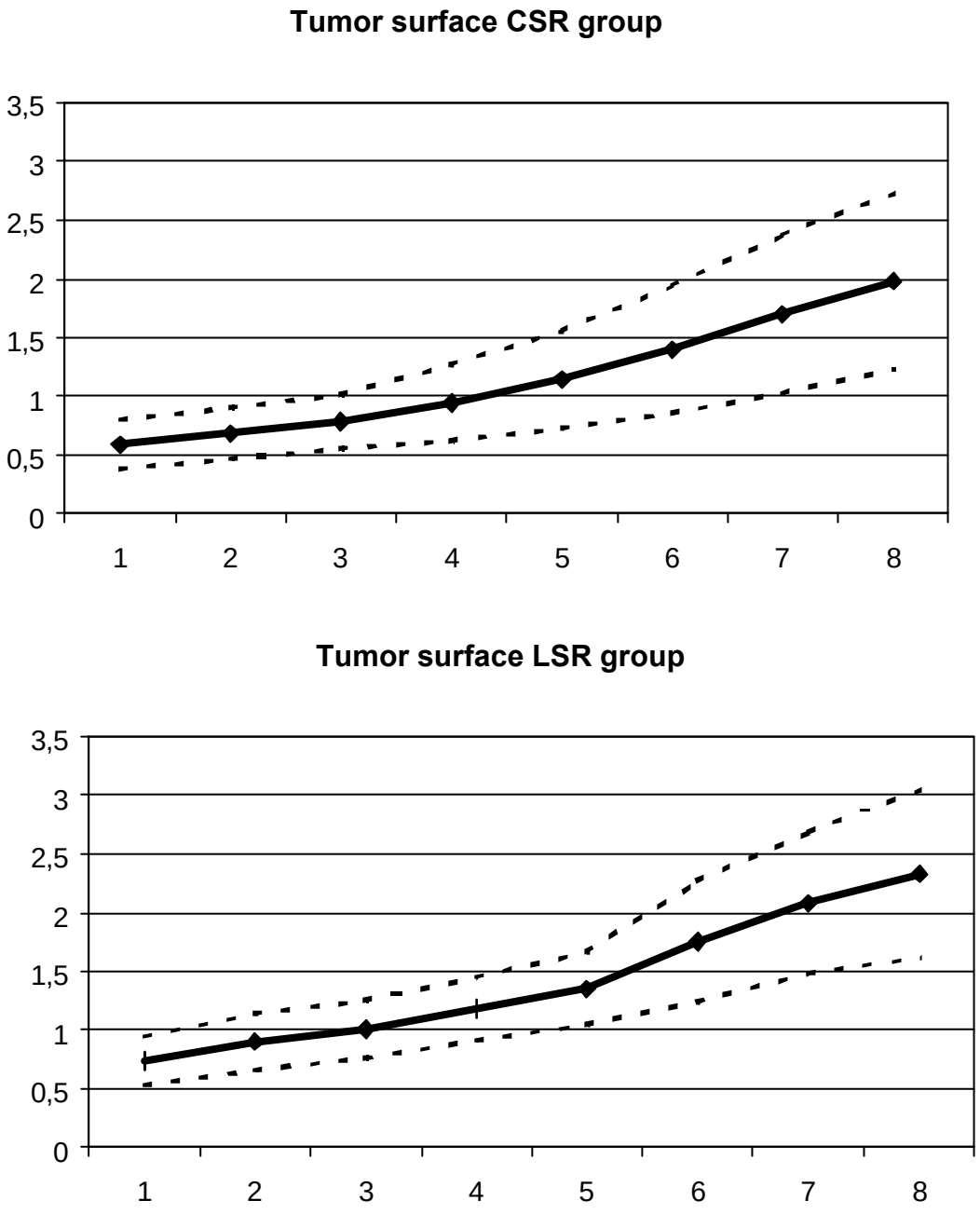

Figure 7d. Tumor growth over the 8 days after tumor implantation, measured by the tumor base surface in cm^2 for both therapy groups (mean ±SD)

With tumor progression partial necrosis and bleeding could be observed. Tumoral necrosis was observed in 59 animals (24 from the CSR and 33 from the LSR groups). Fifty animals had tumor necrosis degree 1 (softening/fluctuation) and 7 had degree 2 (minimal skin defect) as defined in Table 1 on page 10. The necrosis was detected on the 8±1 day (7.8±1.2 for the CSR and 8.2±0.8 for the LSR groups).

Only tumoral bleeding after the 4^{-th} day was regarded as tumor related. Sings of bleeding before this timepoint were attributed to the trauma during inoculation of the cell suspension. Tumoral bleeding was observed in 9 animals (6 from the LSR and 2 from the CSR groups). In all of these cases this was minimal bleeding grade 1 (Table 1, page 10). The bleeding appeared on day 7.4±0.7 (6.7±0.6 for the CSR and 7.8±0.4 for the LSR groups). The earlier appearance of the bleeding in the CSR group was the only parameter that significantly differed between the both groups. Nevertheless the number of animals included in this analysis is too small - only 8.

Of the animals that later developed recurrences at the resection site 3/9 (33.3%) had initially necrosis of the primary tumor grade 1 and 1/9 (11.1%) tumoral bleeding grade 1. Of the animal with local control 54/130 (41.5%) had tumoral necrosis – 47 cases with grade 1 and 7 cases with grade 2. Eight animals of this subgroup of 130 with local control (6.2%) had grade 1 tumor related bleeding.

The tumor recurrences presented with a different macroscopic aspect. While the main tumor could be approximated to a split half spheroid the recurrences were thicker and more rounded – approximation to a whole spheroid. This could be attributed to the way of initiation and the different location. The initial tumors were injected in an artificial pouch with a slit-like form between the mechanically quite resistant cartilage and tense skin, where as the recurrences developed from one centre like a sphere in the proximal part of the auricle, where the soft subcutaneous tissue is much thicker and offers more space for tumor growth.

4.1.3. Histological findings

4.1.3.1. Tumor morphology

The VX2 SCC presents as a solid tumor with characteristic intratumoral patterns and interaction with the surrounding tissue (Fig. 8, page 68). Usually with growth of the

tumoral mass a clear delineation between an active periphery and the centre becomes evident. The centre often shows necrotic changes. Vital tumor cells are usually concentrated around the blood vessels. Intratumoral blood vessels are few if any. In tumors without necrotic changes rarely vessels without erythrocytes and with extremely thin walls comprising only one layer of endothelial cells could be observed - most probably lymphatics. On the contrary the periphery is active and proliferates in the surrounding tissues. Here the active vital tumoral tissue could be surrounded by lymphohistiocytic infiltrate. The perichondrium and the cartilage seem to present a relatively resistant barrier for the tumor growth as their infiltration could occur usually at later stages [111]. On the other hand the tumor infiltrates readily in the overlying epithelium. In the subcutaneous tissue the tumor gives small emboli consisting of multiple well-adhered together cells. These emboli could be found in the lymphatic as well as in the blood vessels (Fig. 8, page 68).

The VX2 carcinoma used in our rabbit model is a typical squamous cell carcinoma with a very low differentiation and anaplastic appearance. The cells are highly pleomorphic. They are polygonal, contain large cytoplasm and enlarged nuclei. The cell borders are usually well delineated and sharp, the nucleus is centrally located. Nuclear enlargement and pleomorphism is pronounced. The nuclear chromatin is markedly hyperchromatic, with a coarsely granular pattern and marked chromatin clumping. Mitotic figures are commonly observed. The prominent nucleoli and the mitotic figures if evident, prove the low differentiation of the tumor.

4.1.3.2. Resection related aspects

Both resection tools used (scalpel and CO_2 laser) produced clearly distinguishable tissue changes. The scalpel resection results in a sharp cut through all tissue layers of the auricle (Fig. 9, page 69). At the moment of sacrifice complete wound healing was observed with fibrosis and covered by epithelium.

The CO_2 laser resection with 10 watts power setting in continuous wave mode results in narrow necrotic zone with minor burn changes. Occasional carbonisation particles could

be found. On the resection surface the soft tissues were covered with a thin coagulation layer that was amorphous. No distinct structures (tissues, cells or matrix) could be identified in this layer. All structures were welded in a homogenous mass. This layer was not present on the cartilage border. Here coagulation was visible only in the perichondrium. In the tumoral tissue similar changes were observed. In some necrotic areas the coagulation layer looked thinner or was partially absent.

At the moment of sacrifice complete wound healing was observed with fibrosis that was covered by epithelium. The fibrous scar was most marked on the curve between the transversal and longitudinal parts of the resection line. Its contraction in the process of maturing of the fibrosis led in some cases to minor deformation of the rest auricle (Fig. 10b, page 70).

4.2. Lymph node metastases

4.2.1. Incidence of lymph node metastases

Of the 139 animals, which entered the second part of the protocol, in 56 (40.3%) LN metastases were present (Fig. 6, page 67). In 56/56 there was metastatic involvement of the first echelon parotid LN (level I). Five animals had two clearly delimited tumoral masses in the parotideal region, each with thick capsule. In 10/56 animals (17.9%) further LNs were involved too. In 9/56 (16.1%) LNs from the caudal mandibular group (level II) and in 1/56 (1.8%) LN from the rostral mandibular group (level III) were involved. Five animals had involvement of more than one LN in level II: in four animals there were 2 and in one animal 3 LN metastases in level II.

All animals with metastases in levels II and III had also metastases in level I. No skip metastases from the tumor to levels II and III were observed. This comes to underline the importance of the first echelon parotideal LN. In 100% of the cases the initial

lymphogenic metastatic spread involves it (Fig. 11).

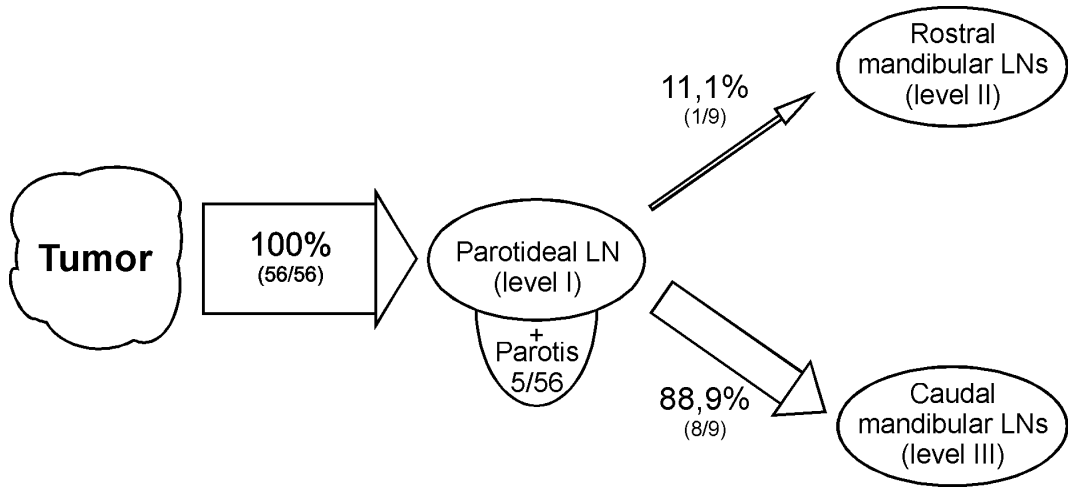

Figure 11. Pathways for the lymphogenic metastatic spread. The width of the arrows is proportional to the relative incidence.

All animals with recurrences at the primary site had LN metastases. Of them in 9/9 (100%) metastatic involvement of the first echelon parotid LN (level I) and in 3/9 (33.3%) of a second LN from the caudal mandibular group (level II) was present. No metastatic involvement of the LNs from the rostral mandibular group (level III) was observed in any of the animals with local tumoral recurrence.

Of the 130 animals, which were proven to be tumor free at the primary site after the resection (complete local control), in 47 (36.2%) LN metastases were present (Fig. 12). In 47/47 there was metastatic involvement of the first echelon parotid LN (level I). In 7/47 (14.9%) further LNs were involved too. In 6/47 (12.8%) LNs from the caudal mandibular group (level II) and in 1/47 (2.1%) LNs from the rostral mandibular group (level III) were involved. Of the 9 animals with local recurrence of the tumor in 9 (100%) LN metastases were present. In 9/9 there was metastatic involvement of the first echelon parotid LN (level I). In 3/9 (33.3%) further LNs were involved to - from the caudal mandibular group (level II).

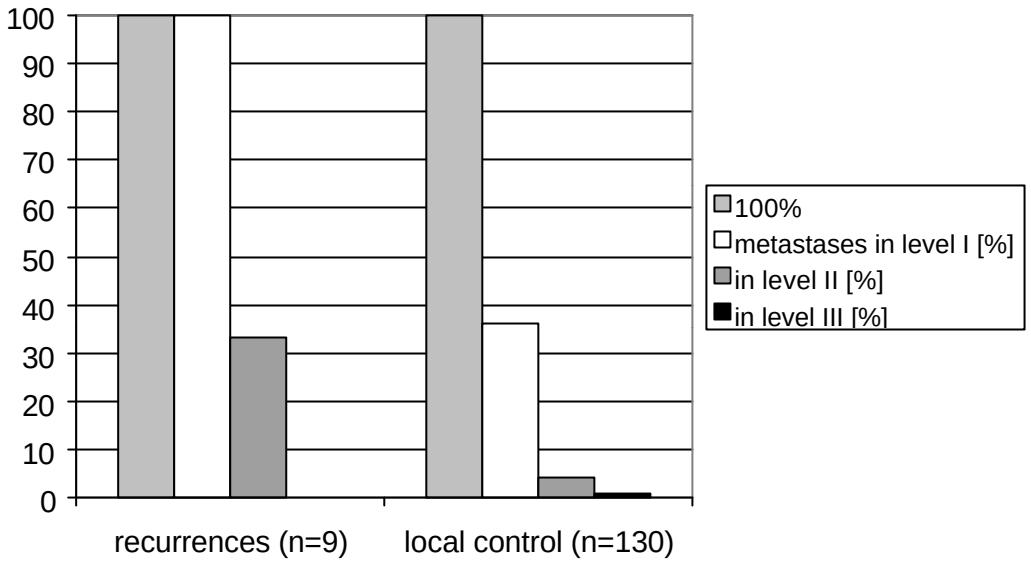

Figure 12. Incidence of LN metastases at levels I, II and III for the animals with local recurrence of the tumor and those with complete local control achieved after the resection, presented as percentage of the total number of the animals in each group.

Compared on the incidence of LN metastases the two therapeutic groups showed significant differences (Fig. 13). Of the animals with en bloc cold steel ablatio in 16/65 (24.6%) metastases to regional LNs were found. In all cases involvement of the first echelon parotideal LN was observed (level I). Two of 65 animals had involvement of LNs from further levels – one of level II (the caudal mandibular group) and one of level III (the rostral mandibular group). Of the animals with piecemeal laser-surgical resection in 31/65 (47.76%) metastases to regional LNs were found. Again in all cases involvement of the first echelon parotideal LN was observed (level I). Five of 65 animals had involvement of LNs from further levels all of level II. The Pearson's Chi-Square test of independence showed that the piecemeal LSR tended to lead to more LN metastases in the echelon parotideal LN (31/65 or 47.7%) as compared to the en bloc cold-steel ablatio (16/65 or 24.6%) p=0.01. This difference appears statistically significant. Similar tendency was observed for the metastases to other neck levels 7.8% for the LSR vs. 3.1% for the CSR group without being statistically significant.

