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Time matters – the fast growth of head and neck squamous cell carcinoma

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-025359
Article Type:	Research
Date Submitted by the Author:	12-Jul-2018
Complete List of Authors:	Dejaco, Daniel; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Steinbichler, Teresa; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Schartinger, Volker; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Fischer, Natalie; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Anegg, Maria; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Dudas, Joszef; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Posch, Andrea; Medical University of Innsbruck, Department of Radiationoncology Widmann, Gerlig; Medizinische Universitat Innsbruck, Department of Radiology Riechelmann, Herbert; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery
Keywords:	head and neck squamous cell carcinoma, tumour volume, tumour growth rate, EGFR, Ki67, CD44

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Time matters – the fast growth of head and neck squamous cell carcinoma

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Word count: 3349

Abstract: 201

References: 30

Figures: 4

Tables: 4

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ABSTRACT

Objectives: Data on tumour growth of head and neck squamous cell carcinoma (HNSCC) is sparse. We calculated specific growth rates (SGR) for the primary tumour (PT-SGR) and largest pathological cervical lymph nodes (LN-SGR) in patients with incident HNSSC treated with primary radiotherapy (RT) or radiochemotherapy (RCT).

Methods: In CT scans obtained at time of diagnosis and subsequent planning CTs immediately prior to RT or RCT, volumes of the primary tumour (PT-volume) and largest pathological cervical lymph node (LN-volume) were retrospectively measured. SGRs were calculated assuming an exponential growth function.

Results: In 123 patients, mean interval between diagnostic and planning CT was 29±21 days. PT-SGR was 1.8%±1.8% (mean±SD) per day and was positively correlated with EGFR, Ki67 and CD44 expression (p=0.02; p=0.02; p=0.03). LN-SGR was 1.7%±2.0% per day and increased with larger initial LN-volume, was lower in laryngeal cancer (p=0.003) and slowed down with time. LN-SGR was not correlated with EGFR, Ki67 or CD44 expression in primary tumours (p>0.12). New cartilage or bone infiltration occurred in 10 patients and new central lymph node necrosis in 8 patients.

Conclusion: HNSCC are fast growing tumours for which treatment must not be delayed. Clinical tumour growth rates are influences by EGFR, KI67 and CD44 expression.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- In patients with incident head and neck squamous cell carcinoma (HNSCC), specific growth rates (SGR) for primary tumours (PT) and largest pathological cervical lymph nodes (LN) were retrospectively calculated.
- SGR in percentage growth per day was calculated from two CT scans obtained at time of diagnosis and subsequent planning CTs immediately prior to radiochemotherapy as previously described (SGR = ln[1^{st} volume*2^{nd} volume]/[t2-t1]).
- Volumes in millilitres for PT and LN were calculated from maximum orthogonal diameters in all three planes applying an ellipsoid formula as previously described (volume = (π *[x*y*z/1000])/6).
- To explore the impact of SGR of PT and LN on overall survival, Kaplan Maier and Cox regression models were used and SGRs were categorized in groups with slow (< 0.3%/day), intermediate (0.3% ≤ 3%/day) and rapid (>3%/day) SGRs.
- To explore the correlation of SGR with EGFR, Ki67 and CD44, Jonckheere-Terpstra tests were used and the percentage of positive cells grouped in 4 groups (0%, 1-30%, 31-60% and more than 60%).

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KEYWORDS

head and neck squamous cell carcinoma; tumour volume; tumour growth rate; EGFR; Ki67; CD44;

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INTRODUCTION

In patients with head and neck squamous cell carcinoma (HNSCC), the median treatment waiting time in the US almost doubled from 19 to 30 days between 1998 and 2011. In a recent cancer registry based study, Murphy and co-authors analysed almost 275.000 patients with HNSCC of the most common cancer sites. The authors observed an independent effect of increased treatment waiting time on overall survival (OS)[1] and calculated 46 to 52 days as threshold for decreased OS[2]. Similar observations were reported for oral cancer in a recent review including 18 studies[3].

A likely reason for the association of treatment waiting time and decreased OS is meantime tumour growth. Mathematical models to approximate tumour growth from imaging data are available since the 1960s[4]. Originally, direct curve fitting to calculate tumor volume doubling time (DT) was the standard method to assess tumour growth. Recently, calculation of specific growth rate (SGR), defined as relative volume increase per unit of time, was proposed instead. It was reported more reliable for short time intervals and minor tumour volume differences.

Data on SGR of HNSCC is limited[5 6]. A median SGR for primary tumours (PT-SGR) of 0.74% per day in patients with oropharyngeal HNSCC waiting for RCT was reported by Murphy and colleagues. The authors assessed the PT-SGR in 85 patients between diagnostic CTs and planning CTs and concluded that rapid PT-SGR may predict treatment failure in these patients[5]. Van Bockel and co-authors reported a significant association between high PT-SGR and decreased OS (p=0.013) in 131 patients with laryngeal HNSCC[6].

In this retrospective study, we calculated SGR of the primary tumour (PT-SGR) and largest pathological cervical lymph node (LN-SGR) of patients with incident HNSCC from CTs obtained at diagnosis and from planning CTs obtained directly before RT/RCT. We investigated the influence of various factors including several biomarkers on PT-SGR and LN-SGR. We were further interested in the influence of SGR on OS and on the development of new lymph node necrosis and bone or cartilage infiltration.

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MATERIALS AND METHODS

Tumour registry population

Patients referred to the Department of Otorhinolaryngology – Head and Neck Surgery, Medical University of Innsbruck, Austria, between 2008 and 2016 with incident histologically confirmed HNSCC were recorded in the clinical tumour registry. Disease was staged according to the seventh edition of the UICC TNM staging system[7] by an interdisciplinary tumour board. Inclusion criteria comprised histologically proven incident HNSCC at any site of the head and neck region including cancer of unknown primary (CUP), any UICC Stage, RT or RCT as primary treatment and availability of both a diagnostic CT and a planning CT. The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants.

Diagnostic CT and planning CT

At the time of clinical diagnosis, diagnostic CT was performed following the standardized CT head & neck imaging protocols at the Department of Radiology, Medical University of Innsbruck. A GE-Medical Systems Light Speed VCT® or Light speed 16 CT scanner® (GE Medical, Vienna, Austria) was used. The scan area ranged from the frontal sinus to the upper mediastinum with a resolution of 512x512 pixels. Slices were calculated from raw data with 2 mm thickness, collimation of 24x1.2 mm and 0.45 pitch. Additional sagittal and coronal images were reconstructed. As contrast medium, Jopamiro 370® (Bracco Austria GmbH, Vienna, Austria) was administered intravenously adjusted to the patient's bodyweight. Planning CT scans were later performed at the Department of Radiation Oncology, Medical University of Innsbruck. Imaging protocols were followed as described above with the same CT scanners, contrast medium, scanning areas, resolutions and calculation protocols. Images from both CT scans were exported in Digital Imaging and Communications in Medicine (DICOM) format using IMPAX EE® (Agfa HealthCare, Bonn, Germany) Picture Archiving and Communication System (PACS®, Cerner, Kansas City USA). LN with a minimal axial diameter >10 mm, a central necrosis >3 mm or if present in neck levels close to the primary tumour in groups of >3 were classified pathological[8].

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Volume approximation, specific growth rate and tumour volume doubling time

Volumes were calculated as previously described[9]. In short, maximum orthogonal diameters in millimetres were measured for the primary tumour (PT) and the largest pathological cervical lymph (LN) in all three planes in axial and coronal sections (Figure 1). Volumes in millilitres were approximated employing an ellipsoid formula (volume = (π *[x*y*z/1000])/6). The largest cervical LN instead of all pathological cervical lymph nodes was considered sufficient for evaluation based on a high correlation previously observed[9]. Central lymph node necrosis and/or cartilage or bone infiltration of the primary tumour was recorded for additional analysis.

SGRs were assumed to be exponential and defined as the relative volume increase given in percent per day. For calculation of SGR, the equation described by Mehrara and co-authors was applied (SGR = ln[1st volume*2nd volume]/[t2-t1])[10]. For comparison with earlier studies, doubling times (DT) for primary tumours (PT-DT) and for largest pathological cervical lymph nodes (LN-DT) were calculated as the time difference*LN(2) divided by the logarithm of the volume ratio of the two observations[11].

Analysis of EGFR, Ki67 and CD44 expression

Tumour biopsies were collected in 4% buffered formalin, fixed overnight and embedded using the ethanol – isopropanol – wax quick 4mm protocol of Histos 5 embedding processor® (Milestone, Bergamo, Italy). Five-micrometre thick paraffin sections were dewaxed and antigen retrieval was performed in a Discovery automated staining system® (Ventana, Tucson, AZ, USA). Primary antibodies were added to the sections by automatic dispensing either as ready-to-use, pre-diluted, stabilized solutions provided by the manufacturers: cyclin dependent kinase inhibitor 2A (p16) INK4® (Ventana, Cat. Nr. 6595294001), Ki67 antigen (Ki67; Linaris E059®, clone MIB-1®, Dossenheim, Germany), CD44 antigen (CD44; Diagnostic Biosystems /Antibodies Online, ABIN1020059, Aachen, Germany) and Epidermal Growth Factor Receptor (EGFR; Invitrogen, Vienna, Austria). Immunohistochemical staining was completed by the Discovery automated staining system® (Ventana, Tucson, AZ, USA) using universal secondary antibody solution, haematoxilin counterstaining and the DAB MAP Kit (all Ventana products, Tucson, AZ, USA) as published previously[12]. All sections were stained with control mouse and rabbit immunoglobulins, using the same highest concentration as for the primary antibodies, and

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these controls were not reactive[13]. The immunohistochemical reactions were observed independently by two blinded observers, who collected 10 representative tumour cell nests from each specimen[14]. These regions were analysed on an Olympus BX50 microscope® (Olympus, Tokyo, Japan) and the staining intensity and representation of tumour cell nests were scored as previously described[15]. The cut-off for p16-positivity was 60% or more positive tumour cells[16].