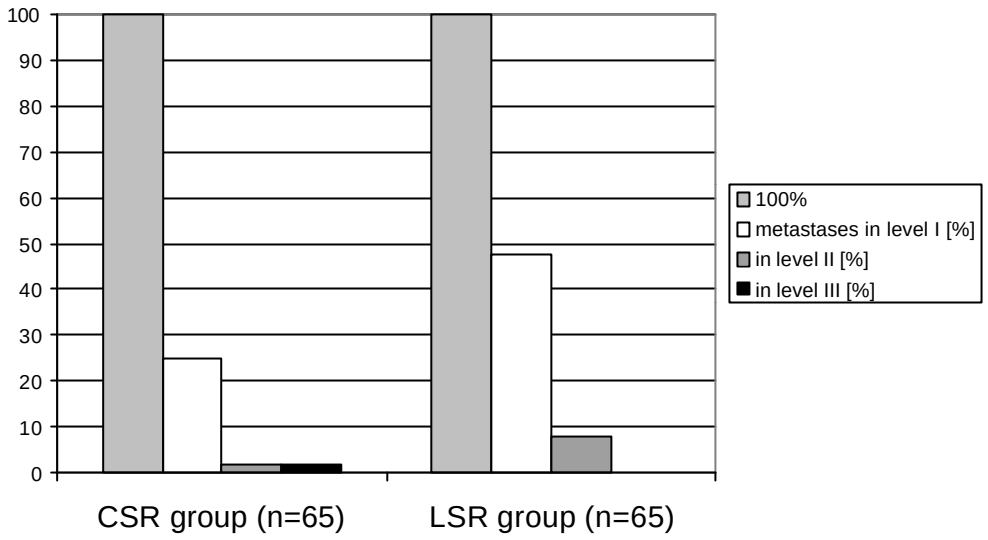

Figure 13. Incidence of LN metastases at levels I, II and III for the cold steel resection group and laser-surgical resection group, presented as percentage of the total number of the animals in each group. Only animals with complete local tumoral control are included.

4.2.2. Pathological evaluation

The parotideal LN metastases had mean maximal diameter of 36.3±16 mm and minimal 25.6±11.7 mm. The LN metastases to other neck levels had a mean maximal diameter of 10.4±3.2 mm, and minimal one 5.7±2.1 mm. The largest dimension of a LN metastasis was 78 mm, the smallest – 3 mm. The metastases presented with a thick fibrous capsule. They were usually firmly connected to the underlying neck structures. In five animals in the parotideal region two tumoral masses were found, each with thick capsule. Though wrapped closely together by the fascia and tumor induced fibrosis they were clearly delimited by own fibrous capsules without signs of tumoral infiltration between them. The LN metastases in the parotideal region lied in a close intimacy with the large collector vein of the auricle - the caudal auricular vein and had an important common contact surface. Nevertheless there were no macroscopic signs of bleeding

from vessel erosion. When sectioned the metastases were filled with caseous material sometimes with partial colliquation.

A total number of 1985 serial histologic cuts revealed 674 LN (Table 1). The mean yield of LN per animal (one neck side) was 4.13±1.37. In all levels I (parotideal region) 152 LN were studied (mean 1.09±0.37, range 0-2). In 15/139 (10.8%) animals in this region 2 LN were found. In all levels II (caudal mandibular region) 209 LN were studied (mean 1.5±0.74, range 0-3). In all levels III (rostral mandibular region) 213 LN were studied (mean 1.53±0.78, range 0-3). The respective incidence of metastases was 59/152 LN in level I plus 5 cases of double LN metastases or a complementary metastasis in the parotis, 15/209 LN in level II and 1/213 LN in level III.

	Parotideal region	**Caudal mandibular LNs**	**Rostral mandibular LNs**	**Total**
Number of LN examined	152	209	213	574
Mean yield pro animal	1.09±0.37	1.5±0.74	1.53±0.78	4.13±1.37
Total number of metastatic LN	61	15	1	77
Metastatic LNs as % from all LNs examined	42.1%	7.2%	0.47%	13.4%

Table 6. Distribution of the LN findings to the three regions of interest (parotideal, rostral and caudal mandibular) for the whole cohort.

4.2.3. Histologic findings

The first gross impression of the metastatic LN is the absence of the typical LN structures. The normal LN has a central hilar part with blood vessels followed by a medullar zone with sinuses and medullar cords all filled with lymphocytes (Fig. 14a, page 71). In the periphery is the cortex of the lymph node. Between the medullar and the cortical zones lies amorphic paracortex. The cortical zone contains the lymphatic nodules. They are characterised by a relatively eosinophilic germinal centre and basophilic mantle zone (towards the hilus) and a cap (towards the periphery). Under

the fine capsule that surrounds the whole LN a narrow subcapsular sinus could be observed.

The histologic picture of the LN metastases is characterised by the absence of all above-mentioned structures. Instead the metastases show several concentric zones (Fig 14b, page 71). The central one is usually necrotic. It is characterised by the presence of abundant granular eosinophilic debris. This is a sign, that the tumor has outgrown its blood supply. Larger metastases often have central cavitation. The zone of active tumoral growth is towards the periphery (or seldom around intratumoral blood vessels). Here strands and nests of vital tumoral cells are observed. The third concentric zone is the LN capsule. A common phenomenon is its thickening. This is due to the diffusion of toxic substances from the metastasis to the surrounding tissues. These substances elicit desmoplastic reaction, characterised by excessive growth of dense fibrous tissue.

Some of the enlarged LN, detected by palpation or at dissection were found tumor free. The histological changes observed were mainly due to reactive processes. In sinus histiocytosis the sinuses are dilated, palely staining and are separated by thin projections of darker staining lymphoid tissue. The activated histiocytes are characterised by their irregular cell form with projecting cytoplasmatic processes and a nucleus with overall vesicular appearance, due to the peripheral clumping of the dispersed chromatin. The cytoplasm may show intense eosinophilia. In cases when in the draining area of a LN lie necrotic tissues rich with lipoid materials, foamy macrophages may be observed (Fig 14d, page 71).

Another type of LN enlargement observed presented the toxic inflammatory changes. The toxic substances produced by the tumor and the tissue necrosis induce local inflammatory process that involves the LNs in the draining area. In such activated LNs the normal structure may be substituted by a diffuse cellular process or by fibrosis. The cellular infiltration is patchy. The medullar and cortical zones and the follicles are replaced by sheets and clusters of large pale cells. Some islands of surviving lymphoid tissue may still be present. Small foci of histiocytic giant cells or necrosis could be

observed too. Important argument for the absence of direct tumoral invasion of the LN is the absence of cellular atypia in such infiltrates (Fig 14c, page 71).

4.3. Incidence of recurrences

Of the 139 animals that survived the anaesthesia and the surgical intervention in 9 (6.5%) local tumoral recurrences developed. In eight cases this was 1 tumoral mass and in one – multiple masses. The tumor recurred at the resection line in 5 (7.1%) animals of the CSR group and in 4 (5.8%) from the piecemeal LSR group (Fig. 16). The intergroup difference is not statistically significant. At the point of sacrifice the tumoral masses had mean longitudinal diameter of 30.2±17.5 mm and transversal one 19.3±10.3 mm. The recurrent tumor if presented as surface area [111] was 6.5±5.6 cm^2 large. The recurrences became clinically evident during the second postoperative week in 5/9 (55.6 %), the third in 3/9 (33.3%) and the fourth week in 1/9 (11.1%) animals. Such a late appearance of recurrences shows, that the VX2 tumoral cells could survive for an important time in the host, before they start to proliferate.

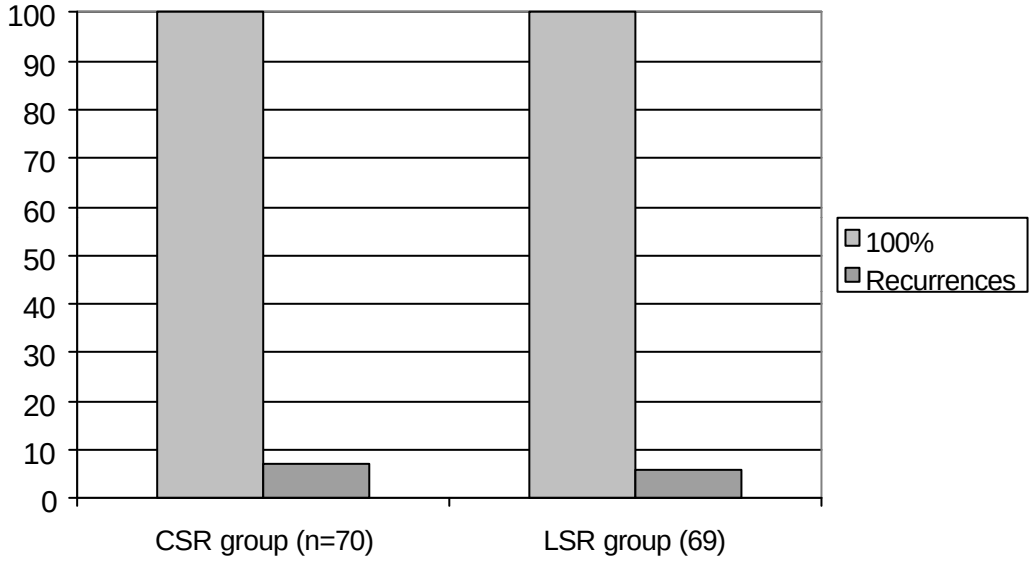

Figure 16. Incidence of local tumor recurrences for the cold steel resection group and laser-surgical resection group, presented as percentage of the total number of the animals in each group.

4.4. Distant metastases

4.4.1. Incidence of distant metastases

At section the abdominal and thoracic cavities and organs were examined for the presence of macroscopic metastases. In no case distant metastases were observed at other locations than lungs. In one animal tumoral mass was found on the parietal pleura, but it was obvious contact involvement by a pulmonary one. That is why in this chapter only lung metastases are reported.

Of the 139 animals, which entered the second part of the protocol in 19 (13.6%) lung metastases were present. In 3/19 the metastases were unilateral and in 16/19 – bilateral. Of the cases with unilateral lung metastases in two the ipsilateral to the tumor and in one the contralateral lung were involved. Animals with tumoral recurrence had significantly higher incidence of lung metastases (6/9, 66.7%) than those with local control (13/130, 10%), $p<0.001$ (Fig. 17).

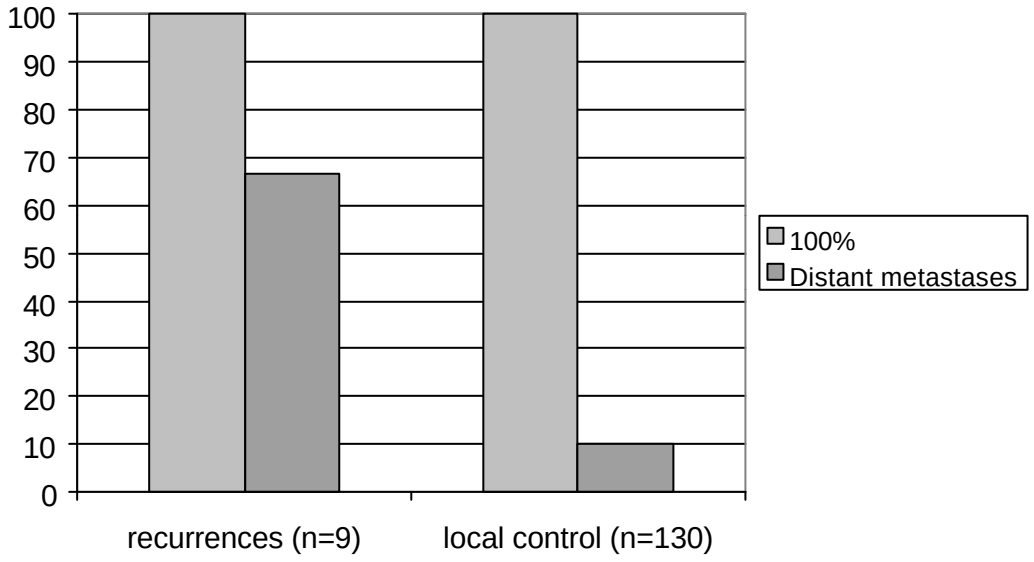

Figure 17. Incidence of distant metastases for animals with and without local tumoral recurrence, presented as percentage of the total number of the animals in each group.

All animals with lung metastases had also neck LN metastases. In 19/19 there was involvement of the first echelon parotideal LN and in 5/19 complementary LN from the caudal mandibular group were involved. Pathologically the metastases were predominantly multiple.

Animals that had no local tumor recurrence and only regional LN metastases had significantly higher incidence of lung metastases (13/47, 27.7%) than animals without regional tumor disease (0/83, 0%) $p<0.001$ (Fig. 18). No cases with skip metastases were observed.

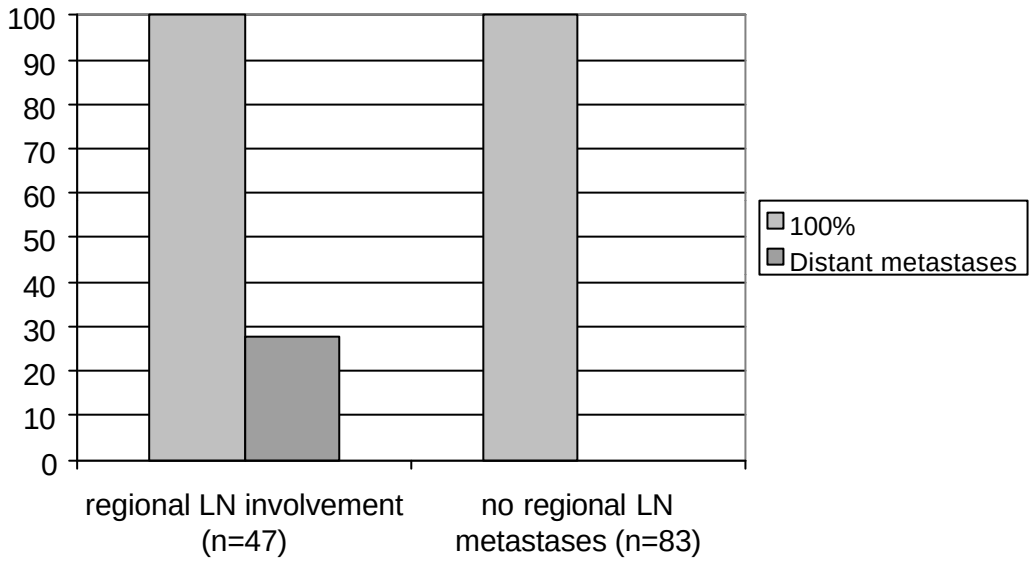

Figure 18. Incidence of distant (pulmonary) metastases for animals with neck metastases, which are tumorfree at the primary localisation, presented as percentage of the total number of the animals in each group.

Of the 130 animals with complete local control thirteen (10%) had lung metastases. The incidence for both treatment groups (CSR and piecemeal LSR) was similar - 8/65 (12.3%) for the CSR and 5/65 (7.7%) for the LSR (Fig. 19).

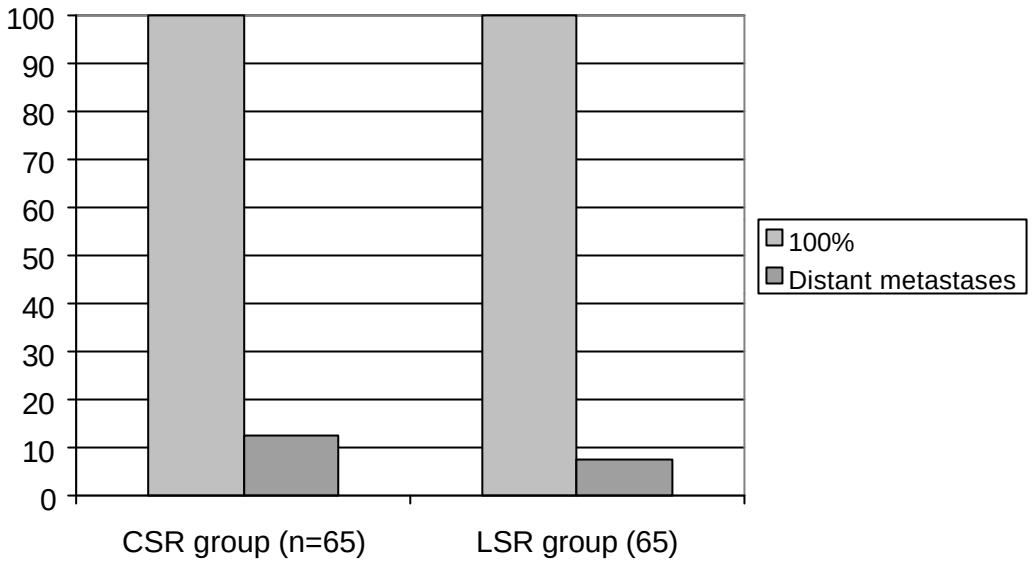

Figure 19. Incidence of distant (pulmonary) metastases for animals with local control for the cold steel resection group and laser-surgical resection group, presented as percentage of the total number of the animals in each group.

4.4.2. Pathological evaluation

Apart of the general incidence, another interesting aspect of the metastaftic spread to the lungs presents the morphology of the metastases – their count and size (Fig. 20, page 73). The mean number of unique seedings pro animal varied in a very large range from 1 to 172 (in both lungs) with a mean value of 52.2±60.8. The total number of lung metastases didn't differ significantly between the group with tumor recurrence (34.83±67.5) and for the group with local control (60.8±58.4). The animals that underwent cold steel resection had significantly less metastatic nests (15.1±18.9) versus 110.4±58.4 for those subjected to transtumoral laser-surgical resection ($p<0.001$). In the animals with local control at the primary site, those from the CSR group had again significantly smaller number of unique seedings in the lungs (18.7±23.1) compared to the LSR group (119.8±33.4 $p<0.01$).

The macroscopic aspect of the lung metastases was quantitatively evaluated by the diameters of the largest and smallest ones for every animal. The largest lung metastasis

had maximal diameter of 35 mm, and the smallest one – 4 mm. The minimal and maximal diameters of the lung metastases in animals with local control were comparable for the both therapy groups.

	CSR	LSR
Maximal diameter of unique lung metastasis [mm]	21.00±10.1	16.6±9.2
Minimal diameter of unique lung metastasis [mm]	9.3±4.8	5.4±3.9

Table 7. Maximal and minimal diameters of the lung metastases in the both therapeutic groups. Only animals with local control are included. The minimal and maximal diameters are measured from different metastases.

Three patterns of metastatic involvement of the lungs were identified (Fig. 21). Type A is characterised by the presence of single or few large metastases with few small ones and large areas of uninvolved lung tissue. In type B the lungs were filled with multiple small metastases, so sometimes normal parenchyma could hardly be found. Type C (mixed pattern) is characterised by the domination of single or few large metastases over fewer metastases of comparatively small size evenly distributed all over the lungs.

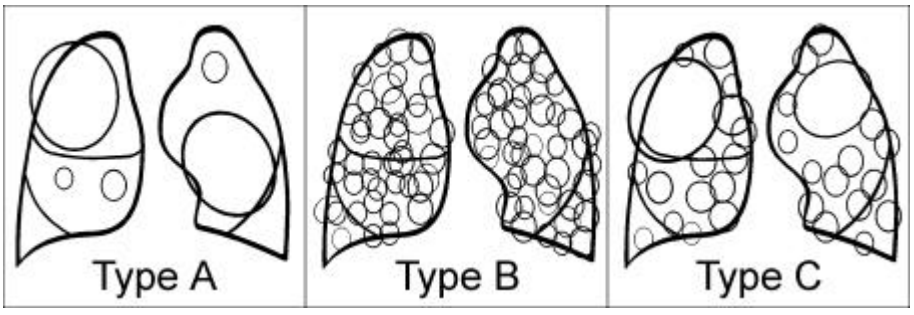

Figure 21. Macroscopic patterns of metastatic involvement of the lungs.

The macroscopic sectioning of the specimens showed, that the metastases were relatively hard, elastic, well differentiated from the surrounding parenchyma. No tendency of the metastases to coadunate was observed. This supposes that the large metastases have probably disseminated at an earlier time point compared to the small ones, and grew more over longer time, rather than they present simply multiple coadunated small ones.

Though statistically not significant the following trend could be observed (Table 8). The distant metastases of the animals with cold-steel ablatio auris tended to be of type A and C with few large dominating metastatic focuses and several smaller ones. The distant metastases of the animals with transtumoral laser surgical resection tended to be of type B with multiple, quite synchronous small metastases.

	A	**B**	**C**	**Total**
CSR	3	0	5	8
LSR	0	4	1	5
Total	3	4	6	13

Table 8. Incidence of the three types of macroscopic lung involvement for animals without local recurrence from both therapy groups

4.4.3. Histologic findings

The lung metastases presented as well delineated tumor masses (Fig. 22, page 74). They were surrounded by a pseudocapsule resulting from the compressed parenchyma and not like in the LN metastases by a fibrous capsule. Nevertheless here again the typical pattern of concentric layers was observed with vital cells predominantly in the periphery and central necrosis. Blood vessels and cartilage containing bronchi seem to be quite resistant to the tumor invasion. In their spatial progression the metastases reached to these mechanical „obstacles“ and bypassed them from the side, rather than to infiltrate and destruct them. Sometimes large vessels or bronchi were found within the tumoral necrosis. In multiple metastases there were well marked septa of pseudocapsule between the individual metastatic seedings. The tumors did not show tendency to coadunate.

5. Discussion

5.1. The auricular VX2 carcinoma as an animal model for HNSCC

The VX2 SCC is an established animal model of neoplastic disease. Initially Shope and Hurst observed spontaneous malignant transformation of a papilloma viral infection in rabbits [101]. Kidd and Rous succeeded to obtain a cell line from this carcinoma, which could be transplanted further to other animals and became gradually virus independent [48]. The difficulties with the low take rate of the tumor, its propagation and slow growth the first investigators had, are nowadays well under control. The actual VX2 SCC cell lines are characterised by aggressivity and predictable induction rates [112]. Reliable reproducible methods have been established for their preservation and propagation [100,111]. The microvascular architecture of the tumor is well studied [62,88,116,124]. Since the 1960s the VX2 tumor has been used in a large variety of experimental studies. Intramuscularly induced tumor has been used for evaluation of techniques for minimally invasive tumor ablation by vacuum-assisted excision [10], MRI-guided focused ultrasound hyperthermia [82]. Herborn et al. reported on the possibilities of the interstitial MS-325 enhanced lymphography for tumours induced in the dorsal footpads. The technique allowed detailed visualization of the lymphatic pathways of drainage and the lymph nodes along them (poplyteal, inguinal, iliac, paraaortal). Though in a totally different localisation and system the tumor showed tendency for predominantly lymphatic metastases [39]. VX2 carcinomas have been successfully induced in parenchymal organs – lung, kidney and liver [32,97]. Here the possibilities of radiofrequency ablation have been tested [68]. VX2 implantation has been described as a model for gastrointestinal tumor [71] and a model of peritoneal dissemination of a gastrointestinal tumor [70]. The possibilities of chemotherapy for cancer with different primary localisations have also been evaluated in VX2 model [20,32,71,97].

Of particular interest are the experiments based on VX2 SCC auricle model. In this experimental setting the relation between the primary tumor and its regional and distant

metastases plays an important role. The primary tumor is easily accessible – completely exposed to be observed and precisely documented. Different diagnostic and therapeutic interventions can be readily carried out. As the ear is a periphery structure far from important organs, vessels and nerves the tumor growth and manipulations on it cause less discomfort for the animals. The ear model is characterised by topographically and timely constant metastatic pathways. Tumor spread occurs initially along the lymphatic vessels, affecting a well-studied cascade of LNs that again could be easily accessed. At later stages the metastatic process involves distant sites – the lungs. This chronologically and topographically reproducible biologic process is in its nature analogue to the process of regional and distant metastatic spread of the SCC of the head and neck in humans [23,111]. Here to every tumor location in the upper aerodigestive tract correspond certain regional lymph nodes that are usual sites of metastases from this primary locations [57,90,117]. Apart of this lymphatic spread tumoral cells may further propagate from the primary site and the involved LN with the blood circulation to distant locations (usually the lungs, liver or bones) [27,34]. From this background the SCC of the head and neck is not just a tumor in this region, but a multilevel pathologic process, determined by the spread from this primary locus to its specific region and in advanced disease stages to distant foci. Another aspect: induced on the auricle the VX2 carcinoma becomes a tumor of the skin of the head and face. In humans malignancies with such localisation give metastases to the LNs within the parotid gland [109]. In the rabbit auricle model the first echelon metastases involves the parotideal LNs too. Some histologic and even biologic similarities on macromolecular level between the VX2 SCC and human HNSCC have also been reported [64]. The VX2 auricle model has been used for evaluation of chemotherapy protocols [20], optimisation of intraarterial tumor embolisation [112-114], immunomodulation [98,110] and study of the metabolism of the tumor [65].

5.2. General study results

By the piecemeal CO_2 laser surgical resection in our animal model we observed

phenomena and results similar to those known from the clinical practice in humans. The laser allowed for extremely precise cutting with minimization of the surgical wound, simplification of the procedure, reduction of the operative time [67]. Here the auricular VX2 carcinoma model proved one of its major advantages – the easily accessible primary tumor. This helped performing the resection in an uniform way without being limited by the endoscopic approach [46,126]. The laser beam was operated in continuous wave mode with power settings of 10 watts. With these working parameters it provided good coagulation of the small vessels – another important advantage for the clinical practice [115]. The cutting properties of the laser beam together with its haemostatic effect allow for cutting with a precision never achieved by another instrument. As a result the secure margins around the tumor could be reduced to only few millimetres, comparable to those used in human endocopic resection. In our experimental setting this approach led to preservation of the central vessel bundle and more than the half of that portion of the auricle, which had to be resected in the scalpel group in order to achieve secure margins of at least 1 cm, as aimed in the classical oncologic surgery. This preserved tissue volume and the relative aesthetic benefit corresponds in the endoscopic laser surgery of the upper aerodigestive tract to function preservation. In anatomically complex organs like the larynx, tongue and (hypo)pharynx every millimetre normal tissue saved is beneficial for the rest respiratory, protective, alimentary and phonatory functions.

The similar incidence of local tumor recurrences between the piecemeal LSR and the CSR groups comes to show that the minimization of the volume healthy tissue resected is feasible and in no way at the cost of less oncologic radicality. The observed recurrences in both groups after a R(0) *in sano* resection could hardly be attributed to the surgical technique. They are probably an expression of particularities in the biologic behaviour of the VX2 SCC. The histologic slides showed that the border between the tumor and the healthy tissue is quite clear. The tumor did not show tendency to infiltrate or give projections far away in the periphery (per continuitatem). It grows as a solid one with active periphery giving multiple emboli of cancer cells into the peritumoral lymph and blood vessels. Lymph stasis in certain areas during and after the resection could lead to a stop in the movement of these emboli and help their local adhesion. In this way

local metastatic colonization develops. After different periods of time some of them could give rise to a recurrence. The interval of time between the resection and the apparition of the recurrent tumoral mass was quite variable. The most early recurrences were detected 14 days after the resection, the latest one - 28 days after the resection.

Without being sutured but just left open instead, the laser surgical wounds healed without complications. No postoperative bleeding, wound dehiscence or infection were observed in the LSR group even though some of the animals used to grate on the ear. The healing process was slowed down and was per secundam intentionem – commonly observed phenomenon after laser surgery [18,36,58]. This resulted in a more marked fibrosis. At the point between the transversal and longitudinal parts of the resection line a pronounced scar developed, which caused in several animals some deformation of the auricle (Fig. 10, page 70).

5.3. Lymphatic metastases – possible influencing factors

The observed incidence of metastases after ablatio auris and longterm local control (24.6%) confirms other reports in the literature with analogue experimental setting. In their experiment Dünne et al. sacrificed the animals on the 7^{th} day after the induction and detected micrometastases in the parotideal LN in 25% of the cases [21]. In our work the primary tumor was resected at this timepoint so no further metastatic spread could develop, but the animals were left alive for further 42 days. During this time the metastases grew and even necrotised. Our setting is analogous to the clinical situation with resected primary tumor (R0) and „wait and see“ approach to the neck. Nevertheless both experiments show the background dissemination, which is dependent only on the time after the tumor induction (i.e. tumor size). This means that at this timepoint in 1/4 of the animals the metastatic spread has involved the first echelon regional LN though it is still not enlarged. It remains unclear whether all micrometastases to the LNs would progress. The incidence of LN metastases in the VX2 auricle SCC model has been shown to be independent from the primary tumor inoculation technique [111]. In this way the VX2 auricle model corresponds to the natural history of the HNSCC in

humans. Here the incidence and site of regional metastases are quite constant for every single primary localisation and degree of histologic differentiation [119]. Important factors related to the metastatic spread are the tumor size (partially function of time since occurrence) and depth of infiltration [17,34,50]. Newer analyses point out, that actually the tumor thickness rather than the tumor size (diameter or surface) is related to the incidence of regional metastases [76,125]. In our VX2 model a little significant correlation was found only between the maximal width of the tumor and the presence of LN metastases ($r=0.18$ $p=0.038$). The other tumor dimension (maximal length, thickness, base surface area) as well as the increase of all these dimensions from day 1 to day 9 do not seem to correlate with the presence of regional metastases.

The observed difference in the incidence of LN metastases between the CSR group 24.6% (16/65 animals) and the piecemeal LSR group 47.7% (31/65 animals) was found statistically significant. This result was both surprising and disturbing. Our original hypothesis was that there would be no difference in these two groups. This study raises the question of whether tumor transection is responsible for this difference. If it is assumed that the metastases in the CSR group are due only to the natural progression of the cancer then the number of metastases above this level in the piecemeal LSR group (15 of 65 or 23.1%) may be due to the tumor transection.

Having accepted this striking difference the most reasonable question seems to be „Why?“ What mechanisms, what kind of interaction between the laser and the tumoral tissue could precipitate metastatic spread?

5.3.1. Characteristics of the tumor microvasculature

An important background for possible explanations presents the anatomic microstructure of the tumors and concretely the VX2 SCC. A tumor without blood supply could not overgrow 200 µm because of the hypoxia [13]. It is the ability of natural tumors to induce angiogenesis and for induced tumors complementary to use the

local vessel network that plays a decisive local (for the tumor growth), regional (for tumor spread) and systemic (paraneoplastic and immunological phenomena) role.

Tumors have the property to induce the growth of new blood vessels in many different ways – physical factors (increased pressure), chemical factors (low pO2, low pH), signal molecules (VEGF, angiopoietin). These new blood vessels are but structurally and functionally abnormal. The endothelial layer is incompetent with tumoral cells exposed [13,92]. Intratumoral vessels have diminished capacity to react to physical stimuli, which could be explained again with the abnormal structure of their walls [19]. Only newly the absolute proangiogenic capabilities of tumors were questioned. Pezzella et al reported on tumor growth based predominantly on existing normal blood vessels without signs of neoangiogenesis [86].

The presence of intratumoral lymphatic vessels was earlier a quite debatable topic [43,77]. The general opinion was that the tumors do not have lymphatics within, but only enlarged peritumoral lymph vessels [13,54]. The most cancers are characterised by high interstitial pressure in the centre, decreasing in the periphery and higher than in the surrounding healthy tissue [44,77]. This again questions the presence of lymphatic vessels within the tumours. Another opinion is that intratumoral lymphatic vessels exist, but they are compressed and occluded by the proliferating tumor whereas at the periphery excess VEGF-C causes lymphatics to enlarge [13].

Recent studies have proved, that tumors can induce lymphangiogenesis in the surrounding tissue with resulting higher incidence of metastases [103]. In human SCC lymphatic vessels have also been clearly proven and some lymphangiogenic properties of the tumor were observed [6]. There are even opinions that SCC compared to other cancer types is particularly active in stimulating lymphangiogenesis [43,77]. On their side lymphatic vessels seem to be capable to actively attract tumor cell and lymphatic metastases [121]. In some models based on xenotransplant tumors, intratumoral lymphatic vessels have been detected. It is but not clear whether they arise in the tumor or are just host lymphatics that become trapped in between the growing tumor foci [47,108].

The lymphatic system and its pathologic involvement differ between the different animal groups and species. The human lymphatic system is predominantly unidirectional – the lymph from a given peripheral region would be transported to the central collector along relatively constant pathways. In the animal world the lymphatic network is mostly multidirectional with large collaterals and anastomoses. This explains the rare tendency of animals to suffer from lymphoedema compared with humans, where drainage impairment along a lymphatic vessel leads to retrograde oedema [31].