Data analysis

Frequency data were presented in tabular form. For continuous data (volumes and growth rates), means and standard deviations (SD) as well as medians and 25th ($p25^{th}$) and 75th percentiles ($p75^{th}$) were provided. The median follow-up time was calculated as described by Schemper and Smith[17]. Logarithmic transformation was used to analyse volumetric data in regression models. Kruskal-Wallis and Jonckheere-Terpstra tests were used to evaluate the univariate influence of ordinal factors on growth rates. For survival analyses, Kaplan Maier and Cox regression models were used. For Kaplan Meier plots, growth rates were categorized in groups with slow, intermediate and rapid growth rates. All calculations were performed with SPSS 23.0 (IBM Corp., Armonk, NY).

Patient and Public Involvement

The development of the research question was based on previous publications exploring tumor volume in HNSCC [9] and its prognostic value if treated primarily with surgery[18]. Neither patients nor the public were involved in the design of the study, the recruitment of the study or the conduct of the study.

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RESULTS

Study population

Between 2008 and 2016, 802 patients with incident HNSCC were recorded in the clinical cancer registry. Of 123 patients treated with primary RT or RCT, PT-volumes and LN-volumes were calculated from diagnostic and planning-CTs. In four patients with CUP-syndrome, no PT-volume and in 27 patients with N0 stage neck, no LN-volume could be measured. Of the 123 patients, 32 were female. The mean (± SD) age was 63±10 years ranging from 38 to 87 years. Median follow up time was 45 months (95% confidence interval 42 to 48 months). Additional clinical data of the 123 included patients is provided in table 1.

Table 1: Clinical data of included 123 HNSCC patients.

		No. of patients
Sex	male	89
	female	32
Age	≤ 50 years	11
	51 – 60 years	42
	61 – 70 years	39
	71 – 80 years	26
	≥ 80 years	5
p16	negative	83
	positive	38
Tumour site	oral cavity	16
	oropharynx	55
	hypopharynx	24
	larynx	21
	other*	5
Clinical UICC stage	stage I	4
	stage II	7
	stage III	17
	stage IVa	75
	stage IVb	16
	stage IVc	2

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Time interval, tumour and lymph node volumes

Mean (± SD) time interval between diagnostic CTs and planning CTs was 29±21 days ranging from 6 to 146 days. For PT, a total of 119 volume sets from diagnostic CT and planning CT were available (Figure 2). Mean PT-volume from diagnostic CTs was 16.3 (±20.4) mL ranging from 0 (CUP-syndrome) to 100.8 mL. Mean PT-volume from planning CTs was 24.5 (±26.8) mL ranging from 0 to 160.3 mL. The mean PT-volume increase of 6.7 (±17.2) mL during the period between 1st and 2nd CT scans was highly significant (p<0.001).

For LN, a total of 96 volume sets were available (Figure 2). Mean LN-volume from diagnostic CTs was 9.2 (±22.2) mL ranging from 0 (N0) to 156.2 mL. Mean LN-volume from planning CTs was 15.2 (±31.5) mL ranging from 0 to 233.5 mL. The mean LN-volume increase of 5.3 (±17.3) mL during the observation period was highly significant (p<0.001). Distributions of these parameters were right skewed. Medians and quartiles for PT-volume and LN-volume are provided in table 2.

Table 2: Volumes of primary tumours and largest pathological cervical lymph nodes in diagnostic and planning CTs in 123 patients with HNSCC. Median time interval between 1st and 2nd CT scans was 24 days. Specific growth rates were calculated as suggested by Mehrara and co-authors[10], tumour doubling-times were calculated as proposed by Schwartz[11].

	Diagnostic CT volume[1)] [ml]	Planning CT volume[1)] [ml]	SGR[2)] [%/day]	DT[3)]
Primary tumour	9.4 (3.3; 21.4)	16.7 (5.4; 31.6)	1.4 (0.6; 2.7)	43 (24; 85)
Lymph node	2.0 (0.2; 9.5;)	3.7 (0.6; 19.4)	1.2 (0.3; 2.5)	41 (25; 80)

1) Median (25th and 75th percentile)

2) Specific growth rate (median, 25th and 75th percentile) percent per day

3) Tumour doubling time in days (median, 25th and 75th percentile)

Specific growth rates (SGR) and tumour doubling times (DT)

Mean (±SD) PT-SGR was 1.8%±1.8%/day ranging from minus 2.6%/day (volume decrease) to 8.6%/day. Mean LN-SGR was 1.7%±2.0%/day ranging from minus 1.5% to 11.0%/day. PT-SGR

and LN-SGR were right-skewed. For medians and quartiles see table 2. Mean PT-SGR of tumours of the oral cavity, oropharynx, hypopharynx and larynx were 3.1%±1.5%/day, 1.8%±1.7%/day, 2.0%±2.0%/day and 1.5%±2.1%/day, respectively. Mean LN-SGR for the primary tumour sites oral cavity, oropharynx, hypopharynx and larynx were 3.4%±3.9%/day, 1.9%±2.2%/day, 1.9%±1.4%/day and 0.8%±1.1%/day, respectively. For tumour sites, medians and quartiles are provided in table 3.

Table 3: Specific growth rates (SGR) for primary tumours (PT) and largest pathological cervical lymph nodes (LN) for common tumour sites of 123 patients with incident HNSCC.

Tumour site	n	PT-SGR[1)]	LN-SGR[1)]
Oral cavity	15	2.4 (1.0; 3.9)	0.8 (0.0; 1.5)
Oropharynx	55	1.4 (0.7; 2.6)	2.5 (0.4; 2.6)
Hypopharynx	25	1.7 (0.6; 2.7)	2.1 (0.6; 2.9)
Larynx	21	1.0 (0.3; 3.1)	0.8 (0.0; 1.4)
Others*	5	0.1 (0.0; 0.1)	1.4 (1.1; 5.2)

1) Median (25th and 75th percentile)

*including nasopharynx, paranasal sinuses and salivary glands

Median PT-DT was 43 days. The 25th percentile was 24 and the 75th percentile 85 days. Median LN-DT was 41 days. The 25th percentile was 25 and the 75th percentile 80 days (Tab. 2). PT-DT and LN-DT were considerably right skewed.

Factors influencing specific growth rates

PT-SGR was independent of the initial PT-volume in diagnostic CT (p=0.19). PT-SGR did also not depend on the interval between 1st and 2nd CT (p=0.14). Moreover, tumour site had no significant impact on SGR (p=0.58; Tab. 3). Interestingly, PT-SGR positively correlated with the expression of EGFR, Ki67 and CD44 (p=0.02; p=0.02 and p=0.03, respectively; Figure 3). The expression of p16 had no influence on PT-SGR (p=0.21).

In contrast, LN-SGR depended on the initial LN-volume measured in diagnostic CT (p=0.003) with higher LN-SGRs in lymph nodes with larger LN-volumes in the 1st CT. Also, the interval

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between the diagnostic CT and planning CT significantly influenced LN-SGR (p=0.003). The longer the interval, the lower the observed LN-SGR. Moreover, LN-SGR was significantly influenced by tumour site (p=0.032; Tab. 3) with smallest growth rates in lymph nodes from laryngeal HNSCC (p=0.003). In contrast to PT-SGR, neither EGFR, Ki67, CD44 nor p16 expression in PTs significantly correlated with LN-SGR (p=0.12; p=0.31; p=0.75 and p=0.81, respectively).

Specific-growth-rate and overall survival

PT-SGR nearly missed significant impact on OS when used as a single covariate in a Cox regression model (log rank p=0.054). For Kaplan Meier analyses, PT-SGR were categorized in 3 groups: slow (< 0.3%/day; n=22); intermediate (0.3% ≤ 3%/day; n=73) and rapid (>3%/day; n=26). Survival curves of these 3 SGR groups did not differ significantly (log rank p=0.45; Figure 4). Likewise, LN-SGR had no significant impact on OS as a covariate in Cox regression (log rank p=0.83) nor in Kaplan Meier analyses (log rank p=0.97; data not shown).

Cartilage or bone infiltration and central lymph node necrosis

Cartilage or bone infiltration was observed in diagnostic CTs of 40/123 (33%) patients and in planning CTs of 50/123 (41%) patients. Thus, during the median 24 days interval between the two CTs, new cartilage or bone infiltration occurred in 10 patients, which was not influenced by PT-SGR (p=0.918). However, cartilage or bone infiltration had a significant negative impact on survival (p=0.003) and was significantly more frequent in p16 negative tumours (p=0.02).

Lymph node necrosis was observed in diagnostic CTs of 74/97 (76%) patients. LN-SGR was significantly higher in patient with lymph nose necrosis (p<0.001), was associated with poorer survival (p=0.03) and did not depend on p16 status. Lymph nose necrosis was observed in planning CTs of 82/97 (85%) patients. Thus, during the median 24 days interval between the two CTs, new lymph node necrosis occurred in 8 patients, which was not influenced by LN-SGR (p=0.818). Central lymph node necrosis had a significant negative impact on survival (p<0.05).

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DISCUSSION

Long waiting times for treatment of head and neck cancer result in decreased overall survival[1-3]. One reason might be meantime tumour growth. We examined tumour growth during the time between the initial diagnostic CT scan and the planning CT for RT or RCT in 123 patients with incident HNSCC. The mean interval between 1st and 2nd CT was 29 days ranging from 6 to 146 days. In this time, diagnostic work up, interdisciplinary tumour board presentation and pre-treatment procedures including dental treatments and application of percutaneous gastrostomies were carried out. PT- and LN-volumes in diagnostic and planning CTs were assessed using identical protocols. PT-SGR and LN-SGR were calculated as proposed by Mehrara and co-authors[10].

Due to the skewness of volumetric data, the median is considered an appropriate measure of central tendency, but the mean is also provided for comparison with previous publications. Mean PT-volume in diagnostic CTs was approximately 16 mL (median≈9 mL). In planning CTs of the same patients 3-4 weeks later, mean PT-volume was approximately 25 mL (median≈17 mL; Tab. 2). These PT-volumes are in line with previous publications reporting mean volumes of 11-37 mL[18-21]. Mean LN-volume measured in diagnostic CTs was 9 mL (median≈2 mL) and approximately 15 mL (median≈4 mL) in planning CTs (Tab 2). Previously reported mean LN-volumes were considerably larger with 22-25 mL[22 23]. However, in these studies the volume of all pathological cervical lymph nodes was measured instead of only the largest one as in this study.

Tumour and pathological lymph node growth can be reported as doubling time (DT) or specific growth rates (SGR). DT is the number of days the tumour needs to double its volume[4]. The lower the DT, the faster the tumour growth. Since DT was frequently reported in earlier studies, it was provided for comparison. SGR is defined as relative increase of volume in percent per day. The resulting variable is constant, linear and independent of the initially measured volumes[10]. SGR is considered less affected by measurement uncertainties of short time intervals and minor volume differences than DT[10]. For both, DT and SGR, tumour volumes are required. Since the reference method of slice-by-slice segmentation to measure PT- and LN-volumes is time-

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consuming, a volume approximation method applicable over a wide range of PT and LN sizes was used. This approximation is based on the measurement of maximum orthogonal diameters employing an ellipsoid formula resulting in a slight underestimation of 8% of the PT-volume measured with the reference method[9]. A similar approximation method has also been used by Jensen and co-authors[24].

Mean primary tumour specific growth rates (PT-SGR) in incident HNSCC was 1.8%/day (Tab. 2), unimodally distributed, slightly right skewed and independent of the initial PT-volume, which supports the basic concept underlying SGR calculation. The interval between diagnostic CT and planning CT did not influence PT-SGR (p=0.1), suggesting that no marked growth deceleration occurred with longer waiting times. Interestingly, PT-SGRs did not significantly differ by tumour site (p=0.6; Tab 3). Murphy and colleagues observed a lower median SGR of 0.74%/day in patients with oropharyngeal cancer[5]. However, the authors did not include sites with faster tumour growth such as the oral cavity (median SGR 2.4%/day) and hypopharynx (median SGR 2.0%/day). Moreover, most of the patients reported by Murphy and colleagues had T1 or T2 HNSCC and were p16 positive.

The median PT-DT in this study was 43 days. The 25th percentile was 24 and the 75th percentile 85 days. Median LN-DT was 41 days. The 25th percentile was 25 and the 75th percentile 80 days (Tab. 2). Jensen and co-authors reported a slower growth rate in 61 patients with HNSCC with a median PT-DT of 99 days. The authors however additionally stated that half of the patients showed a faster growth rate with a PT-DT of 30 days[24]. When compared with DT of other solid tumours, this means that HNSCC reveal rapid tumour growth. For breast cancer, Ingebly and co-authors reported a median DT of 285 days[4]. For lung cancer, DTs vary depending on histology between median 42 days for metastases as reported by Loeffler and colleagues and 181 for non-small-cellular lung cancer as reported by Winer-Muram and co-workers[4 25]. For pancreatic adenocarcinoma, Furkawa and colleagues reported a mean PT-DT of 144 days[25]. For Sarcomas median PT-DTs of 35 days were reported by Blomqvist and co-authors[25]. Additional median PT-DTs for additional solid tumours are provided in table 4.

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Table 4: Reported tumour volume doubling times for selected solid tumours*.

Tumour	Median DT [days]	n
Present study	47	123
HNSCC[24]	99**	61
Breast cancer[4]	285	16
Lung bronchioalveolarcarcinoma[25]	181	9
Non small-cell lung carcinoma[25]	181	6
Lung metastasis[4]	42	24
Pancreatic adenocarcinoma[25]	144	9
Sarcoma[25]	35	21

*modified after Mehrara and co-authors[25]

**Jensen and co-workers also stated tumour volume doubling times for the faster growing half of the patients with a median PT-DT of 30[24].

PT-SGRs significantly correlated with the expression of EGFR, Ki67 and CD44 (p=0.021; p=0.018 and p=0.031, respectively) with higher PT-SGRs in patients with higher expression of the three biomarkers (Figure 3). These correlations appear biologically sound. A correlation of clinical tumour growth rates and expression of these biomarkers within the tumour of the same patients was not yet reported. EGFR is a cell surface receptor which promotes proliferation, invasion, angiogenesis and metastatic spread in HNSCC, if overexpressed[26]. Ki-67 is a nuclear protein expressed on cells in all phases of the cell cycle except in G0-phase. Thus, its expression marks the total fraction of proliferating cells in a tumour[27]. In laryngeal and hypopharyngeal SCC, an association between Ki67 expression and advanced tumour stages has been reported[28] [29]. In line with our observation of a correlation of CD44 expression and PT-SGR, an association between advanced T categories and high CD44 expressions has been reported in a meta-analysis including thirty studies with 2102 patients[30]. No significant correlation between PT-SGR and p16 expression was observed (p=0.81). This observation differs from previous observations made by Murphy and colleagues. The authors observed that p16 expression correlated well with

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PT-SGR with faster growth rates in p16 negative HNSCCs. However, the authors included oropharyngeal HNSCC only and the majority (79%) were p16 positive tumours[5]. When used as a covariate in a Cox model, PT-SGR had no significant effect on OS (log rank p=0.054). No significant differences of survival curves were also observed, if PT-SGR were categorized in groups of low, medium and high growth rates (log rank p=0.5; Figure 4). In contrast, van Bockel and co-worker observed a significant association between PT-SGR and OS in laryngeal cancer[6].

To our knowledge, no data on lymph node specific growth rates (LN-SGR) in HNSCC have been previously reported. In the investigated patients, the mean LN-SGR was 1.7%/day, similar to mean PT-SGR (Tab. 2). In contrast to PT-SGR, LN-SGR did depend on initial LN-volumes in diagnostic CT scans (p=0.003) with higher LN-SGRs in lymph nodes with larger initial LN-volumes. Also, the interval between diagnostic CT and planning CT significantly influenced LN-SGR (p=0.003). The longer the interval, the lower the observed LN-SGR, suggesting growth slowdown. Moreover, LN-SGR was significantly influenced by tumour site (p=0.03; Tab. 3), with smallest growth rates in lymph nodes from laryngeal HNSCC. In contrast to PT-SGR, LN-SGR did not depend on expressions of EGFR, Ki67, CD44 nor p16 expression in primary tumours (p=0.115; p=0.311; p=0.746 and p=0.809 respectively). In LN, specific growth rates had no significant impact on OS either (p>0.05).

New cartilage or bone infiltrations during the observation period were observed in 10 patients and new central lymph node necroses were observed 8 patients. Specific growth rates had no significant impact on the development of either of them. Both, initial cartilage or bone infiltration and central lymph node necrosis were associated with worse survival. However, probably due to the low number of events, no significant impact of new cartilage or bone infiltration nor new central lymph node necrosis on survival was observed in Kaplan Meier analysis (both log rank p>0.05).

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CONCLUSION

Head and neck squamous cell carcinomas are rapidly growing malignancies. Primary tumours and lymph nodes grow more than 1% per day. Consequently, time matters, and treatment must not be delayed.

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ABBREVIATIONS

CD44	CD44 antigen
CT	computed tomography
DICOM	Digital Imaging and Communications in Medicine
DT	tumour volume doubling time
EGFR	epidermal growth factor receptor
HNSCC	head and neck squamous cell carcinoma
Ki67	Ki67 antigen
LN	largest pathological cervical lymph node
LN-DT	largest pathological cervical lymph node doubling time
LN-SGR	largest pathological cervical lymph node specific growth rate
p16	Cyclin-dependent kinase inhibitor 2A
PACS	Picture Archiving and Communication System
PT	primary tumour
PT-DT	primary tumour volume doubling time
PT-SGR	primary tumour specific growth rate
RCT	radiochemotherapy
RT	radiotherapy
SD	standard deviation
SGR	specific growth rate
TNM	tumour, node, metastasis
UICC	Union internationale contre le cancer

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REQUIRED STATEMTENTS

Acknowledgements

This work has not yet been previously presented at a conference or as a conference abstract.

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Ethics statement

The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Competing interest and funding statement

All authors have completed the Unified Competing Interest form and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

Contributorship statement

Daniel Dejaco has written major parts of the manuscript and performed the calculations of tumour and lymph node volumes. Teresa Steinbichler was involved in establishing the study concept, design and the internal review process of the manuscript. Schartinger Volker established the population based tumour registry from which patients were enrolled. He further obtained the ethics committee approval for the study. Natalie Fischer performed additional tumor and pathological cervical lymph node measurements and was involved in the internal

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review process of the manuscript. Maria Anegg has performed additional calculations and data base work in the population based tumour registry. Joszef Dudas performed the immunohistochemical staining of the tumour biopsies and was involved in establishing the study concept, design and the internal review process of the manuscript. Andrea Posch provided the radiotherapy-planning CT scans and performed performed additional tumor and pathological cervical lymph node measurements. Gerlig Widmann supervised the tumor and pathological cervical lymph node measurements as an experienced head and neck radiologist. Herbert Riechelmann supervised the writing process of the manuscript and performed all statistical calculations in the manuscript.

Transparency declaration

The Corresponding Author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study have been explained.

Details of ethical approval and patient consent

The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants.

Data sharing

No additional data from the study is available.

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FIGURE LEGENDS

Figure 1: Tumour growth assessment using maximal orthogonal tumour diameters from axial contrast enhanced diagnostic CT scans (A) and subsequent planning CT scans (B)

Axial contrast enhanced diagnostic CT scan (A) and subsequent planning CT scan (B) 46 days later of a cT4a cN2b cM0 squamous cell carcinoma of the oral cavity. The maximum anterior-posterior and medio-lateral tumour diameters (white lines) were measured from axial scans, the cranio-caudal tumour diameters were measured from corresponding coronal scans (not depicted). The tumour volume was assessed as previously described using an ellipsoid formula[9]. PT-volume from diagnostic CT was 14.8 mL, PT-volume from planning CT was 51.0 mL translating to a PT-SGR of 2.8%/day.

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Figure 2: Correlation of primary tumour volume (A) and largest pathological lymph node volume (B) measured from diagnostic CT scans and a planning CT scans

Scattergram of primary tumour volumes (A) and largest pathological lymph node volume (B) measured from diagnostic CT scans (x-axis) planning CT scans (y-axis). Both axes are on log scale. The diagonal line represents the line of identity. Dots above this line indicate volume increases.