Microvascular structure of the VX2 carcinoma

Experimental xenotransplant tumors comprise two blood networks: coopted vessels from the preexisting network of the host vasculature and vessels resulting from the angiogenic response of host vessels to cancer. In terms of angiogenesis the VX2 seems to behave like the most xenotransplant tumors. Already at the first day after transplantation the tumor induces neovascular growth. At the seventh day there is a clear delineation between well perfused by the new vessels periphery and the already partially avascular and necrotic centre [62,124]. The peripheral vascularisation exceeds far that of the surrounding normal tissues and the avascular centre [39,88]. The VX2 tumor induces changes in the interstitial diffusion coefficients and the microvascular permeability [45]. The acidity in the tumor interstitium is increased with a lower spatial pH gradient away from the vessels [65]. This on its turn may influence the intratumoral blood flow by changing the blood viscosity [116]. The intratumoral vessels have an up to 8-fold increased permeability for macromolecules [35,75]. Our clinicopathological observations support part of these findings. We observed the gradient of the vasculature and the tumor vitality from the centre to the periphery in all specimens studied – primary tumor, recurrences, LN and lung metastases. In larger tumors with dominant central necrosis the tumor was viable only around the few intratumoral vessel and the periphery. It is but impossible to state whether these vessels were coopted old ones or induced by the tumor. We also found lymphatic vessels both intra and peritumoral. They were identified by the absence of erythrocytes, the monolayer walls of endothelial cells, absence of musculature and an incomplete or lacking basal lamina. These

lymphatics often contained tumor emboli. It was easy to identify lymphatic vessels in the tumor periphery. Intratumoral lymphatics were seldom observed.

5.3.2. Possible mechanisms of laser-tumor interaction

On the basis of this short overview on the tumoral angiogenic and lymphangiogenic properties and microcirculation in natural and transplanted tumors and particularly the VX2 carcinoma and our own clinical and histological findings we can hypothesize two mechanisms of laser-tumor interaction, which could probably explain the observed higher incidence of LN metastases after piecemeal laser-surgical resection.

1. Dissemination through the opened lymphatics at the resection line

The major argument of many proponents of piecemeal laser resection has been that the laser beam seals the lymphatics in this zone thus making such dissemination impossible [67,104]. This assumption is based on studies reporting occlusion of lymphatics by the laser beam [36,37]. Some animal experiments have supported this thesis [78,115,120]. Another potential causative factor - the locally produced vapour was shown incapable to propulse particles or cells along the lymphatic network [38]. However, other studies support the contrary thesis that only the blood vessel are occluded by the laser, where as lymphatics remain open. Experimenting with CO_2 laser on the skin lymphatics Schenk and Ehrenberg found that lymphatics are more resistant to laser „sealing“ than blood vessels and remain wide open [99]. Laser similarly to freezing destroys cells but is relatively protective for the connective tissue matrix [59]. Fligny et al. observed some „sealing“ capabilities of laser on transected neurons. Nevertheless they pointed out that this effect remains debatable [29]. In a small patient group Frühling et al. found no evidence that CO_2 laser beam obliterates the lymphatic drainage of the surgical wound [30]. If this is true then the laser-cut end of a lymphatic could present a gateway for precipitated tumor dissemination.

2. Dissemination through lymphatics away from the resection line

2a. Dissemination through the walls of the intratumoral lymphatics

Penetration of the tumor emboli trough openings in the walls of a lymph vessel is another mechanism that actually corresponds to the natural (spontaneous) mechanism of this process. The cross-section opening of a cut lymphatic vessel should present a difficult target for the tumor emboli because of the lymphatic vessel's small cross-section. With some approximation the lymphatic vessel could be regarded as a cylinder (Fig. 20). The surface of its luminal cross-section would be $S_{cs} = \pi r^2$. A segment with length equal to only the half of the radius ($b = S_{cs}/2\pi r = \pi r^2/2\pi r = r/2$) has already the same surface. The endothelial lining of the vessel wall is incompetent because of biochemical alterations in the basement membrane [84,92]. In this way it presents multiple „openings" which have a cumulative surface exceeding many times a normal vessel's cross-section.

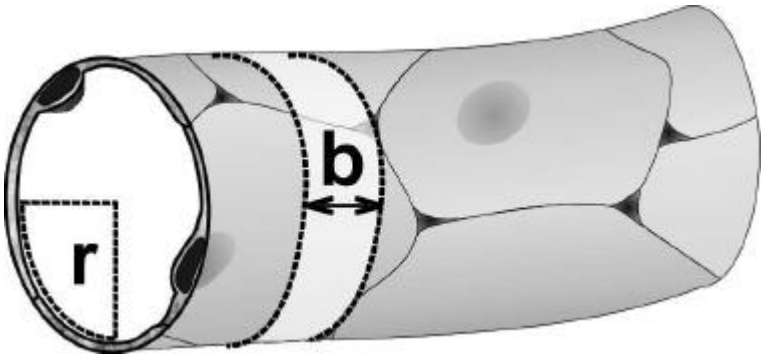

Figure 20. The cross-section of the vessel has a much smaller surface than its walls. Penetration of tumor cells through intercellular or larger defects in the walls is another potential mechanism for initialisation of metastases.

2b. Dissemination along the peritumoral lymphatics

This is another natural mechanism of propagation of the neoplastic disease from the primary localisation to the regional LNs.

Both mechanisms of spontaneous metastasing - through the walls of the intratumoral lymphatics and along the peritumoral lymphatics could be non-specifically precipitated by piecemeal LSR in one or more of the following ways:

- Pressure decrease. The tumor (Latin *tumor,* a swelling) is actually a compressing mass. Due to its growth and the increased interstitial pressure, which is higher than in the surrounding healthy tissue it could compress and partially obliterate the intratumoral and peritumoral lymphatics [44,77]. The partial decompression after the transtumoral laser incision may release these vessels, causing a flood of tumor emboli. When cutting through the tumor with the laser we have often seen this potential „release of pressure". This is especially the case in tumors with necrotic and partially liquefied centres where the incision causes expulsion of these cancer fragments.

- Changes in the permeability and the lymph/blood flow caused by the local heat. When it meets tissue the beam energy of the laser is converted to heat in explosive manner [2,18,36,83]. This is the physical mechanism of the laser cutting. The technical developments of the last years provided the clinical practice with laser devices operated in modes that dramatically reduce the locally produced heat. As a result of his experiments Harenberg supposes that the locally produced vapour could not propulse particles or cells along the lymphatic network [38]. In our experiment a CO_2 laser with a continuous wave mode and a 10 watt power settings was used, similar to clinical practice in order to perform larger and deeper incisions. Clearly local heating occurs. There is carbonization, liquefaction and a visible laser plume. Additionally, surrounding tissues were warm on palpation. This warming will lead to an enlargement of the lymphatics which is associated with a changed flow. So this mechanism appears reasonable and could not be excluded.

- A further potential mechanism for tumor spread is the mechanical trauma to the tumor mass itself. Massage – the basic treatment of many conditions with lymph stasis - could probably precipitate metastases if performed directly or indirectly on a tumoral mass. In

our experiment group, laser tumor resection was associated with a mechanical effect (holding, pulling and stretching the ear near the tumor by hand during the resection). The resection-associated mechanical impact on the tumor was smaller in the CSR group. With this technique the tumor was hardly touched – with one cut of the scalpel the distal part of the auricle was detached. Mechanically stimulated intraoperative tumor spread seems to be less likely. Although the approach in the LSR group was maximally atraumatic again without touching the tumor itself, the manual fixation of the auricle presented a comparatively greater indirect mechanical stress. This is often the case in the endoscopic laser surgery for larger tumors. Here in the process of the preparation parts of the tumor are grasped with the instrument. Additionally, traction on the specimen which is done with laser resection produces mechanical pressure. Already the process of exposure of the operating field through the laryngoscope could hypothetically cause pressure on the surrounding tissues with modification of the lymph flow.

The above-described hypothetical mechanisms apply to our study model and could probably explain the observed higher incidence of metastases after piecemeal LSR. The laser-tissue interaction on the cutting edge is only one factor, which was in the centre of past scientific discussions. The above-mentioned processes concerning the entering of tumor cells into the lymphatics were mostly based on processes associated with pressure or flow. Mechanical and thermal processes in the tumor and its periphery could definitively not be neglected. They seem to be relevant to the actual conditions in the everyday laser surgery as it is practically impossible to completely separate the cutting effect of the laser from the mechanical impacts of the whole surgical intervention. Further studies are needed to test these hypotheses. The possibility of explosion-like tumor cell spread into the lymphatic network must be discussed, which would then be dependent on the applied laser energy. Till then the old rule of the thumb applying for any kind of oncologic surgery (open surgery, endoscopic cold steel or laser) – minimal contact trauma on the tumor – should be obeyed.

5.4. Distant metastases

Distant metastases were found only in the lungs. In no case macroscopic metastases to the trachea, liver or peritoneum were found. This corresponds to other reports, in which only pulmonary metastases were found and only one single case of a tracheal metastasis [111]. In the same article van Es describes another single case of a skip metastasis from the primary tumor to the lungs at day 10 after implantation. In our group we found no skip metastases. All animals with pulmonary involvement had nodal disease too.

The relationships between the incidence and characteristics of the lung metastases and some clinical and pathological factors were analysed with Pearson product moment correlation coefficient [69]. The presence of distant metastases correlated significantly with the local recurrences ($r=0.41$ $p<0.001$), with the involvement of the first echelon LN ($r=0.48$ $p<0.001$) and further nodal involvement ($r=0.38$ $p<0.001$). In order to check which of these factors could be the main indicator for distant metastases partial correlation and linear regression analysis were performed. After controlling for any one or combination of these variables again all were found to correlate with pulmonary involvement. This means that in our experimental setting both the local control (R0 resection) and the regional involvement appear equally important factors for distant tumoral spread. On the other hand distant metastases seem not to be related to the tumor growth speed and maximal size, the presence of necrotic changes in the tumor, the presence of tumoral bleeding in advanced disease, the count and the maximal size of the LN metastases. These finding correspond to the results of other investigators [111]. No significant correlation of lung disease was found for the surgical technique (piecemeal LSR or en bloc CSR) or for the intraoperative bleeding.

Apart of the general incidence the other parameters of the distant metastases - count of pulmonary seedings, largest diameter, type of distribution (A, B, C – see page 36) were analysed for the same clinical and pathologic factors. Statistically significant high correlation was found for the number of pulmonary metastases and the resection technique ($r=0.79$ $p<0.001$) but not for intraoperative bleeding. The number of pulmonary foci was not related to the tumor size, growth, the count and size of LN

metastases. No relationships between the diameter and/or the type of lung metastases with any clinical or pathological factor was found.

The correlation between the presence of lung metastases and bad prognosis (early death) was moderate ($r=0.55$ $p<0.001$). Only 9/19 (47.4%) of the animals with lung metastases survived till the 51^{-st} day. There was a very high negative relationship between the number of pulmonary metastases and survival ($r=-0.92$ $p<0.001$).

Two mechanisms for distant tumor seeding have been discussed for the VX2 SCC in NSW rabbit model. The one is the natural propagation of the disease from the primary tumor or the neck metastases via the circulation. The other one is immediate transvascular seeding already at the moment of tumoral injection. Here small portions of the cell suspension could enter directly the blood vessels and build the early lung metastases as well lymph node metastases [32]. This mechanism is characteristic for the models of parenchymal injection of the cell suspension [66,111]. In intramuscular and subcutaneous injection there is no evidence for such dissemination [111]. To evaluate this hypothesis we analysed the incidence of distant metastases with the occurrence of bleeding at the point of injection of the tumoral cell suspension. Such a bleeding is a sign of injury to minor vessels, which could be a gate for direct haematogenous dissemination. We observed bleeding during the injection in 6 animals (1 from the CSR and 5 from the LSR groups). No one of these 6 animals presented afterwards distant metastases. On this base we could state, that occasional direct intravenous injection is unlikely to induce metastases.

Similar analysis showed that bleeding as a sign of trauma at the place of injection is not related to local recurrences or metastases to the LNs.

Weight loss appears to be strongly associated with the presence of lung metastases, a phenomenon also observed in humans. For the VX2 model van Es reported a significant correlation between weight loss and primary tumour-size and between weight loss and size of lung metastases [111]. In our study cohort the animals free of pulmonary metastases gained weight (21.4±15.3% from the body mass at resection), whereas the

animals with pulmonary involvement lost weight (-1.4±13.3%). Weight loss after the 3^{-rd} postoperative week was a significant predictor for the presence of lung metastases ($p<0.01$).

Without doubt the distant lung metastases are a sign of haematogenous dissemination. Their clinical and pathological incidence is lower than that of lymphogenic metastases, but this does not mean that the primary SCC „releases“ less tumoral cells in the blood circulation than along the lymphatic collectors. We could speculate that actually the lymphatic metastases present only the top of the iceberg of the total tumoral dissemination. The phenomenon of a metastasis is a result of many complex factors including release of tumoral cells/emboli from the primary (intravasation), characteristics of the transport medium (blood/lymph) and vessels, microangioarchitecture (lymphatic sinuses in the LNs; capillaries in the lungs) and metabolism of the host organ [3,81,85]. In histologic sections from the primary tumor we detected metastatic emboli both in the lymph and blood vessels. Predominantly the lymphatics were involved and only occasionally the blood circulation. But this difference could be an artefact – the blood net flow is about two to three orders of magnitude higher than the flow rate of the lymph [108]. The tumor cells are just swept away - we see the arch, but not the flying arrow. On the contrary - the clearance of the tumoral cells in the lymphatics is lower and so they are often found on the histologic slides. The composition of lymph is similar to that of interstitial fluid – the milieu from witch the cells actually come. In contrast the blood is a concentrate of soluble active macromolecules and cells that counteract the circulating metastases. Tumor emboli here would experience serum and cell toxicity and high mechanical stress [8,108]. It is estimated that only 1% of the circulating blood metastases could survive and extravasate at the distant site where again only a small proportion would grow [51,84]. The regional LNs present a net where metabolic, cellular and last but not least mechanical factors offer a „better“ milieu for the tumor cells.

With the background of the observed increased lymphogenic metastatic spread after piecemeal LSR compared to en bloc CSR one might expect a higher incidence of haematogenous (distant) metastases as well. In our experiment this incidence was

similar for the both treatment groups - 8/65 (12.3%) for the CSR and 5/65 (7.7%) for the piecemeal LSR. This low incidence could be due to ligation of the main auricular artery at the beginning of the resection. This likely influenced the intra and peritumoral circulation. In spite of this, however after this manipulation the operating field was far from being dry. There was always bleeding over the resection line from collateral vessels, which had to be coagulated or compressed by the skin sutures. The CO_2 laser provided much better haemostasis in the operating field. The bleeding was relatively less. If bleeding from larger vessels occurred the beam was reapplied over them but from an increased instance – the handpiece was pulled about 1 cm away from its optimal working distance. In this way the beam was defocused, which resulted in increase of its coagulating properties and minimisation of the sharp cutting effect. This technique was effective for achieving complete haemostasis in 60/69 (87%) resections. Only in 9/69 animals monopolar coagulation of sutures were necessary for controlling the bleeding. This is another possible explanation for the observed similarity of the haematogenous distant metastases between the groups. As it was already mentioned there is no uniform opinion if the leaser occludes the lymphatics [30,36,37,59,78,99,115]. On the contrary all of these authors agree that the CO_2 laser closes smaller blood vessels. Our results could be regarded as an experimental proof for these morphological observations.

5.5. The CO_2 laser in the otorhinolaryngologic surgery

In the last three decades the CO_2 laser became the working horse in the field of otorhinolaryngology. This is only one member of the large family of different laser types based on different lasing materials (gases, solid state substances, organic dyes). Soon after the discovery of the principle of LASER (light amplification by stimulated emission of radiation) by Maiman in 1960 the exploration for possible applications of this new tool started. Already at the beginning the medical researchers played an important role. The first two laser types used in oncologic experimental setting - ruby and Nd-in-glass achieved quite disappointing results – their mainly thermal effect caused viable cancer cells to be propelled in the organism. 1965 devices emitting a

focused CO_2 laser beam were developed. The advantages of this new type of laser were recognised soon after – it allowed not only for tissue destruction by evaporation, but for extremely precise and bloodless cutting [102]. Strong and Jako were the first to introduce the CO_2 laser into microsurgery of the larynx in the early 1970s. Initially used for resection of benign conditions the laser soon proved its advantages for the oncologic surgery too [118]. The endoscopic laser surgical resection of early stage glottic carcinomas has the advantage of very good oncologic results with minimal trauma to the healthy tissues of this highly specialised organ. The phonatory function is preserved in an optimal way [16,41,93]. Small malignancies of the epiglottis are easily extirpated again with minimal lost of function [15,28]. In general for laryngeal neoplasms the endoscopically visible mucosal part is predictable for the direction and extent of the deep infiltration, as well for the infiltration of the cartilages of the framework [11]. The classical surgical approach to malignancies of the upper aerodigestive tract comprises well-established open surgical procedures, which follow well-defined resection lines – predefined blocks are removed. Even by minimal tumoral invasion of a certain area the whole block should be resected. On the contrary – the extent of the laser surgical resection comprises only the tumor and a minimal healthy tissue around it. The endoscopic microscopic laser surgery allows tailored resections, containing only the actual tumor volume with the appropriate healthy margins, allowing for maximal preservation of the skeleton of the organ, and respectively better functional results [2,127]. The unique properties of a CO_2 laser coupled to a surgical microscope allow for extremely fine preparation of small hypopharyngeal carcinomas, which usually extend over a considerable surface by a limited depth of infiltration [24,96,105]. After laser surgical resection the wound should not be covered by mucosal or other flaps. Postoperative irradiation could be started early after the resection, despite the observation that laser wounds heal slower [59]. The carbonisation area at the resection line is thin and allows the pathologist to judge, if the resection is in sano.