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Figure 3: Specific growth rates of the primary tumours and percentage of Ki67 positive cells

Percentage of Ki67 positive immunohistochemistry in tumour samples of HNSCC patients and according primary tumour specific growth rates. The percentage of Ki67 positive cells was grouped in 0%, 1 to 30%, 31 to 60% and more than 60% of cancer cells (x-axis). Mean specific growth rates of HNSCC primary tumour (y-axis) were obtained 119 patients. Small bars represent standard deviation. PT-SGR positively correlated with the expression of Ki67 (Jonckheere-Terpstra p=0.02).

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Figure 4: Kaplan Maier plot for specific growth rates of primary tumours grouped by low, medium and high growth rate

Kaplan Maier plot of specific growth rates (SGR) of primary tumours grouped by low (black line; SGR< 0.3%/day; n=22); medium (dark grey line; 0.3% ≤ SGR < 3%/day; n=73) and high (pale grey line; SGR >3%/day; n=26) growth rates. X-axis represents time in months, Y-axis overall survival. The survival curves of the 3 specific growth rate groups did not differ significantly (log rank p=0.45).

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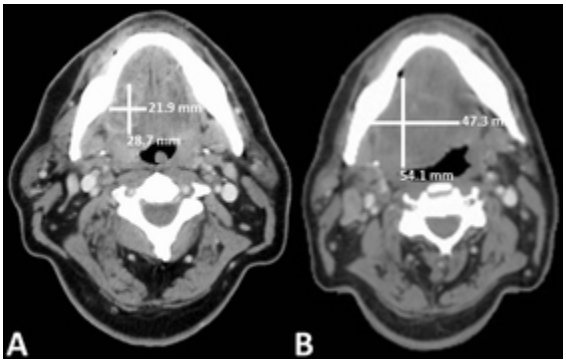

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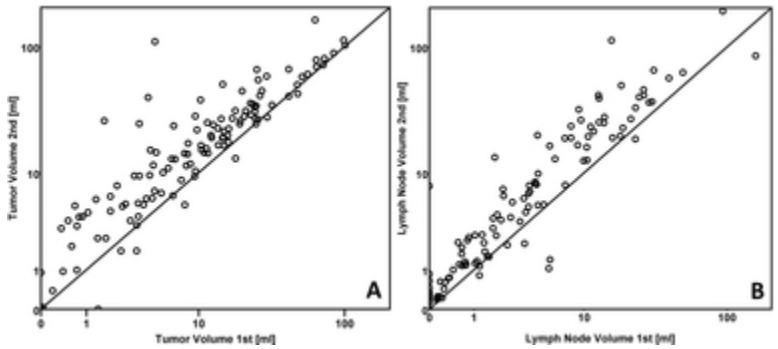

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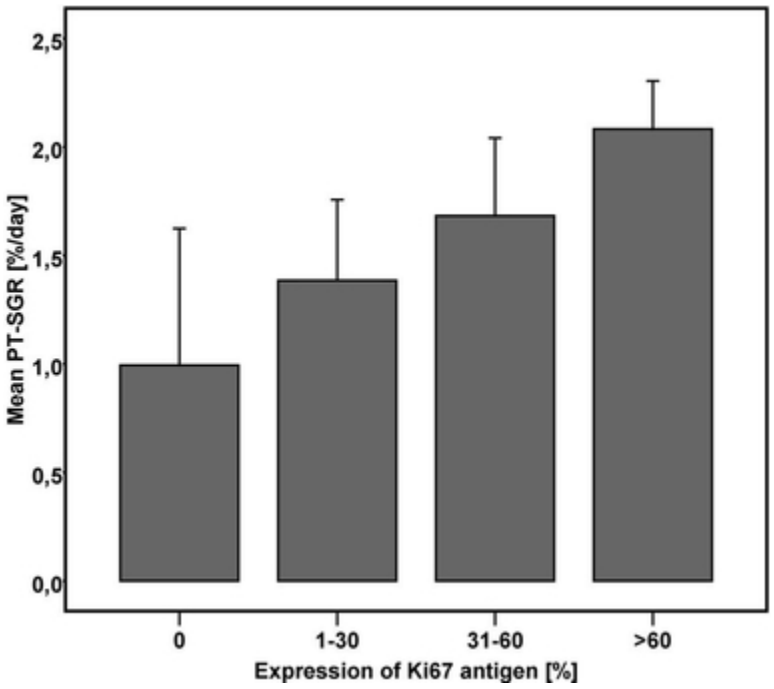

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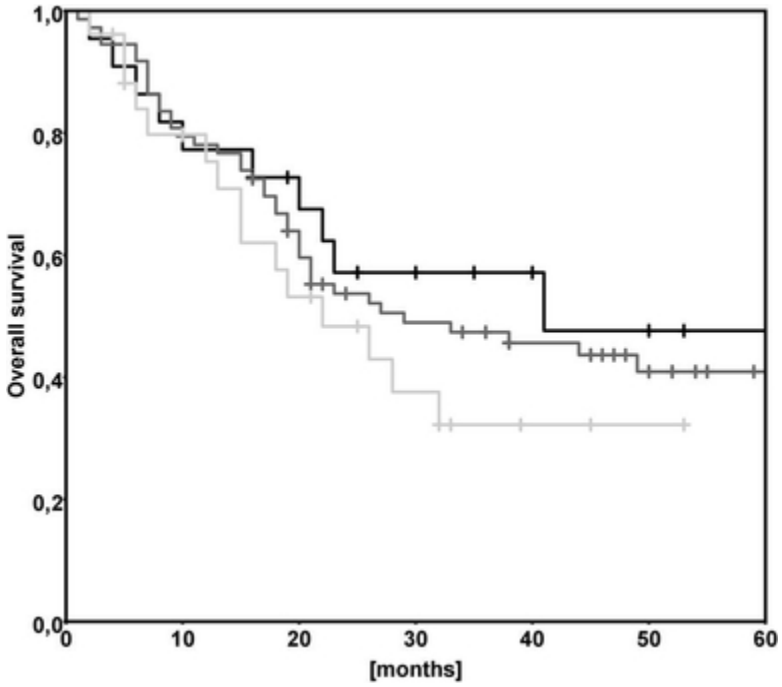

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STROBE Statement: "Time matters – the fast growth of head and neck squamous cell carcinoma"

	Item No	Answer
Title and abstract	1	(*a*) The retrospective study design was described in the "Abstract" section, page 2, line 7, the "Strengths and Limitations of the Study" section, page 3 and the "Introduction" section, page 5.
		(*b*) An informative and balanced summary of what was done was provided in the "Abstract" section, page 2.
Introduction		
Background/rationale	2	The scientific background and rational for the investigation was reported in the "Introduction" section of the manuscript, page 5.
Objectives	3	The objective of the study was specified in the "Abstract" section, page 2 and at the end of the "Introduction" section, page 2.
Methods		
Study design	4	Key elements of the study design were mentioned early in the "Abstract" section, page 2 and the "Introduction" section, page 5 of the manuscript. Additional details of the study design were outlined in the "Methods", page 6 to 8.
Setting	5	The setting, location, relevant dates, including period of recruitment and data collection was included in the "Methods" section, page 6 and in the "Results" section, page 9.
Participants	6	(*a*) *Cohort study*— The eligibility criteria and the sources and methods of selection of participants are provided in detail in the "Methods" section, page 6.
		(*b*) *Cohort study*— No matching was performed in this study.
Variables	7	All outcomes, predictors, potential confounders and effect modifiers were defined, presented and discussed in the "Methods" section, page 6 to 8, "Results" section, page 9 to 12and "Discussion" section, page 13 to 16. Diagnostic criteria were provided in the "Methods" section, page 6, when applicable.
Data sources/ measurement	8*	Source of data and details of methods was provided in the "Methods" section, page 6 and 8.
Bias	9	Potential sources of bias were addressed in the "Discussion" section, page 13 to 16.
Study size	10	An explanation how the study size was arrived at was provided in the "Method" section, page 6.
Quantitative variables	11	An explanation how quantitative variables were handled in the analyses was provided in the "Methods" section, page 8.
Statistical methods	12	(*a*) All statistical methods were described in the "Methods" section, page 8.
		(*b*) All methods for subgroup analyses of the presented study were described in the "Methods" section, page 8.
		(*c*) An explanation how missing data was addressed was provided in the "Methods" section, page 8, "Results" section, pages 9 to 12 and "Discussion" section, pages 13 to 16.
		(*d*) *Cohort study*—No loss to follow-up occurred due to the study design.
		(*e*) No sensitivity analyses were performed in the study.

Continued on next page

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Results		
Participants	13*	(a) Numbers of individuals at each stage of the study was reported in the "Results" section, page 9 and the "Discussion" section, page 13.
		(b) No non-participation occurred due to the study design.
		(c) No flow diagram was used for this study.
Descriptive data	14*	(a) Clinical characteristics of study participants were provided in table 1, page 9.
		(b) Number of participants with missing data for each variable of interest was provided in the "Results" section, page 9 and in table 1, page 9.
		(c) *Cohort study*—Follow-up time was summarised as time between diagnostic computed tomography scan and planning computed tomography scan in this study provided in the "Results" section, page 9.
Outcome data	15*	*Cohort study*— Numbers of outcome events was provided in the "Results" section, page 9 to 12.
Main results	16	(*a*) Means, medians, standard deviations, 95% confidence intervals and 25th and 75th percentiles were provided in the "Results" section, page 9 to 12, if applicable.
		(*b*) Category boundaries for continuous variables were specified in the "Methods" section, page 8 and "Results" section, pages 9 to 12.
		(*c*) No relative risk estimations were performed in this study.
Other analyses	17	All additional other analyses performed in the study were outlined in the "Methods" section, page 8 and "Results" section, pages 9 to 12.
Discussion		
Key results	18	Key results with reference to previously outlined study objectives were summarised in the "Discussion" section, pages 13 to 16.
Limitations	19	Limitations of the study, taking into account sources of potential bias and imprecision were outlined in the "Discussion" section, pages 13 to 16.
Interpretation	20	A cautious overall interpretation of the results was given in the "Discussion", pages 13 to 16and the "Conclusion" section, page 17.
Generalisability	21	The external validity of the study results was outlined in the "Discussion" section, pages 13 to 16 and table 4, page 15.
Other information		
Funding	22	No financial support for any of the work presented in the present manuscript was obtained, provided on page 19.

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Specific growth rates calculated from computed tomographies in patients with head and neck squamous cell carcinoma: a retrospective study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-025359.R1
Article Type:	Research
Date Submitted by the Author:	27-Nov-2018
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Primary Subject Heading:	Oncology
Secondary Subject Heading:	Ear, nose and throat/otolaryngology, Diagnostics, Radiology and imaging
Keywords:	head and neck squamous cell carcinoma, tumour volume, tumour growth rate, EGFR, Ki67, CD44

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Specific growth rates calculated from computed tomographies in patients with head and neck squamous cell carcinoma: a retrospective study

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Word count: 3848
Abstract: 238
References: 32
Figures: 4
Tables: 4

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ABSTRACT

OBJECTIVE: To provide data on specific growth rates (SGR) of primary tumours (PT-SGR) and largest pathological cervical lymph nodes (LN-SGR) for head and neck squamous cell carcinoma (HNSCC). To explore PT-SGR's and LN-SGR's correlation with selected biomarkers EGFR, Ki67 and CD44.

DESIGN AND SETTING: Retrospective study performed at a tertiary oncologic referral centre in Innsbruck, Austria.

PARTICIPANTS: Adult patients with incident HNSCC treated with primary radiotherapy (RT) or radiochemotherapy (RCT).

OUTCOME MEASURES: Volumes of the primary tumour (PT-volume) and largest pathological cervical lymph node (LN-volume) were measured in computed tomography (CT) scans obtained at time of diagnosis and subsequent planning CTs immediately prior to RT or RCT. SGRs were calculated assuming an exponential growth function. PT-SGR's and LN-SGR's correlation with EGFR, Ki67 and CD44 were explored.

RESULTS: In 123 patients, mean interval between diagnostic and planning CT was 29±21 days. PT-SGR was 1.8%±1.8% (mean±SD) per day and was positively correlated with EGFR, Ki67 and CD44 expression (p=0.02; p=0.02; p=0.03). LN-SGR was 1.7%±2.0% per day and increased with larger initial LN-volume, was lower in laryngeal cancer (p=0.003) and slowed down with time. LN-SGR was not correlated with EGFR, Ki67 or CD44 expression in primary tumours (p>0.12). New cartilage or bone infiltration occurred in 10 patients and new central lymph node necrosis in 8 patients.

CONCLUSIONS: HNSCC are fast growing tumours for which treatment must not be delayed. Clinical tumour growth rates are influences by EGFR, KI67 and CD44 expression.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- In patients with incident head and neck squamous cell carcinoma (HNSCC), specific growth rates (SGR) for primary tumours (PT) and largest pathological cervical lymph nodes (LN) were retrospectively calculated.
- SGR in percentage growth per day was calculated from two CT scans obtained at time of diagnosis and subsequent planning CTs immediately prior to radiochemotherapy as previously described (SGR=ln[1stvolume*2ndvolume]/[t2-t1]).
- Volumes in millilitres (mL) for PT and LN were calculated from maximum orthogonal diameters in all three planes applying an ellipsoid formula as previously described (volume=(π*[x*y*z/1000])/6).
- To explore the impact of SGR of PT and LN on overall survival, Kaplan Maier and Cox regression models were used and SGRs were categorized in groups with slow (< 0.3%/day), intermediate (0.3% ≤ 3%/day) and rapid (>3%/day) SGRs.
- To explore the correlation of SGR with EGFR, Ki67 and CD44, Jonckheere-Terpstra tests were used and the percentage of positive cells grouped in 4 groups (0%, 1-30%, 31-60% and more than 60%).
- Limitations include retrospective study design, small number of patients, small interval of observation, lack of more modern imaging- and segmentation techniques.

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KEYWORDS

head and neck squamous cell carcinoma; tumour volume; tumour growth rate; EGFR; Ki67; CD44;

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INTRODUCTION

In patients with head and neck squamous cell carcinoma (HNSCC), the median treatment waiting time in the US almost doubled from 19 to 30 days between 1998 and 2011. In a recent cancer registry based study, Murphy and co-authors analysed almost 275.000 patients with HNSCC of the most common cancer sites. The authors observed an independent effect of increased treatment waiting time on overall survival (OS)[1] and calculated 46 to 52 days as threshold for decreased OS[2]. Similar observations were reported for oral cancer in a recent review including 18 studies[3].

A likely reason for the association of treatment waiting time and decreased OS is meantime tumour growth. Mathematical models to approximate tumour growth from imaging data are available since the 1960s[4]. Originally, direct curve fitting to calculate tumour volume doubling time (DT) was the standard method to assess tumour growth[4]. Recently, calculation of specific growth rate (SGR), defined as relative volume increase per unit of time, was proposed instead. It was reported more reliable for short time intervals and minor tumour volume differences[5].

Data on SGR of HNSCC is limited[6-7]. A median SGR for primary tumours (PT-SGR) of 0.74% per day in patients with oropharyngeal HNSCC waiting for RCT was reported by Murphy and colleagues. The authors assessed the PT-SGR in 85 patients between diagnostic CTs and planning CTs and concluded that rapid PT-SGR may predict treatment failure in these patients[6]. Van Bockel and co-authors reported a significant association between high PT-SGR and decreased OS (p=0.013) in 131 patients with laryngeal HNSCC[7].

In this retrospective study, we calculated SGR[5] of the primary tumour (PT-SGR) and largest pathological cervical lymph node (LN-SGR) of patients with incident HNSCC from CTs obtained at diagnosis and from planning CTs obtained directly before RT/RCT. We investigated the influence of various factors including several biomarkers on PT-SGR and LN-SGR, which were previously observed to be associated with tumour proliferation[8-12]. We were further interested in the influence of SGR on OS and on the development of new lymph node necrosis and bone or cartilage infiltration.

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MATERIALS AND METHODS

Tumour registry population

Patients referred to the Department of Otorhinolaryngology – Head and Neck Surgery, Medical University of Innsbruck, Austria, between 2008 and 2016 with incident histologically confirmed HNSCC were recorded in the clinical tumour registry. Disease was staged according to the seventh edition of the UICC TNM staging system[13] by an interdisciplinary tumour board. Inclusion criteria comprised histologically proven incident HNSCC at any site of the head and neck region including cancer of unknown primary (CUP), any UICC Stage, RT or RCT as primary treatment and availability of both a diagnostic CT and a planning CT. The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants.

Diagnostic CT and planning CT

At the time of clinical diagnosis, diagnostic CT was performed following the standardized CT head & neck imaging protocols at the Department of Radiology, Medical University of Innsbruck. A GE-Medical Systems Light Speed VCT® or Light speed 16 CT scanner® (GE Medical, Vienna, Austria) was used. The scan area ranged from the frontal sinus to the upper mediastinum with a resolution of 512x512 pixels. Slices were calculated from raw data with 2 millimetres (mm) thickness, collimation of 24x1.2mm and 0.45 pitch. Additional sagittal and coronal images were reconstructed. As contrast medium, Jopamiro 370® (Bracco Austria GmbH, Vienna, Austria) was administered intravenously adjusted to the patient's bodyweight. Planning CT scans were later performed at the Department of Radiation Oncology, Medical University of Innsbruck. Imaging protocols were followed as described above with the same CT scanners, contrast medium, scanning areas, resolutions and calculation protocols. Images from both CT scans were exported in Digital Imaging and Communications in Medicine (DICOM) format using IMPAX EE® (Agfa HealthCare, Bonn, Germany) Picture Archiving and Communication System (PACS®, Cerner, Kansas City USA). LN with a minimal axial diameter >10mm, a central necrosis >3mm or if present in neck levels close to the primary tumour in groups of >3 were classified pathological[14].

Volume approximation, specific growth rate and tumour volume doubling time

Volumes were calculated as previously described[15]. In short, maximum orthogonal diameters in mm were measured for the primary tumour (PT) and the largest pathological cervical lymph (LN) in all three planes in axial and coronal sections (Figure 1). Volumes in millilitres (mL) were approximated employing an ellipsoid formula (volume=(π*[x*y*z/1000])/6). The largest cervical LN instead of all pathological cervical lymph nodes was considered sufficient for evaluation based on a high correlation previously observed[15]. Central lymph node necrosis and/or cartilage or bone infiltration of the primary tumour was recorded for additional analysis.

SGRs were assumed to be exponential and defined as the relative volume increase given in percent per day. For calculation of SGR, the equation described by Mehrara and co-authors was applied (SGR=ln[1stvolume*2ndvolume]/[t2-t1])[5]. For comparison with earlier studies, doubling times (DT) for primary tumours (PT-DT) and for largest pathological cervical lymph nodes (LN-DT) were calculated as the time difference*LN divided by the logarithm of the volume ratio of the two observations[16].

Analysis of EGFR, Ki67 and CD44 expression

Tumour biopsies were collected in 4% buffered formalin, fixed overnight and embedded using the ethanol – isopropanol – wax quick 4mm protocol of Histos 5 embedding processor® (Milestone, Bergamo, Italy). Five-micrometre thick paraffin sections were dewaxed and antigen retrieval was performed in a Discovery automated staining system® (Ventana, Tucson, AZ, USA). Primary antibodies were added to the sections by automatic dispensing either as ready-to-use, pre-diluted, stabilized solutions provided by the manufacturers: cyclin dependent kinase inhibitor 2A (p16) INK4® (Ventana, Cat. Nr. 6595294001), Ki67 antigen (Ki67; Linaris E059®, clone MIB-1®, Dossenheim, Germany), CD44 antigen (CD44; Diagnostic Biosystems /Antibodies Online, ABIN1020059, Aachen, Germany) and Epidermal Growth Factor Receptor (EGFR; Invitrogen, Vienna, Austria). These three biomarkers were chosen because an association with tumour proliferation had been reported[8-12]: 1) EGFR is a cell surface receptor that promotes proliferation, invasion, angiogenesis and metastatic spread in HNSCC, if overexpressed[8]. Thus, high expression of EGRF might suggest higher PT-SGR. 2) Ki67 is a nuclear protein expressed on cells in all phases of the cell cycle except in G0-phase. Thus, its expression marks the total

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fraction of proliferating cells in a tumour[9-11], which might be correlated with PT-SGR. 3) Positive correlations between CD44 expression and advanced T categories were previously reported in a meta-analysis including thirty studies with 2102 patients[12]. Since T category is primarily based on maximal tumour diameter, a possible positive correlation between CD44 expression and PT-SGR was suggested. Immunohistochemical staining was completed by the Discovery automated staining system® (Ventana, Tucson, AZ, USA) using universal secondary antibody solution, haematoxilin counterstaining and the DAB MAP Kit (all Ventana products, Tucson, AZ, USA) as published previously[17]. All sections were stained with control mouse and rabbit immunoglobulins, using the same highest concentration as for the primary antibodies, and these controls were not reactive[18]. The immunohistochemical reactions were observed independently by two blinded observers, who collected 10 representative tumour cell nests from each specimen[19]. These regions were analysed on an Olympus BX50 microscope® (Olympus, Tokyo, Japan) and the staining intensity and representation of tumour cell nests were scored as previously described[20]. The cut-off for p16-positivity was 70% or more positive tumour cells[21].