In the beginning the indications for laser tumoral resection in the upper aerodigestive tract were limited to small tumors with clearly identifiable margins [12,107]. In advanced tumoral disease laser was seen only as a palliative temporary measure for tumor debulking [52,67,93]. Later with increasing experience of the surgeons and the

technical improvements of the laser systems the extent of the endoscopic resections increased. It is often the case, that larger tumors could not be well exposed through the surgical laryngoscope. In order to resect them they have to be divided with the CO_2 laser in several parts, which are excised separately [15,106]. Using this technique the surgeon could see under the microscope the depth of tumoral growth at the resection line [2,95].

At first glance it could appear strange, that to cut through the tumor became so widely accepted. Dividing the tumor with the laser seems opposed to the classical approach of the oncologic surgery – resection as a whole block with the safety margins around and even together with the regional LN groups. Particular laser-specific adverse effects have also been described. The vapour produced locally under the laser beam could transfer infectious material [33]. Tumor cells have been detected in laser plume. Though some seemed morphologicaly intact they showed no viability signs and could not induce tumor growth [79]. The idea to cut the tumor into pieces with the laser is a consequence of the history of its clinical applications. The first laser systems were used to cause tumor destruction in advanced disease. Only later the "cutting" properties of the CO_2 laser were discovered. Today cutting through the tumor in the context of palliative tumor debulking is widely accepted in the routine clinical practice [94].

The concept of tumor transection to include "piecemeal" resection is not limited to head and neck C $\hat{I}_2$ laser surgery only. This has been done with other surgical techniques and other tools as well. If we have a vague glance at the history of the oncologic surgery we will see that impairing the integrity of a tumor is a part of the usual oncologic practice, especially concerning the head and neck tumors. Manipulation of a tumor including incising it or partial resection is performed both with diagnostic and therapeutic intent. Biopsy for histological verification is such an intervention. Mohs' chemosurgery, or even skull base resections, are other examples [7,25,74,80]. There is not any significant medical literature to suggest regional or distant metastasis is higher in these groups. At least since the Virchow's époque (second half of the 18^{th} century) taking a biopsy for histological examination before attempting treatment had became a standard [4]. Then the biopsy was necessary to differentiate between cancer, tuberculosis, lues and other

benign conditions [26]. Nowadays not taking a biopsy is regarded as medical malpractice. In cases with HNSCC this is the second most often allegation that leads to suits after the failure to diagnose [60,61]. On the other hand lately in the literature appeared reports, which prove that cutting the tumor for example for incisional biopsy of HNSCC could cause haematogenous spread [51,81].

Piecemeal resection of laryngeal tumors with curative intent was one of the earliest interventions carried out routinely after the newly introduced indirect laryngoscopy gave the surgeon the possibility to see the larynx in the 60-ties of the 18-th century. At that time this was performed with cold steel instruments or with electrocautery. A „victim“ of this approach became the Prince Frederick of Prussia. In 1887 he presented with hoarseness, which was resistant to conservative therapy. The examination revealed laryngeal tumoral mass. The clinical diagnosis of carcinoma of the left vocal fold was quite obvious. The several attempts to resect the tumor with minimally invasive galvanocautery performed by C. Gerhard – outstanding professor of internal medicine from the University of Berlin – led to postponing of the radical surgical treatment and obvious propagation of the local disease in this 56-years old VIP patient [26]. Piecemeal resection of laryngeal tumors with an electrocautery had its proponents till the second half of the 20^{-th} century [49].

Piecemeal resection of HNSCC with palliative intent is another widely accepted approach in patients with advanced disease. Here the objective is debulking the tumor mass in order to enlarge the lumen of the involved organ and provide better quality of life for the patient. The intervention is used in incurable disease to improve the respiratory and/or alimentary status of the patients with cancer of the larynx, trachea, large bronchi, hypopharynx and oesophagus [1,15,94].

Partial laser-surgical tumor resection as a temporary measure for relief of dyspnea before definitive treatment is an accepted alternative to tracheotomy in patients with advanced laryngeal cancer. Laser tumor mass reduction for obstructing laryngeal tumors, followed by other definitive treatment does not lead to stomal recurrences [67] as known with tracheostomy in interval before laryngectomy [72].

As we could see from this short overview the piecemeal resection of malignancies of the upper aero-digestive tract precedes the apparition of the surgical lasers. This approach has been but rarely analysed in the concrete light of perioperative tumoral dissemination. In the past the main therapeutic aim of the surgery has been the complete removal of the tumoral mass. The current concept yet remains that the goal of the surgery is again complete removal of the tumor mass. This has its clinical rationale. In our experiment we also observed, that the presence of residual tumor at the primary localisation is one of the most strong bad prognostic factors. Only lately there is an important concern about the iatrogenic potentiation of the metastatic spread, both regional and distant. This coincides with the introduction of the laser surgical systems. Whereas current purely clinical studies do not suggest any increased metastatic rates due to tumor transection before total removal in the CO_2 laser endoscopic cancer treatment model, our study does raise some concern [15,67,94,106]. The lack of reported increased metastases could be explained with the preselection of the patients for laser surgery, and even more importantly, with the fact, that most of the patients subjected to laser resection were also treated by neck dissection and/or postoperative irradiation. In advanced cancer regional treatment is a compulsory step, as the majority of patients already have at presentation clinical or occult neck disease. This therapy to the neck could mask a hypothetical increase of the lymphogenic metastasing after piecemeal laser surgical resection. If this is indeed true, then postoperative irradiation in tumors large enough to require endoscopic transection versus endoscopic en bloc resection is indeed reasonable.

We recognize that there was a difference in the width of the resection margin between the en bloc and tumor transection models. This was intentionally chosen so as to be analogous to partial versus total laryngectomy in patients. This difference in resection margin itself could explain the difference in the extent of regional metastasis if it is assumed that “in-transit” tumor spread beyond the narrow margin of the laser resected animals occurred, but was yet within the margin of tissue removed with en bloc resection. This is an important consideration, as partial laryngeal surgery, especially in the glottic larynx, will by definition not have wide margins. It is an interesting hypothetical question whether perioperative chemotherapy, or even postoperative irradiation would destroy these “in-transit” tumor cells. It is further interesting to

speculate whether staged or delayed neck dissection would have lowered the metastastic rate in the “piecemeal” surgical group. Further animal studies could address this issue.

While the stimulus in doing this study was our interest in transoral CO_2 laser surgery, the study actually has relevance to any CO_2 laser surgical approval where “piecemeal” resection has been or could be used (eg. rectal, uterine, or esophageal surgery). These concepts of en bloc versus “piecemeal” resection together with questions regarding the use of chemotherapy and/or irradiation with this type of surgery will become even more important as minimally invasive surgery techniques are further developed.

5.6. New considerations on the VX2 auricle SCC model

5.6.1. Hematogenic metastases

It was a generally accepted opinion, that the VX2 auricle model gives predominantly lymphatic metastases [21,23,111]. Now we have morphologic evidence, that the primary tumor could give direct hematogenic metastases (Fig. 8, page 68). This finding did not affect the clinical and end pathological result, which corresponded to the established concepts. We did not observe any cases of skip metastases from the primary to the lungs. The distant metastases appear to follow the initial lymphogenic ones. All this supposes the presence of minimal or may be even massive process of intravasation of tumor emboli at the primary tumor, which remains but hidden for the study models implemented by now, because of its high speed and low take-rate in the periphery. Nevertheless this aspect requires further investigations. If such increased haematogenic dissemination with subclinicaly low take rate really exists a similar process could be suspected for SCC in humans as well [51,84].

5.6.2. Parotideal lymph nodes

Another generally accepted opinion was that there is only one LN in the parotideal region [21,23,111]. In some older reference books on the rabbit anatomy this LN is even missing, whereas the other LN groups along the neck are presented in details [87]. Of the 139 animals included in our study in 15 (10.8%) an evidence of a second LN in this region was found. Five of them had 2 separate macrsocopic metastases. Actually they lied closely to each other and presented as one spheroid. There was but a clear equatorial line in the thick fibrous capsule surrounding this complex. Dissection along this line showed that there are actually 2 distinct metastases. Unfortunately the advanced tumoral and necrotic changes did not allow to histologically find traces of the pretumoral architecture of these LN. In one case an intact LN was found aside to a large necrotic metastasis in the parotideal region (Fig. 15b, page 72). These observations suggest possible metastasing directly into the paroideal parenchyma. Such a mechanism could not be excluded, having in mind the characteristic absence of compartimentalisation between the epithelial and mesenchymal (including lymphoid tissue) components in the early stages in their development. As a result from such an embryologic development the parotis and its lymph nodes represent a particular complex [63]. The variability in the number of the parotideal LNs that we observed, corresponds better to the notion of varying number of LNs in the parotideal gland of humans [109]. The parotid gland is the only salivary gland to contain LNs. This anatomical premise and the characteristic gland/lymph node symbiosis explains the frequency of head and neck metastases to the parotis [5]. These LNs are a common place for metastases from the skin of the head and face. In our experimental model the VX2 tumor is induced on the auricle, and similarly to humans spreads to the parotideal region.

5.6.3. Technique of tumor injection

In our study cohort we achieved successful transplantation in 99.3% of the animals. This figure differs significantly from the results reported in the literature – 78%-83%

[21,22,111]. This is partially due to the improved inoculation technique – a factor, which has been evaluated and found quite important by other studies [111]. We found the technique of tumor transplantation in previously prepared by gas décollement subcutaneous pocket very useful. It allowed injecting the cell suspension with ease at considerably lower pressures compared to the direct injection. This reduced the lost of cell suspension volume. On the other hand injecting at lower pressure causes less mechanical stress to the tumoral cells, so the number of viable ones does not decrease. The technique resulted as well into a better hygiene at the targeted area.

The technique of injection determines to some extent the tumor size. As the needle is introduced through the subcutaneous plane it forms a channel. During the withdrawal this channel gets contaminated with tumoral cells, which give rise to complementary tumoral growth. In this way the length of this channel could add to the longitudinal diameter of the initial tumor. As a results further calculated values (base surface – ellipsoid, tumor volume – half spheroid) are perturbed.

Other possible explanation for the good take rate in our experiment includes the early time of the resection – 8 days after the inoculation. Spontaneous regression of the VX2 SCC in rabbits has been described, but at relatively later time points – after the 21 day [111]. Nevertheless in our experimental setting the differentiation between „takes“ and „non-takes“ was clear. In all but one animal the tumor mass had increased its dimensions by the time of resection and the tumoral masses had a vital aspect, that was consecutively proven histologically. The only case of an animal with shrinkage of the tumor and atypical macroscopic aspect (to small, pale, hard, resembling a scar) was excluded from the analysis. In this case the histological examination showed small nests of absolutely vital proliferating tumor that probably would have grown with the time. An argument in favour of this prognosis present the few cases we have observed in another experiment, where atypically late tumor reappearance and growth occurred in animal, considered by as „non-takers“. In these animals the tumor reappeared about 14 days after it was considered for a „non-take“ or regression.

Originally the VX2 was derived from the V1 SCC cell line by multiple passages during

which the take rate in the late generations increased [48]. Such a shift in the properties of our VX2 line compared to those of the original one obtained from Prof. Dr. Robert J.J. van Es (Department of Oral and Maxillofacial Surgery, University Medical Center Utrecht, Utrecht, Niederlande) could not be excluded [111].

5.6.4. Technical note on the animal handling.

As mentioned earlier 3 animals were lost as a result of peri- and postoperative complications. One died during the operation, probably of anaesthesia overdosage. One animal was found dead in the transporting case after successful operation. The transport from the operating theatre to the cages lasts about 2-3 minutes. The third animal was found dead in the cage after successful operation. Initially all these cases were attributed to anaesthesia overdosage. Later in similar situations we observed that in certain body positions the anaesthetised animal could compress and occlude its nose so it could not breathe. In several further animals we tried temporary manual obstruction of the nose with the animal already under anaesthesia with the usual combination 5mg/kg xylazinhydrochlorid and 100mg/kg ketaminhydrochlorid The animals could again not breathe and were unable to change the body position. It appears that the compression of the nose could lead to suffocation. Even though that the transporting box and the cages were specially constructed with the appropriate openings there are dangerous corners, where the animal‘s nose could get occluded.

6. Conclusion

The aim of the present study was to compare the piecemeal laser surgical (R0) resection with cold steel (R0) resection en bloc. For this purpose the auricle VX2 squamous cell carcinoma model in New Zealand White rabbits was used. The major study parameter that were to be evaluated included the incidence of tumoral recurrences (local control), incidence and distribution of regional lymphatic metastases and the incidence of distant visceral metastases.

In this concrete experimental setting both surgical techniques achieved comparable local control. The laser-surgical resection resulted in preservation of a larger amount of healthy tissues and structures. The piecemeal laser-surgical resection lead to more regional lymphatic metastases than the cold steal en bloc resection (47.7% vs. 24.6%). This difference was found statistically significant. However, it is not possible to state that tumor cells disseminated from the resection line itself caused this difference or it was due to malignant cell dissemination from deeper tumoral and peritumoral layers. The incidence of distant pulmonary metastases was comparable for the both groups with minor differences, which did not prove to be significant in the observed small group. The presence of local recurrence showed to be a decisive factor for both lymphatic and haematogenous metastatic spread and the overall prognosis.

It is quite probable, that piecemeal laser surgical resection of HNSCC in humans is also associated with increase in lymphatic metastases. The designs of the reported in the literature morphological and clinical studies have been insensitive to answer this specific question. The morphological data on „sealed lymphatics“ is controversial. In the clinical trials preselected patients were included and adjuvant therapy for the regional disease (neck dissection and/or radiotherapy) has been performed. Piecemeal resection has been the approach mainly in advanced tumoral disease. In such cases involvement of the regional LN was usually present or assumed and required therapy.

Our experimental result and the clinical experience underline the need for elective treatment of the neck after piecemeal laser-surgical resection of extended HNSCC. Cutting through the tumor when not necessary just to see the depth of infiltration could be hazardous.

In this experiment it was impossible to distinguish between the role of the laser itself and the concomitant mechanical impact associated with the surgery as the factors leading to lymphatic metastases. In the endoscopic laser surgery these two factors are again difficult to separate. Further studies should address the question for the cost/benefit ratio of laser resection of large tumors en bloc (with more mechanical manipulation of the resectat) compared to piecemeal laser surgical resection (as a measure to minimize the mechanical trauma during the dissection and extirpation). This study will lead to a renewed investigation of whether tumor transection leads to increased metastasis with laser surgery, and further what interventions are possible to alter or eliminate the phenomenon. The role of perioperative chemotherapy, postoperative irradiation, or even staged neck dissection can, and should be investigated.

Deeper knowledge of the tool LASER would not change its essence, but will allow us to use it in a better way. For the good of the patient.