Data analysis

Frequency data were presented in tabular form. For continuous data (volumes and growth rates), means and standard deviations (SD) as well as medians and 25th ($p25^{th}$) and 75th percentiles ($p75^{th}$) were provided. The median follow-up time was calculated as described by Schemper and Smith[22]. Logarithmic transformation was used to analyse volumetric data in regression models. Kruskal-Wallis and Jonckheere-Terpstra tests were used to evaluate the univariate influence of ordinal factors on growth rates. For survival analyses, Kaplan Maier and Cox regression models were used. For Kaplan Meier plots, growth rates were categorized in groups with slow, intermediate and rapid growth rates. All calculations were performed with SPSS 23.0 (IBM Corp., Armonk, NY).

Patient and Public Involvement

The development of the research question was based on previous publications exploring tumor volume in HNSCC[15] and its prognostic value if treated primarily with surgery[23]. Neither patients

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nor the public were involved in the design of the study, the recruitment of the study or the conduct of the study.

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RESULTS

Study population

Between 2008 and 2016, 802 patients with incident HNSCC were recorded in the clinical cancer registry. Of 123 patients treated with primary RT or RCT, PT-volumes and LN-volumes were calculated from diagnostic and planning-CTs. Tumour sites included oral cavity, oropharyngeal, hypopharyngeal and laryngeal HNSCC. No patients with tumours of the nasopharynx, the paranasal sinuses or salivary glands were included. In five patients with CUP-syndrome, no PT-volume and in 27 patients with N0 neck, no LN-volume could be measured. Of the 123 patients, 32 were female. The mean±SD age was 63±10 years ranging from 38 to 87 years. Median follow up time was 45 months (95% confidence interval 42 to 48 months). Additional clinical data of the 123 included patients is provided in table 1.

Table 1: Clinical data of included 123 HNSCC patients.

		No. of patients
Sex	male	89
	female	32
Age	≤ 50 years	11
	51 – 60 years	42
	61 – 70 years	39
	71 – 80 years	26
	≥ 80 years	5
p16	negative	83
	positive	38
Tumour site	oral cavity	16
	oropharynx	55
	hypopharynx	24
	larynx	21
	carcinoma of unknown primary	5
Clinical UICC stage	stage I	4
	stage II	7
	stage III	17
	stage IVa	75
	stage IVb	16
	stage IVc	2

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Time interval, tumour and lymph node volumes

Mean±SD time interval between diagnostic CTs and planning CTs was 29±21 days ranging from 6 to 146 days. For PT, a total of 119 volume sets from diagnostic CT and planning CT were available (Figure 2). Mean PT-volume from diagnostic CTs was 16.3±20.4mL ranging from 0 (CUP-syndrome) to 100.8mL. Mean PT-volume from planning CTs was 24.5±26.8mL ranging from 0 to 160.3mL. The mean PT-volume increase of 6.7±17.2mL during the period between 1st and 2nd CT scans was highly significant (p<0.001).

For LN, a total of 96 volume sets were available (Figure 2). Mean LN-volume from diagnostic CTs was 9.2±22.2mL ranging from 0 (N0) to 156.2mL. Mean LN-volume from planning CTs was 15.2±31.5mL ranging from 0 to 233.5mL. The mean LN-volume increase of 5.3±17.3mL during the observation period was highly significant (p<0.001). Distributions of these parameters were right skewed. Medians and quartiles for PT-volume and LN-volume are provided in table 2.

Table 2: Volumes of primary tumours and largest pathological cervical lymph nodes in diagnostic and planning CTs in 123 patients with HNSCC. Median time interval between 1st and 2nd CT scans was 24 days. Specific growth rates were calculated as suggested by Mehrara and co-authors[5], tumour doubling-times were calculated as proposed by Schwartz[16].

	Diagnostic CT volume[1)] [ml]	Planning CT volume[1)] [ml]	SGR[2)] [%/day]	DT[3)]
Primary tumour	9.4 (3.3; 21.4)	16.7 (5.4; 31.6)	1.4 (0.6; 2.7)	43 (24; 85)
Lymph node	2.0 (0.2; 9.5;)	3.7 (0.6; 19.4)	1.2 (0.3; 2.5)	41 (25; 80)

1) Median (25th and 75th percentile)

2) Specific growth rate (median, 25th and 75th percentile) percent per day

3) Tumour doubling time in days (median, 25th and 75th percentile)

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Specific growth rates (SGR) and tumour doubling times (DT)

Mean±SD PT-SGR was 1.8%±1.8%/day ranging from minus 2.6%/day (volume decrease) to 8.6%/day. Mean LN-SGR was 1.7%±2.0%/day ranging from minus 1.5% to 11.0%/day. PT-SGR and LN-SGR were right-skewed. For medians and quartiles see table 2. Mean PT-SGR of tumours of the oral cavity, oropharynx, hypopharynx and larynx were 3.1%±1.5%/day, 1.8%±1.7%/day, 2.0%±2.0%/day and 1.5%±2.1%/day, respectively. Mean LN-SGR for the primary tumour sites oral cavity, oropharynx, hypopharynx and larynx were 3.4%±3.9%/day, 1.9%±2.2%/day, 1.9%±1.4%/day and 0.8%±1.1%/day, respectively. For tumour sites, medians and quartiles are provided in table 3.

Table 3: Specific growth rates (SGR) for primary tumours (PT) and largest pathological cervical lymph nodes (LN) for common tumour sites of 123 patients with incident HNSCC.

Tumour site	n	PT-SGR[1)]	LN-SGR[1)]
Oral cavity	15	2.4 (1.0; 3.9)	0.8 (0.0; 1.5)
Oropharynx	55	1.4 (0.7; 2.6)	2.5 (0.4; 2.6)
Hypopharynx	25	1.7 (0.6; 2.7)	2.1 (0.6; 2.9)
Larynx	21	1.0 (0.3; 3.1)	0.8 (0.0; 1.4)
Carcinoma of unknown primary	5	0.0 (0.0; 0.0)	1.4 (1.1; 5.2)

1) Median (25th and 75th percentile)

Median PT-DT was 43 days. The 25th percentile was 24 and the 75th percentile 85 days. Median LN-DT was 41 days. The 25th percentile was 25 and the 75th percentile 80 days (Tab. 2). PT-DT and LN-DT were considerably right skewed.

Factors influencing specific growth rates

PT-SGR was independent of the initial PT-volume in diagnostic CT (p=0.19). PT-SGR did also not depend on the interval between 1st and 2nd CT (p=0.14). Moreover, tumour site had no significant impact on SGR (p=0.58; Tab. 3). Interestingly, PT-SGR positively correlated with the expression of EGFR, Ki67 and CD44 (p=0.02; p=0.02 and p=0.03, respectively; Figure 3). The expression of p16 had no influence on PT-SGR (p=0.21).

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In contrast, LN-SGR depended on the initial LN-volume measured in diagnostic CT (p=0.003) with higher LN-SGRs in lymph nodes with larger LN-volumes in the 1st CT. Also, the interval between the diagnostic CT and planning CT significantly influenced LN-SGR (p=0.003). The longer the interval, the lower the observed LN-SGR. Moreover, LN-SGR was significantly influenced by tumour site (p=0.032; Tab. 3) with smallest growth rates in lymph nodes from laryngeal HNSCC (p=0.003). In contrast to PT-SGR, neither EGFR, Ki67, CD44 nor p16 expression in PTs significantly correlated with LN-SGR (p=0.12; p=0.31; p=0.75 and p=0.81, respectively).

Specific-growth-rate and overall survival

PT-SGR nearly missed significant impact on OS when used as a single covariate in a Cox regression model (log rank p=0.054). For Kaplan Meier analyses, PT-SGR were categorized in 3 groups: slow (< 0.3%/day; n=22); intermediate (0.3% ≤ 3%/day; n=73) and rapid (>3%/day; n=26). Survival curves of these 3 SGR groups did not differ significantly (log rank p=0.45; Figure 4). Likewise, LN-SGR had no significant impact on OS as a covariate in Cox regression (log rank p=0.83) nor in Kaplan Meier analyses (log rank p=0.97; data not shown).

Cartilage or bone infiltration and central lymph node necrosis

Cartilage or bone infiltration was observed in diagnostic CTs of 40/123 (33%) patients and in planning CTs of 50/123 (41%) patients. Thus, during the median 24 days interval between the two CTs, new cartilage or bone infiltration occurred in 10 patients, which was not influenced by PT-SGR (p=0.918). However, cartilage or bone infiltration had a significant negative impact on survival (p=0.003) and was significantly more frequent in p16 negative tumours (p=0.02).

Lymph node necrosis was observed in diagnostic CTs of 74/97 (76%) patients. LN-SGR was significantly higher in patient with lymph nose necrosis (p<0.001), was associated with poorer survival (p=0.03) and did not depend on p16 status. Lymph nose necrosis was observed in planning CTs of 82/97 (85%) patients. Thus, during the median 24 days interval between the two CTs, new lymph node necrosis occurred in 8 patients, which was not influenced by LN-SGR (p=0.818). Central lymph node necrosis had a significant negative impact on survival (p<0.05).