Appendix

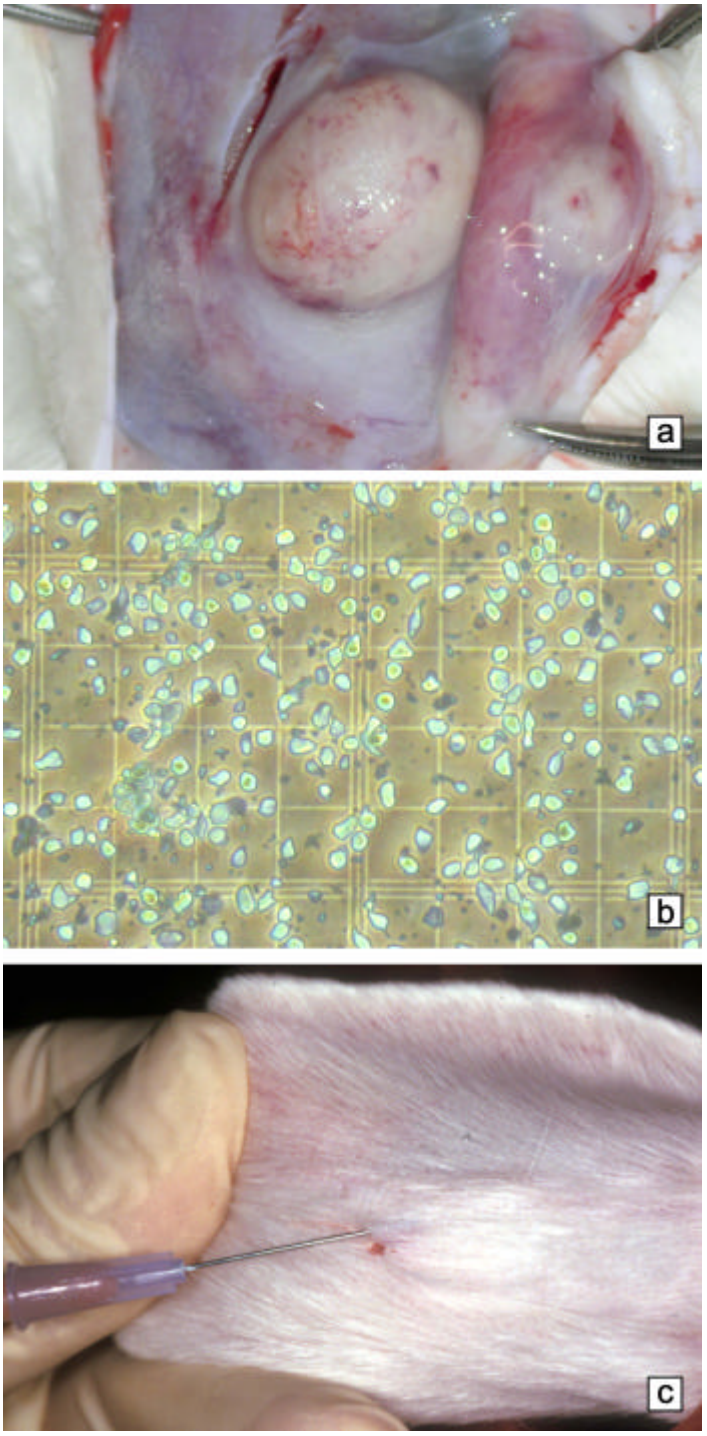

Figure 2: Tumor induction. ***a)*** *VX2 SCC in the gluteal musculature 14 days after induction;* ***b)*** *Trypan blue staining of the cells in the suspension. Live cells exclude the blue dye, and appear clear. Dead cells take up the dye and appear blue;* ***c)*** *Subcutaneous inoculation of 0.3 cc tumor cell suspension.*

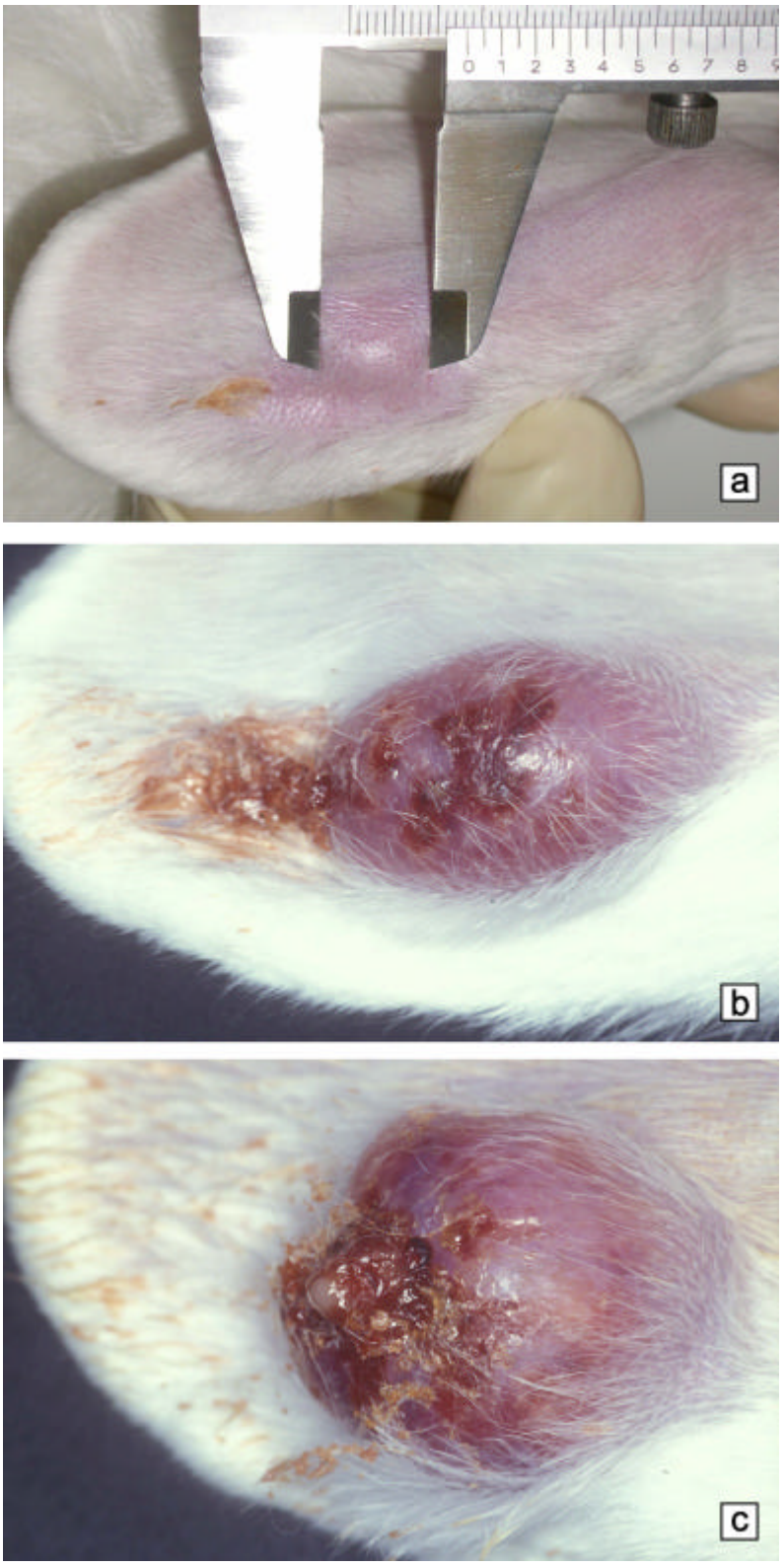

Figure 3: Tumor growth. ***a)*** *Measurement of the tumor 4 days after inoculation; Different macroscopic aspects of the VX2 tumor at the 8-th day after the induction:* ***b)*** *No necrosis, bleeding grade 1;* ***c)*** *Necrosis grade 2.*

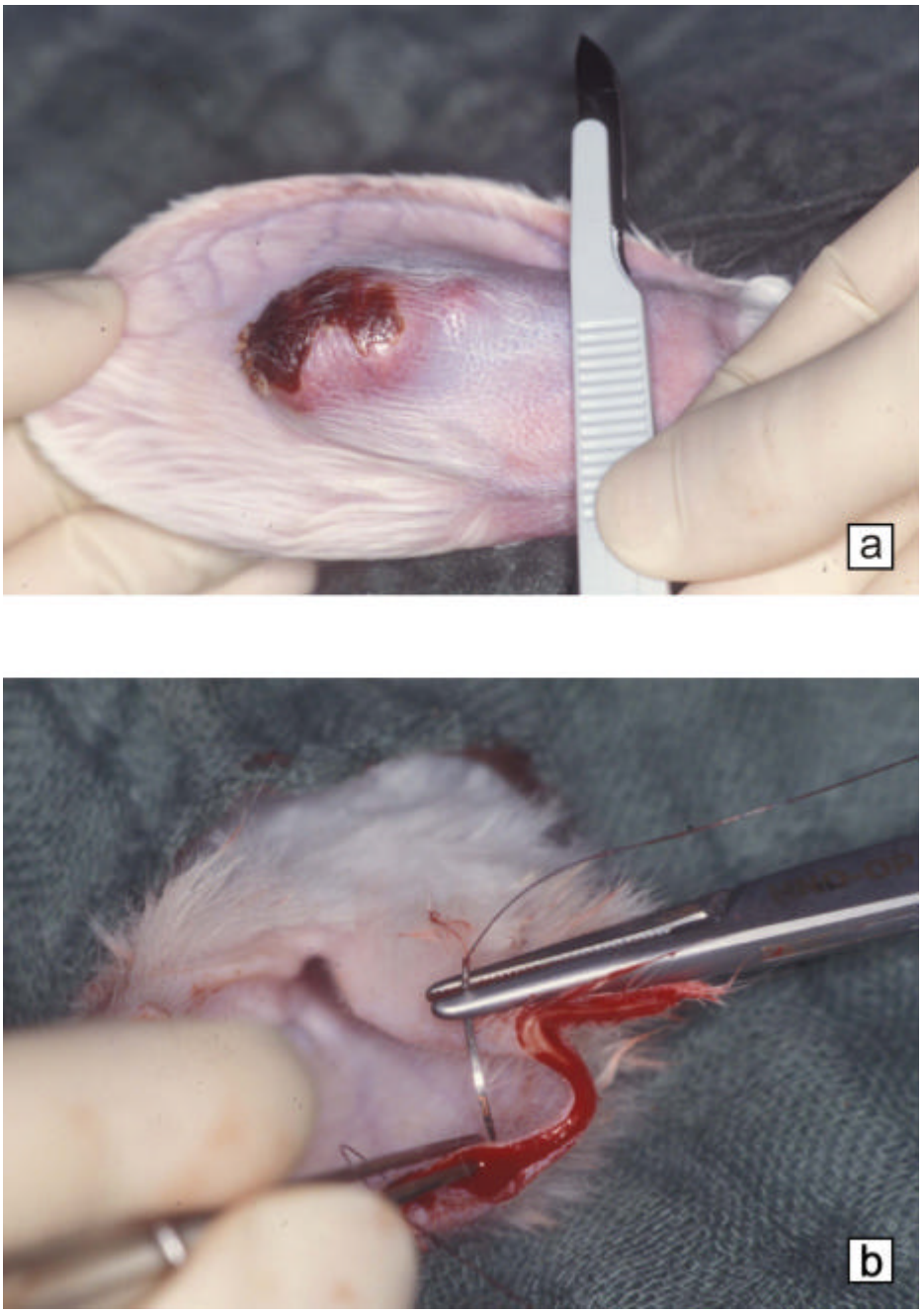

Figure 4: Conventional cold steel resection (CSR) of the tumor. ***a)*** *The resection line goes through macroscopically healthy tissue 1.5-2.5 cm away from the visible tumor border. Complete ablation of the distal part of the auricle is performed;* ***b)*** *The wound is covered by small skin flaps meticulously sutured without perforating the cartilage.*

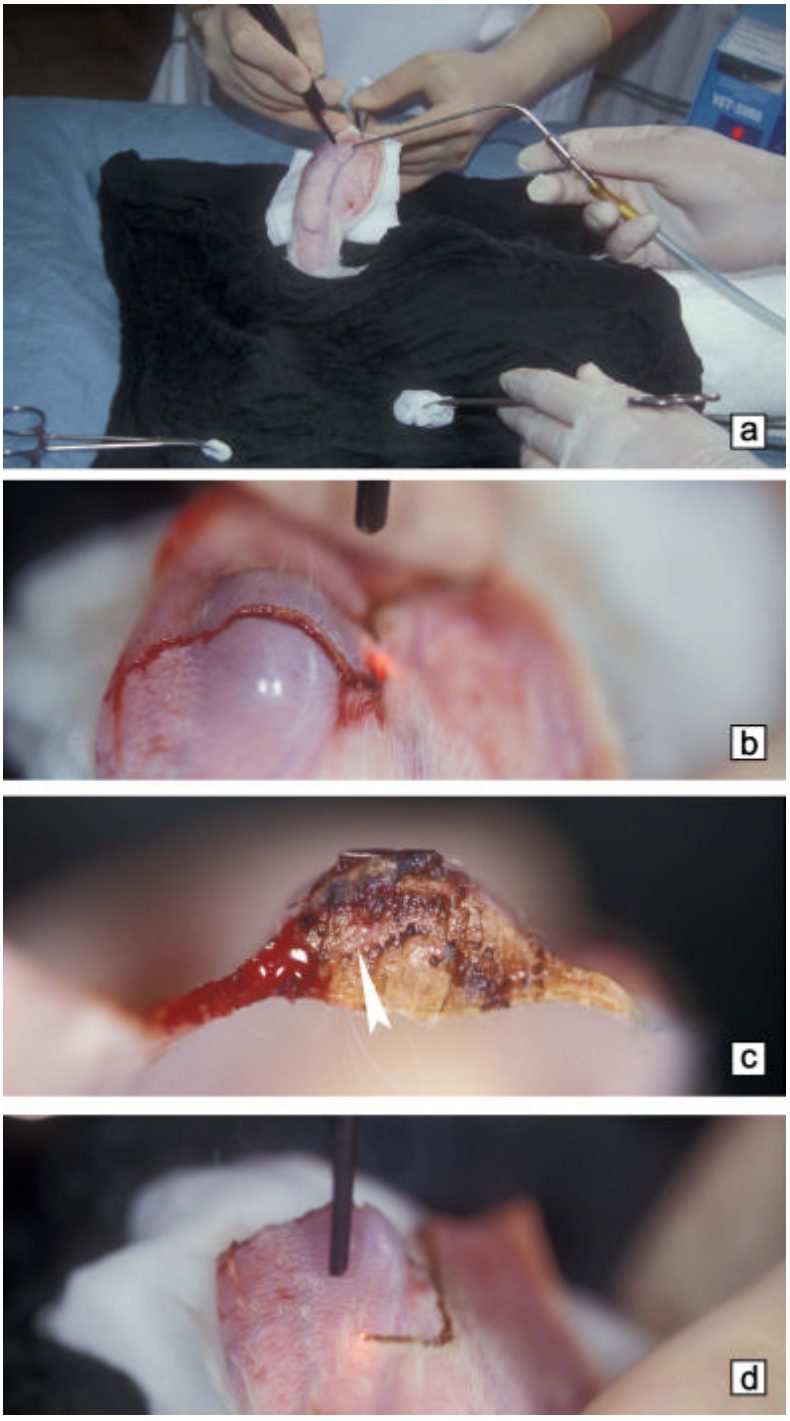

Figure 5: Piecemeal laser-surgical resection (LSR) of the tumor. ***a)*** *The resection is performed with 10 W in continuous wave mode;* ***b)*** *The first cut goes through the tumor;* ***c)*** *The proximal aspect of the cutting surface with minor bleeding and carbonisation traces. In the centre is a small necrotic area (arrow);* ***d)*** *The second cut goes through macroscopically healthy tissue 0.5 cm away from the visible tumor border.*