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DISCUSSION

Long waiting times for treatment of head and neck cancer result in decreased overall survival[1-3]. One reason might be meantime tumour growth. We examined tumour growth during the time between the initial diagnostic CT scan and the planning CT for RT or RCT in 123 patients with incident HNSCC. The majority of the patients suffered from incident stage IVa oropharyngeal HNSCC.

The mean interval between 1st and 2nd CT was 29 days ranging from 6 to 146 days. In this time, diagnostic work up, interdisciplinary tumour board presentation and pre-treatment procedures including dental treatments and application of percutaneous gastrostomies were carried out. PT- and LN-volumes in diagnostic and planning CTs were assessed using identical protocols. PT-SGR and LN-SGR were calculated as proposed by Mehrara and co-authors[5]. This mean interval is comparable to previously reports[1]. Moreover, it is considerably shorter than intervals of 46 to 52 days, which were reported as threshold for decreased OS[2]. From a clinical perspective, this is considered positive. However, in terms of exploring SGR's in advanced stage HNSCC, this interval may not be sufficient to accurately determine great changes in PT-volume or LN-volume.

Due to the skewness of volumetric data, the median was considered an appropriate measure of central tendency, but the mean is also provided for comparison with previous publications. Mean PT-volume in diagnostic CTs was approximately 16mL (median≈9mL). In planning CTs of the same patients 3-4 weeks later, mean PT-volume was approximately 25mL (median≈17mL; Tab. 2). These PT-volumes are in line with previous publications reporting mean volumes of 11-37mL[23-26]. Mean LN-volume measured in diagnostic CTs was 9mL (median≈2mL) and approximately 15mL (median≈4mL) in planning CTs (Tab 2). Previously reported mean LN-volumes were considerably larger with 22-25mL[27, 28]. However, in these studies the volume of all pathological cervical lymph nodes was measured instead of only the largest one as in this study.

Tumour and pathological lymph node growth can be reported as doubling time (DT) or specific growth rates (SGR). DT is the number of days the tumour needs to double its volume[4]. The lower

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the DT, the faster the tumour growth. Since DT was frequently reported in earlier studies, it was provided for comparison. SGR is defined as relative increase of volume in percent per day. The resulting variable is constant, linear and independent of the initially measured volumes[5]. SGR is considered less affected by measurement uncertainties of short time intervals and minor volume differences than DT[5].

For both, DT and SGR, tumour volumes are required. A volume approximation method from CT scans, which reflects frequently available diagnostics, was used here. This approximation was based on measurements of maximum orthogonal diameters, similar to a formula described in 1990 by MacDonald and co-workers to approximate volumes of brain tumours[29]. The method employed an ellipsoid formula, which was applicable over a wide range of PT and LN sizes and resulted only in a slight underestimation of 8% of the PT-volume measured with the reference method[15]. A similar approximation method has also been used by Jensen and co-authors[30].
More sophisticated segmentation- and more modern imaging techniques, may have allowed for better tumour margin delineation. However, they first require specific workstations with limited availability[15] (i.e. semi-automated or automated slice-by-slice segmentation) and large inter-observer variations may remain[31]. The latter may allow better visualisation of oropharyngeal and oral HNSCC (i.e. magnetic resonance imaging MRI) or better visualisation of locally advanced tumours (i.e. fluordesoxyglucose positron emission tomography FDG-PET). However, both imaging techniques are not as frequently available as CT scans and other limitations apply. For MRIs blurred tumour margins may be observed if patients swallow or breathe, for FDG-PET a lack of spatial resolution may be disadvantageous in smaller HNSCC tumours[31].

Mean primary tumour specific growth rates (PT-SGR) in incident HNSCC was 1.8%/day (Tab. 2), unimodally distributed, slightly right skewed and independent of the initial PT-volume, which supports the basic concept underlying SGR calculation. The interval between diagnostic CT and planning CT did not influence PT-SGR (p=0.1), suggesting that no marked growth deceleration occurred with longer waiting times. Interestingly, PT-SGRs did not significantly differ by tumour site (p=0.6; Tab 3). Murphy and colleagues observed a lower median SGR of 0.74%/day in patients with oropharyngeal cancer[6]. However, the authors did not include sites with faster tumour

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growth such as the oral cavity (median SGR 2.4%/day) and hypopharynx (median SGR 2.0%/day). Moreover, most of the patients reported by Murphy and colleagues had T1 or T2 HNSCC and were p16 positive.

The median PT-DT in this study was 43 days. The 25th percentile was 24 and the 75th percentile 85 days. Median LN-DT was 41 days. The 25th percentile was 25 and the 75th percentile 80 days (Tab. 2). Jensen and co-authors reported a slower growth rate in 61 patients with HNSCC with a median PT-DT of 99 days. The authors however additionally stated that half of the patients showed a faster growth rate with a PT-DT of 30 days[30]. When compared with DT of other solid tumours, this means that HNSCC reveal rapid tumour growth. For breast cancer, Ingebly and co-authors reported a median DT of 285 days[4]. For lung cancer, DTs vary depending on histology between median 42 days for metastases as reported by Loeffler and colleagues and 181 for non-small-cellular lung cancer as reported by Winer-Muram and co-workers[4,32]. For pancreatic adenocarcinoma, Furkawa and colleagues reported a mean PT-DT of 144 days[32]. For Sarcomas median PT-DTs of 35 days were reported by Blomqvist and co-authors[32]. Additional median PT-DTs for additional solid tumours are provided in table 4.

Table 4: Reported tumour volume doubling times for selected solid tumours*.

Tumour	**Median DT [days]**	**n**
Present study	47	123
HNSCC[30]	99**	61
Breast cancer[4]	285	16
Lung bronchioalveolarcarcinoma[32]	181	9
Non small-cell lung carcinoma[32]	181	6
Lung metastasis[4]	42	24
Pancreatic adenocarcinoma[32]	144	9
Sarcoma[32]	35	21

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*modified after Mehrara and co-authors[32]

**Jensen and co-workers also stated tumour volume doubling times for the faster growing half of the patients with a median PT-DT of 30[30].

PT-SGRs significantly correlated with the expression of EGFR, Ki67 and CD44 (p=0.021; p=0.018 and p=0.031, respectively) with higher PT-SGRs in patients with higher expression of the three biomarkers (Figure 3). These correlations appear biologically sound. A correlation of clinical tumour growth rates and expression of these biomarkers within the tumour of the same patients was not yet reported. EGFR is a cell surface receptor which promotes proliferation, invasion, angiogenesis and metastatic spread in HNSCC, if overexpressed[8]. Ki-67 is a nuclear protein expressed on cells in all phases of the cell cycle except in G0-phase. Thus, its expression marks the total fraction of proliferating cells in a tumour[9]. In laryngeal and hypopharyngeal SCC, an association between Ki67 expression and advanced tumour stages has been reported[10-11]. In line with our observation of a correlation of CD44 expression and PT-SGR, an association between advanced T categories and high CD44 expressions has been reported in a meta-analysis including thirty studies with 2102 patients[12]. No significant correlation between PT-SGR and p16 expression was observed (p=0.81). This observation differs from previous observations made by Murphy and colleagues. The authors observed that p16 expression correlated well with PT-SGR with faster growth rates in p16 negative HNSCCs. However, the authors included oropharyngeal HNSCC only and the majority (79%) were p16 positive tumours[6]. When used as a covariate in a Cox model, PT-SGR had no significant effect on OS (log rank p=0.054). No significant differences of survival curves were also observed, if PT-SGR were categorized in groups of low, medium and high growth rates (log rank p=0.45; Figure 4). In contrast, van Bockel and co-worker observed a significant association between PT-SGR and OS in laryngeal cancer[7].

To our knowledge, no data on lymph node specific growth rates (LN-SGR) in HNSCC have been previously reported. In the investigated patients, the mean LN-SGR was 1.7%/day, similar to mean PT-SGR (Tab. 2). In contrast to PT-SGR, LN-SGR did depend on initial LN-volumes in diagnostic CT scans (p=0.003) with higher LN-SGRs in lymph nodes with larger initial LN-volumes. Also, the interval between diagnostic CT and planning CT significantly influenced LN-SGR (p=0.003). The

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longer the interval, the lower the observed LN-SGR, suggesting growth slowdown. Moreover, LN-SGR was significantly influenced by tumour site (p=0.03; Tab. 3), with smallest growth rates in lymph nodes from laryngeal HNSCC. In contrast to PT-SGR, LN-SGR did not depend on expressions of EGFR, Ki67, CD44 nor p16 expression in primary tumours (p=0.115; p=0.311; p=0.746 and p=0.809 respectively). In LN, specific growth rates had no significant impact on OS either (p>0.05).

New cartilage or bone infiltrations during the observation period were observed in 10 patients and new central lymph node necroses were observed 8 patients. Specific growth rates had no significant impact on the development of either of them. Both, initial cartilage or bone infiltration and central lymph node necrosis were associated with worse survival. However, probably due to the low number of events, no significant impact of new cartilage or bone infiltration nor new central lymph node necrosis on survival was observed in Kaplan Meier analysis (both log rank p>0.05).

Some limitations of the present study need to be addressed. Firstly, this small numbered retrospective study predominantly exploring patients with advanced stage oropharyngeal HNSCC should be supplemented by a larger, prospective investigation, which also included patients with limited disease of all common HNSCC tumour sites. Secondly, the approximation method[15] proposed here may be easily performed from frequently available CT scans over a wide range of different PT- and LN-volumes. However, more sophisticated segmentation- and more modern imaging techniques may have allowed for better tumour margin delineation and hence for more accurate SGRs. Thirdly, the comparably short interval between diagnostic CT scans and planning CT scans may be considered positive from a clinical perspective. However, in terms of exploring SGRs in advanced stage HNSCC, this interval may not be sufficient to accurately determine great changes in PT- and LN-volume.

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CONCLUSION

Head and neck squamous cell carcinomas are rapidly growing malignancies. Primary tumours and lymph nodes grow more than 1% per day. Consequently, time matters, and treatment must not be delayed.