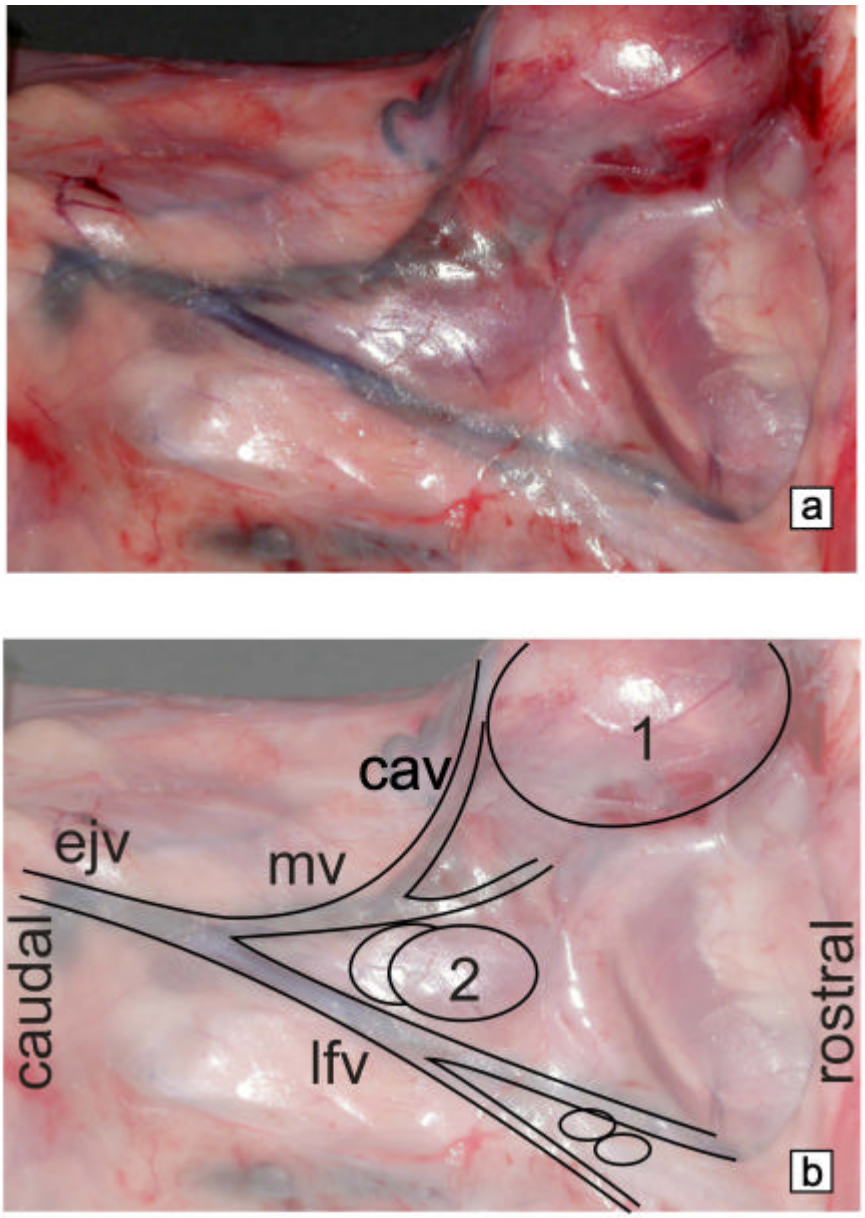

Figure 6: Topography of the lateral neck of the NSW rabbit. ***1)*** *parotideal LN with a large metastasis.* ***2)*** *enlarged caudal LNs under the fascia at the bifurcation of the external jugular vein (ejv). The rostral mandibular LNs* ***3)*** *are usually located at the bifurcation of the linguofacial vein (lfv). mv - mandibular vein; cav - caudal auricle vein.*

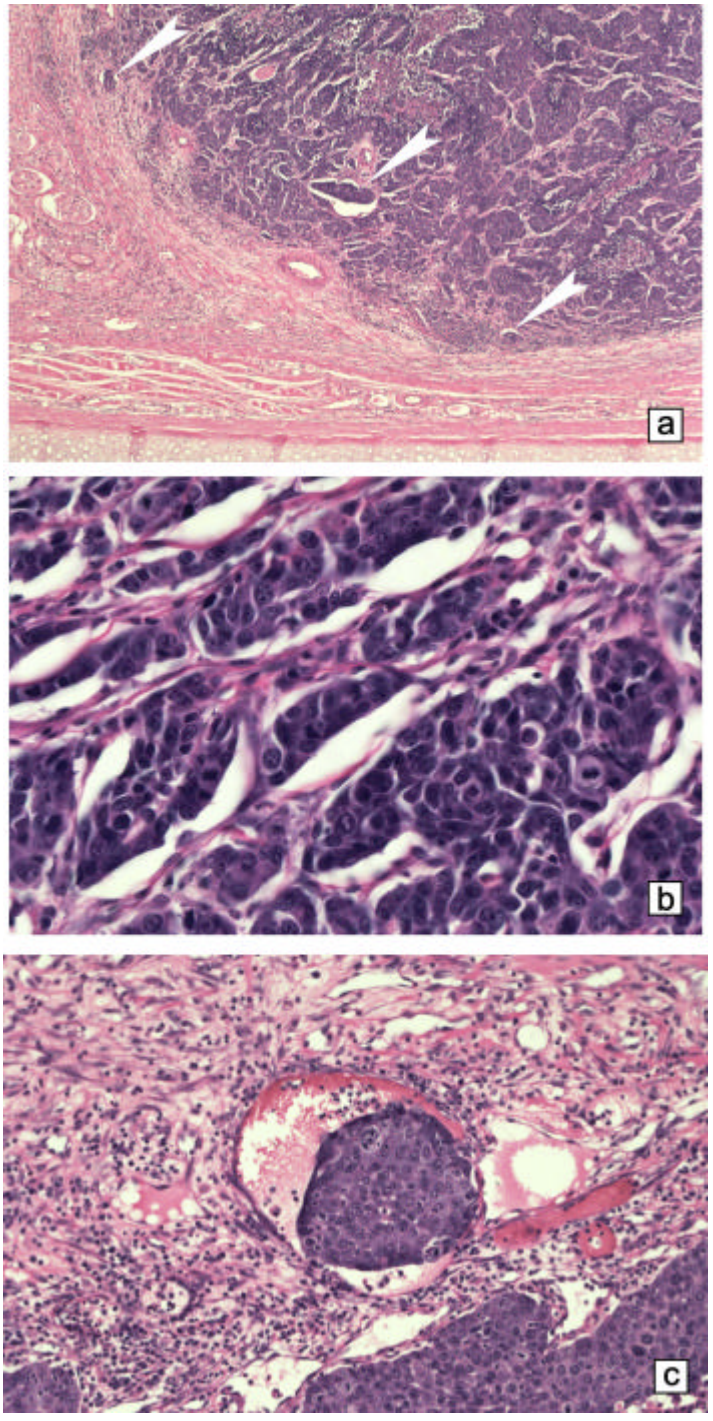

Figure 8: ***a)*** *General aspect of the primary tumor (H&E x5). In detail another lymphatic metastasis (H&E x50). ;* ***b)*** *Periphery of the tumor with 3 metastatic emboli in lymphatic vessels (arrows) Note the necrotic changes towards the tumor's centre (H&E x5);* ***c)*** *Microscopic aspect of the tumor cells (H&E x100);* ***d)*** *A rare finding - metastatic embolus in a blood vessel in the tumor's periphery. (H&E x50). Are haematogenous metastases really that rare?*

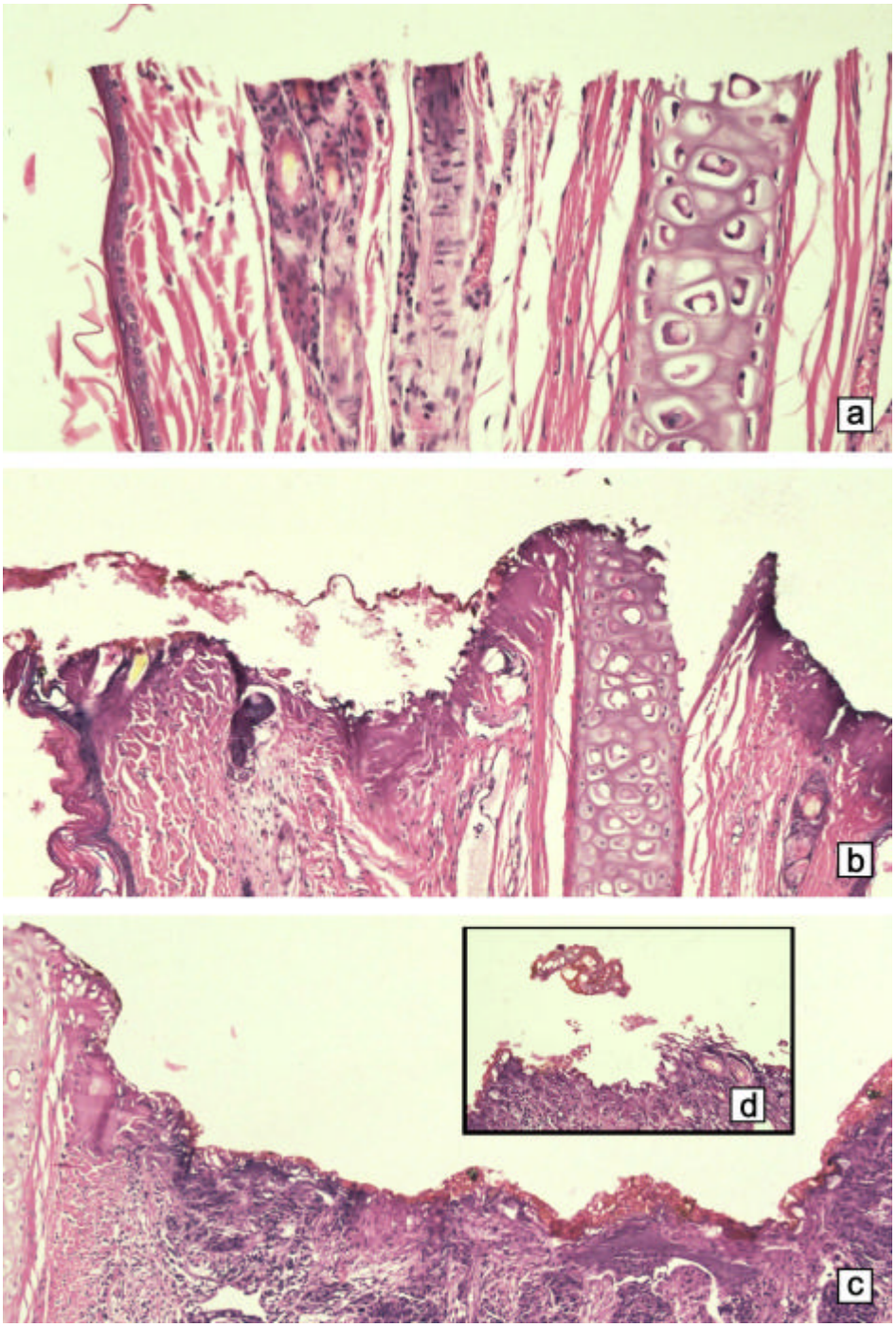

Figure 9: Histologic characteristics of the resection line. ***a)*** *A cut with scalpel (H&E x25).* ***b)*** *Laser cut through healthy tissue. The wound is sealed by a coagulation zone (H&E x25).* ***c)*** *Cut with laser through the tumor (H&E x50).* ***d)*** *In areas with tumor necrosis the coagulated layer is thinner and could be easily detached. (H&E x100).*

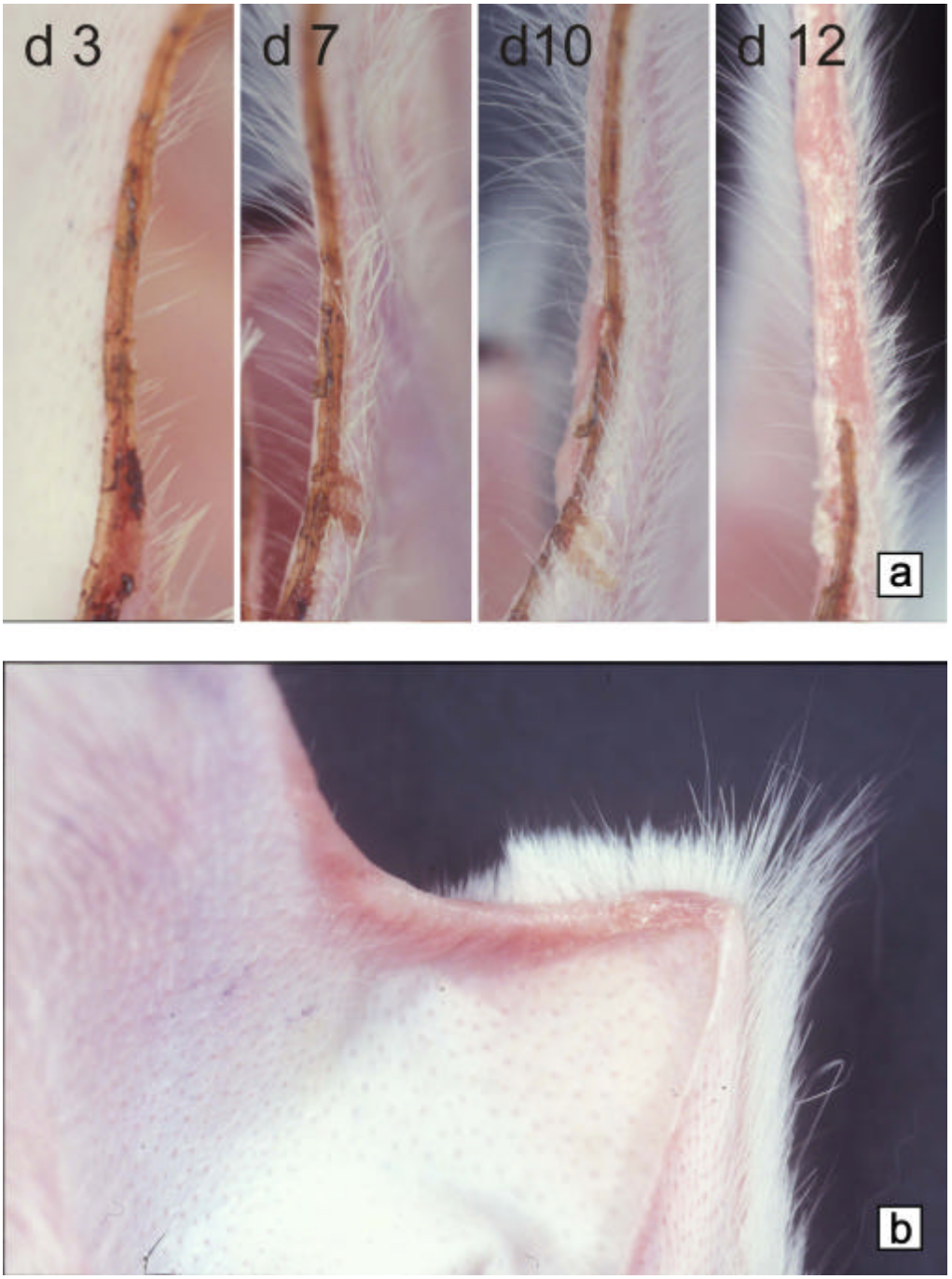

Figure 10: Macroscopic aspects of the wound after laser surgical resection. ***a****) Wound healing at days 3, 7, 10 and 12;* ***b****) Fibrous scar on the curve between the transversal and longitudinal parts of the resection line. During maturation it contracts and causes a deformation of the auricle.*

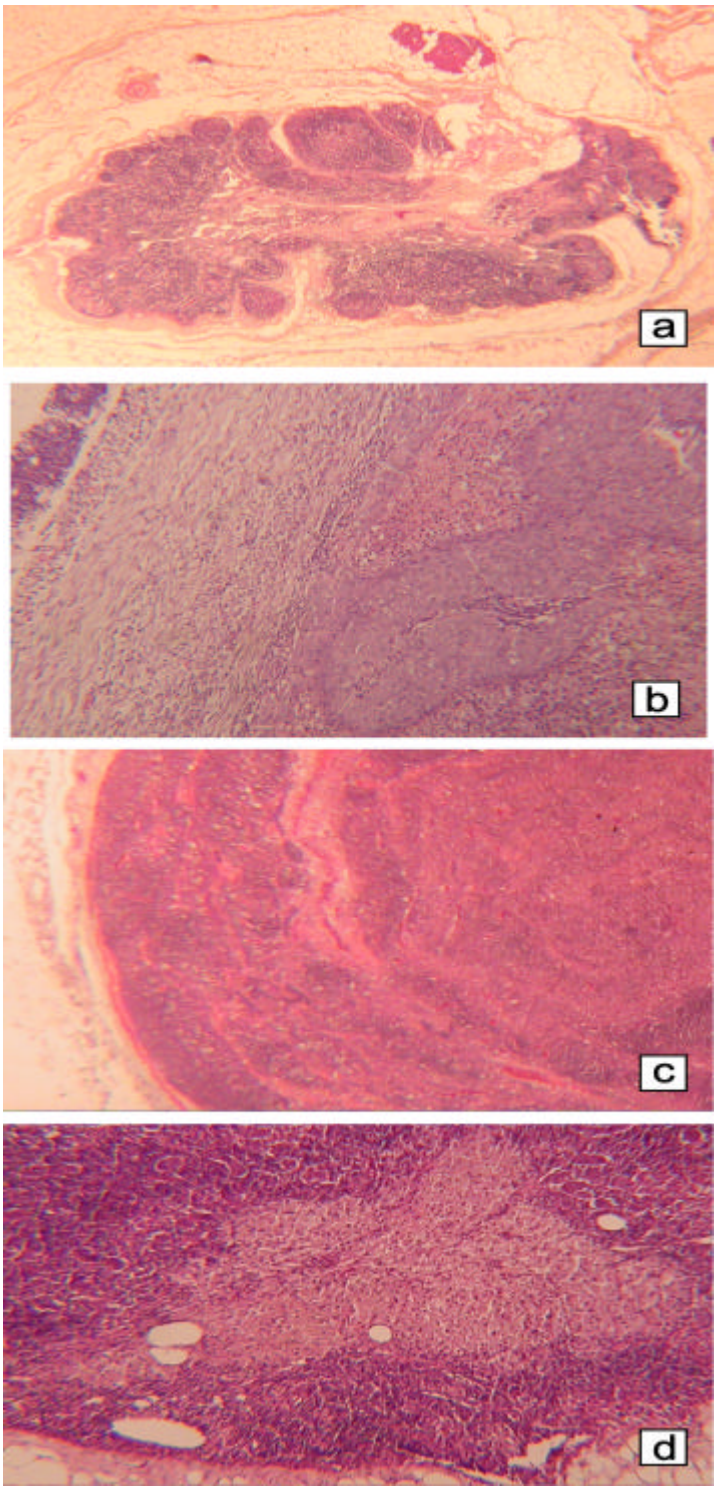

Figure 14: ***a)*** *A LN form the parotideal region with normal structure (H&E x25).* ***b)*** *Large LN metastasis with thickened fibrous capsule, peripheral and perivascular vital tumor and necrosis towards the centre (H&E x50).* ***c)*** *Enlarged (27x19 mm) activated LN without tumoral involvement (H&E x25).* ***d)*** *Enlarged LN with foamy macrophages. Again no tumoral involvement is present (H&E x25).*

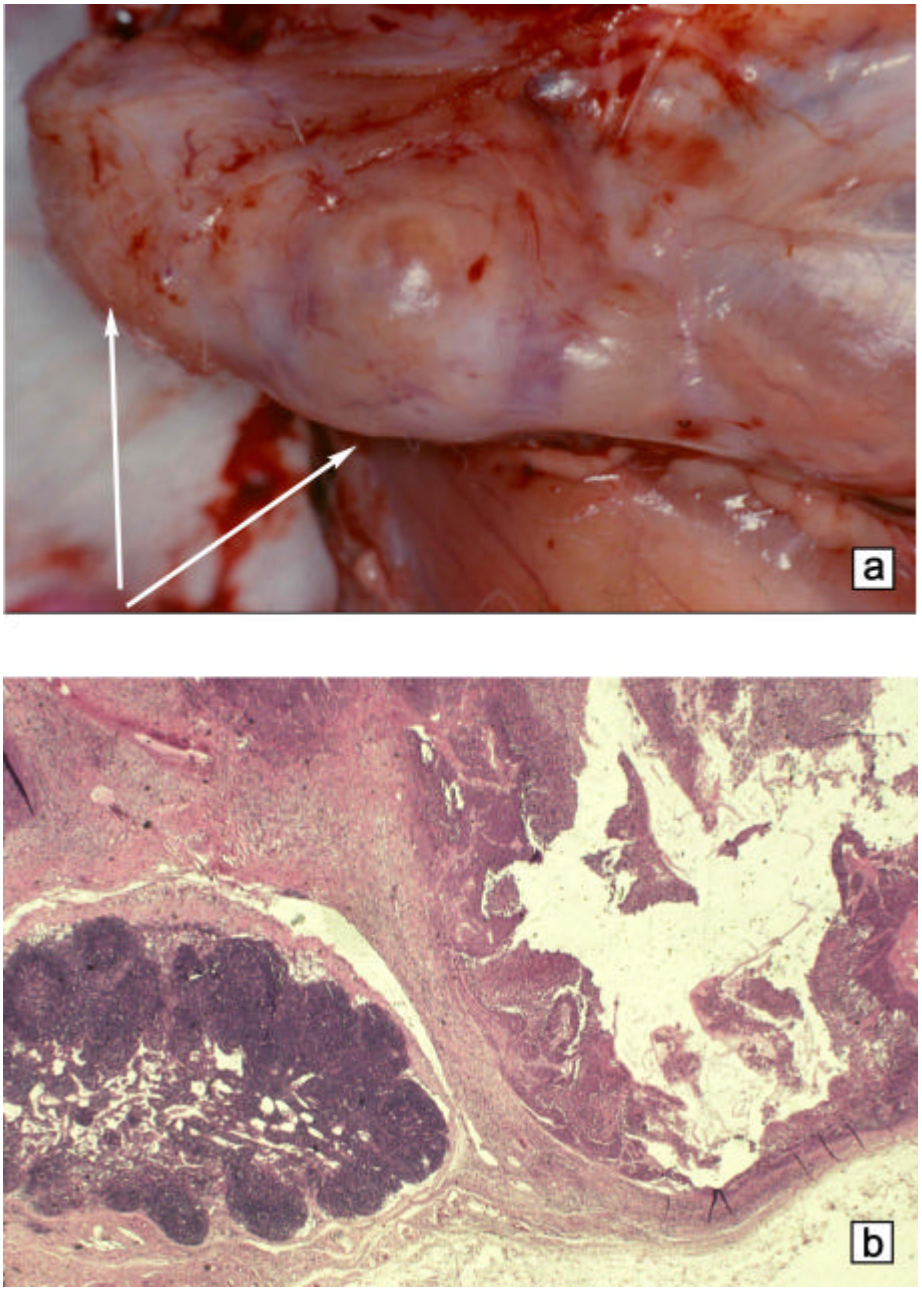

Figure 15: ***a)*** *Two metastases in the parotideal region.* ***b)*** *A tumoral mass in the parotideal region with large central necrosis and active cancer cells only in the periphery (H&E x5). Note the intact LN on its side. The finding suggests the presence of more than one LN in this region or direct metastases to the parotideal gland. The advanced necrosis did not allow to identify in what kind of initial structure (LN or gland) the metastasis developed;*

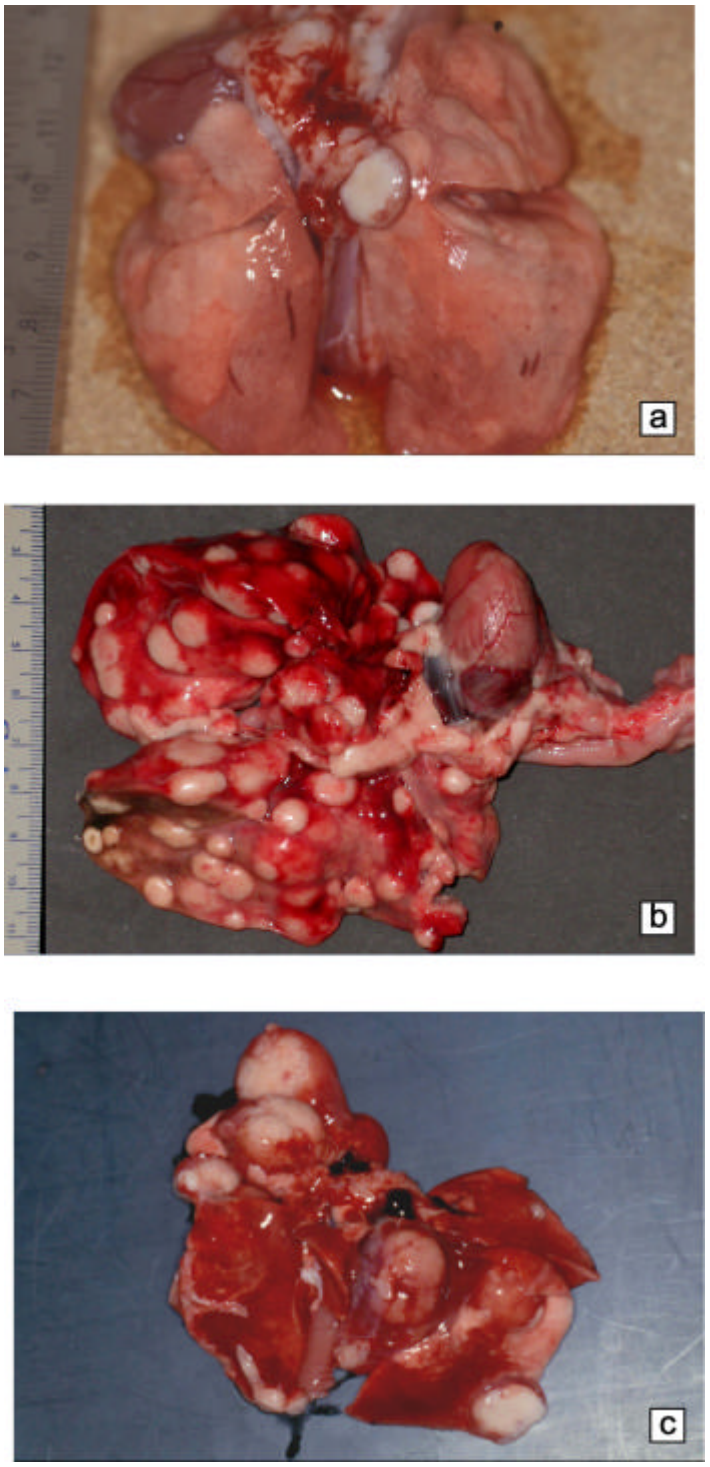

Figure 20: Macroscopic aspect of the lung metastases. ***a)*** *Single metastasis type A;* ***b)*** *Multiple metastases type B;* ***c)*** *Multiple metastases type C.*

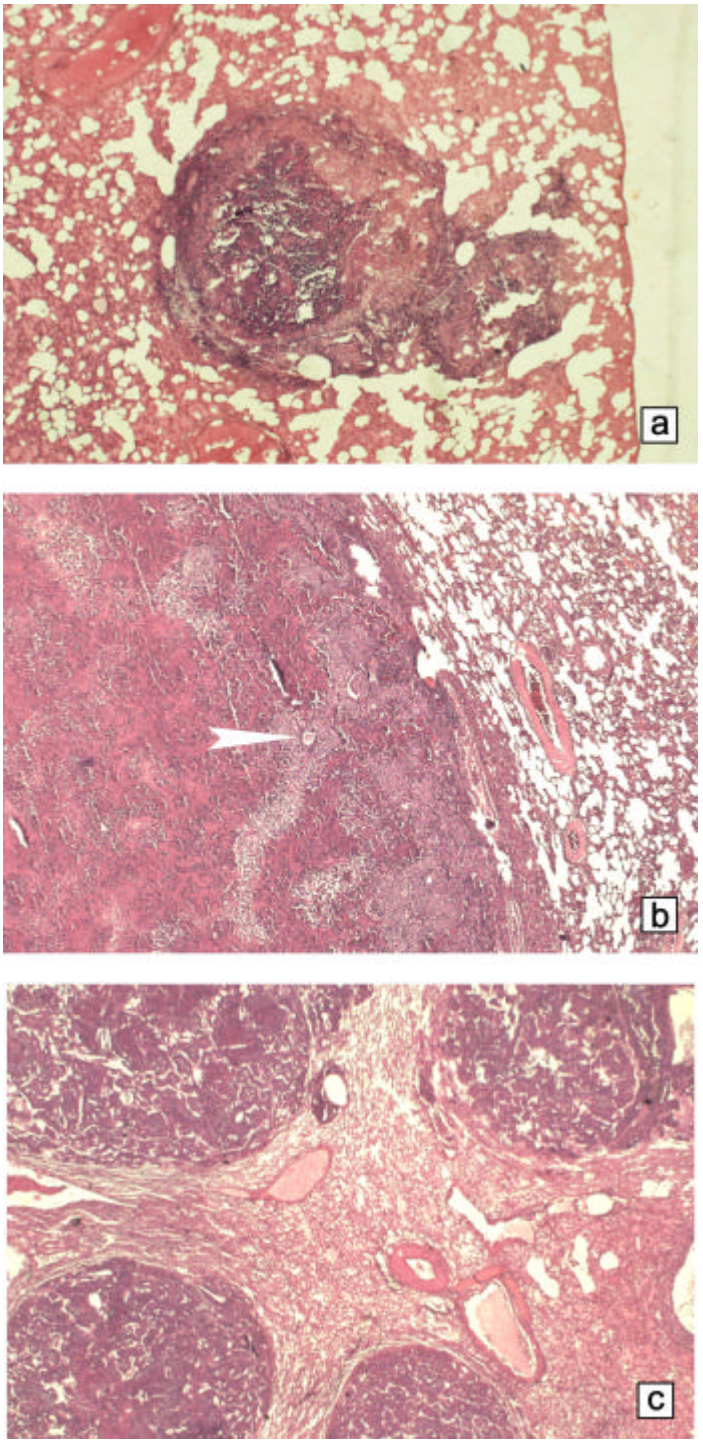

Figure 22: Histologic aspect of the lung metastases. ***a)*** *Small central VX2 metastasis;* ***b)*** *The large metastases show typical concentric layers. The centre is necrotic, full with leucocytes. Live active tumoral cells are to be observed only in the periphery or around small blood vessels (arrow). The tumor periphery is well delineated by a pseudocapsule of compressed parenchyma without fibrosis;* ***c)*** *Multiple lung metastases. Note the compressed parenchyma between them.*

Gr:		Tier:	

	TU-Induktion	Resektion	Faeden ex	End	Tot
Plan	22.06.2003	30.06.2003	07.07.2003	11.08.2003	

VERLAUF

Datum	Gew.	t	TU	L	B	D	B	N	K	LK	Notiz		
22.06.2003													
23.06.2003													
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25.06.2003													
26.06.2003													
27.06.2003													
28.06.2003													
29.06.2003													
30.06.2003													

Datum	Gew.	t	TU	L	B	D	B	N	K	LK	Notiz		
30.06.2003													
01.07.2003													
02.07.2003													
03.07.2003													
04.07.2003													
05.07.2003													

Datum	Gew.	t	TU	L	B	D	B	N	K	LK	Notiz		
07.07.2003													
14.07.2003													
21.07.2003													
28.07.2003													
04.08.2003													
11.08.2003													

INTERVENTIONEN

TU-Induktion	Spender	Notiz			

Resektion	Gewicht	Narkose			
		Notiz			

Laser					

Skalpell					

Faeden ex	Notiz			

Temperatur Normbereich 38,5 - 39,5 C; Gewicht soll in Kg gemessen werden

Obduktion			Befund		Preparat		
		Gewicht	Groesse	Beurt.			
Nachresektat	02						
Ln. parotideus	03						
Lnn. n. cand.	04						
Lnn. n. retr.	05						
Pulmo ipsi.	06						
Pulmo contra.	07						
Leber	08						
Hirn	13						
Lnn. tracheales	15						

Gr:		Tier:	

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Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Incidence of lymph node metastases after piecemeal laser-surgical and en bloc cold steel resection of auricular VX2 carcinoma. A comparative study.“ im Medizinischen Zentrum für Hals-, Nasen-, Ohrenheilkunde unter der Leitung von Professor Dr. J. A. Werner mit Unterstützung durch Frau Priv. Doz. Dr. A.A. Dünne ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine andere als die in der Dissertation angeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Vorliegende Arbeit wird in folgenden Publikationsorganen veröffentlicht:

Lymph node Metastasis in an Animal model: Effect of piecemeal laser surgical resection. Sapundzhiev N, Ramaswamy A, Dünne AA, Sitter H, Davis RK, Werner JA. Eingereicht an Lasers in Surgery and Medicine. Lasers Surg Med. 2005 May 12;36(5):371-376

Teile der Arbeit sind in folgenden Publikationsorganen veröffentlicht:

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