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ABBREVIATIONS

CD44	CD44 antigen
CT	computed tomography
DICOM	Digital Imaging and Communications in Medicine
DT	tumour volume doubling time
EGFR	epidermal growth factor receptor
FDG-PET	fluordesoxyglucose positron emission tomography
HNSCC	head and neck squamous cell carcinoma
Ki67	Ki67 antigen
LN	largest pathological cervical lymph node
LN-DT	largest pathological cervical lymph node doubling time
LN-SGR	largest pathological cervical lymph node specific growth rate
MRI	magnetic resonance imaging
p16	Cyclin-dependent kinase inhibitor 2A
PACS	Picture Archiving and Communication System
PT	primary tumour
PT-DT	primary tumour volume doubling time
PT-SGR	primary tumour specific growth rate
RCT	radiochemotherapy
RT	radiotherapy
SD	standard deviation
SGR	specific growth rate
TNM	tumour, node, metastasis
UICC	Union internationale contre le cancer

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REQUIRED STATEMTENTS

Acknowledgements

This work has not yet been previously presented at a conference or as a conference abstract.

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Ethics statement

The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Competing interest and funding statement

All authors have completed the Unified Competing Interest form and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

Contributorship statement

Daniel Dejaco has written major parts of the manuscript and performed the calculations of tumour and lymph node volumes. Teresa Steinbichler was involved in establishing the study concept, design and the internal review process of the manuscript. Schartinger Volker established the population based tumour registry from which patients were enrolled. He further obtained the ethics committee approval for the study. Natalie Fischer performed additional tumor and pathological cervical lymph node measurements and was involved in the internal

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review process of the manuscript. Maria Anegg has performed additional calculations and data base work in the population based tumour registry. Joszef Dudas performed the immunohistochemical staining of the tumour biopsies and was involved in establishing the study concept, design and the internal review process of the manuscript. Andrea Posch provided the radiotherapy-planning CT scans and performed performed additional tumor and pathological cervical lymph node measurements. Gerlig Widmann supervised the tumor and pathological cervical lymph node measurements as an experienced head and neck radiologist. Herbert Riechelmann supervised the writing process of the manuscript and performed all statistical calculations in the manuscript.

Transparency declaration

The Corresponding Author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study have been explained.

Details of ethical approval and patient consent

The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants.

Data sharing

No additional data from the study is available.

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FIGURE LEGENDS

Figure 1: Tumour growth assessment using maximal orthogonal tumour diameters from axial contrast enhanced diagnostic CT scans (A) and subsequent planning CT scans (B)

Axial contrast enhanced diagnostic CT scan (A) and subsequent planning CT scan (B) 46 days later of a cT4a cN2b cM0 squamous cell carcinoma of the oral cavity. The maximum anterior-posterior and medio-lateral tumour diameters (white lines) were measured from axial scans, the cranio-caudal tumour diameters were measured from corresponding coronal scans (not depicted). The tumour volume was assessed as previously described using an ellipsoid formula[15]. PT-volume from diagnostic CT was 14.8mL, PT-volume from planning CT was 51.0mL translating to a PT-SGR of 2.8%/day.

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Figure 2: Correlation of primary tumour volume (A) and largest pathological lymph node volume (B) measured from diagnostic CT scans and a planning CT scans

Scattergram of primary tumour volumes (A) and largest pathological lymph node volume (B) measured from diagnostic CT scans (x-axis) planning CT scans (y-axis). Both axes are on log scale. The diagonal line represents the line of identity. Dots above this line indicate volume increases.

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Figure 3: Specific growth rates of the primary tumours and percentage of Ki67 positive cells

Percentage of Ki67 positive immunohistochemistry in tumour samples of HNSCC patients and according primary tumour specific growth rates. The percentage of Ki67 positive cells was grouped in 0%, 1 to 30%, 31 to 60% and more than 60% of cancer cells (x-axis). Mean specific growth rates of HNSCC primary tumour (y-axis) were obtained 119 patients. Small bars represent standard deviation. PT-SGR positively correlated with the expression of Ki67 (Jonckheere-Terpstra p=0.02).

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Figure 4: Kaplan Maier plot for specific growth rates of primary tumours grouped by low, medium and high growth rate

Kaplan Maier plot of specific growth rates (SGR) of primary tumours grouped by low (black line; SGR< 0.3%/day; n=22); medium (dark grey line; 0.3% ≤ SGR < 3%/day; n=73) and high (pale grey line; SGR >3%/day; n=26) growth rates. X-axis represents time in months, Y-axis overall survival. The survival curves of the 3 specific growth rate groups did not differ significantly (log rank p=0.45).

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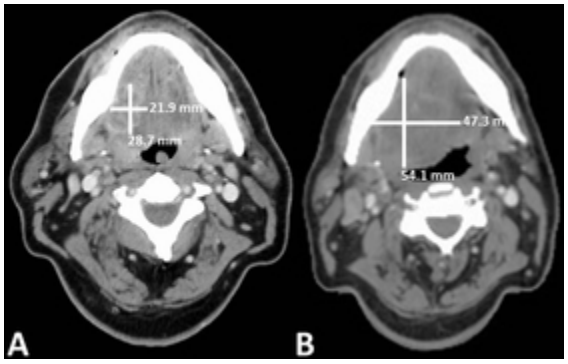

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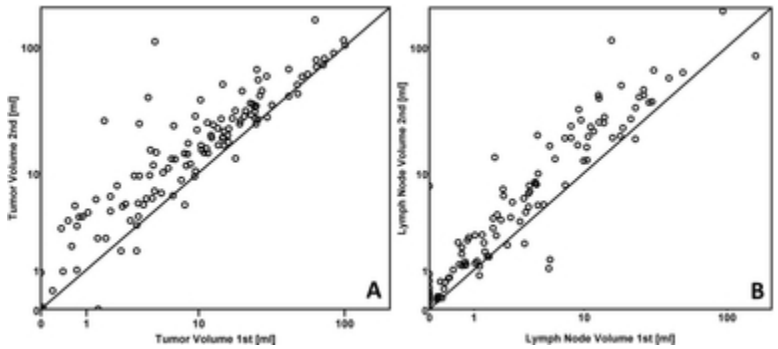

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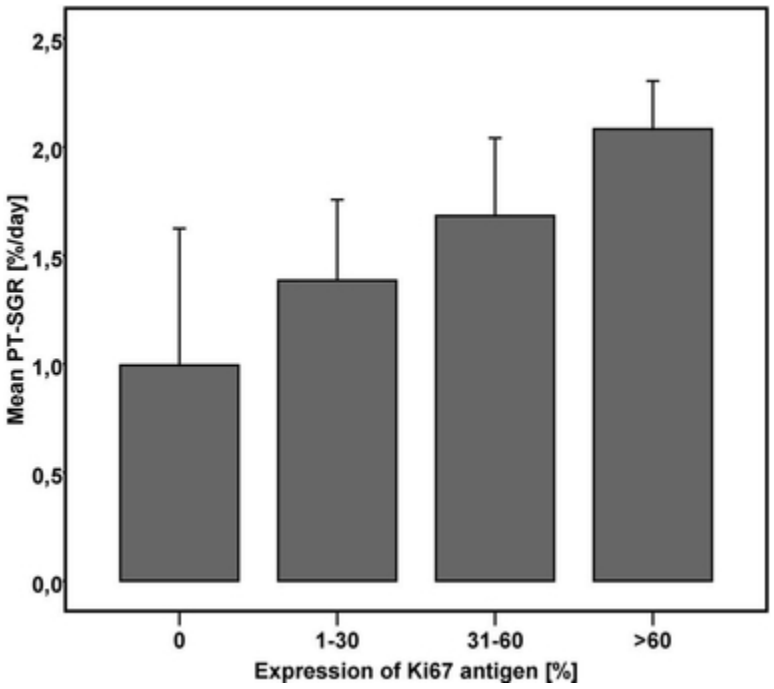

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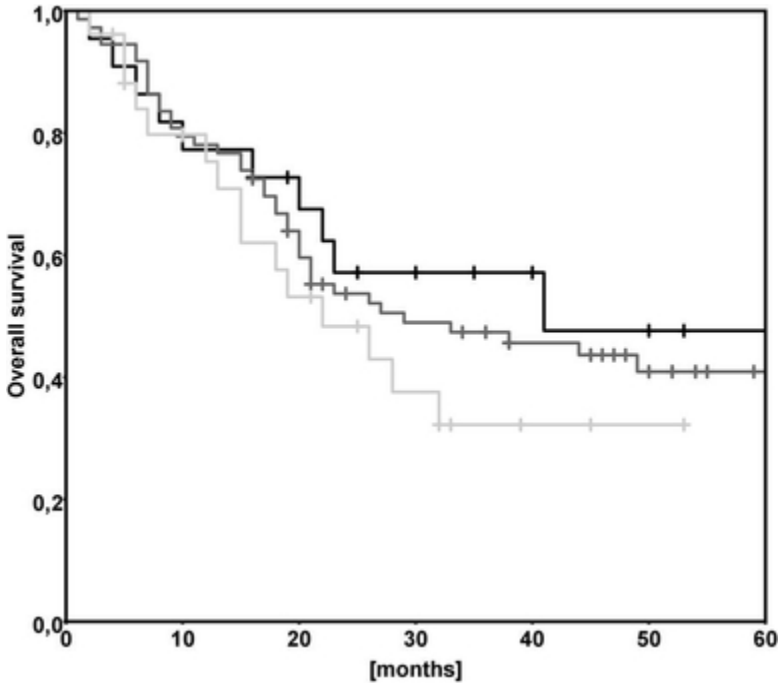

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STROBE Statement: "Time matters – the fast growth of head and neck squamous cell carcinoma"

	Item No	Answer
Title and abstract	1	(*a*) The retrospective study design was described in the "Abstract" section, page 2, line 7, the "Strengths and Limitations of the Study" section, page 3 and the "Introduction" section, page 5.
		(*b*) An informative and balanced summary of what was done was provided in the "Abstract" section, page 2.
Introduction		
Background/rationale	2	The scientific background and rational for the investigation was reported in the "Introduction" section of the manuscript, page 5.
Objectives	3	The objective of the study was specified in the "Abstract" section, page 2 and at the end of the "Introduction" section, page 2.
Methods		
Study design	4	Key elements of the study design were mentioned early in the "Abstract" section, page 2 and the "Introduction" section, page 5 of the manuscript. Additional details of the study design were outlined in the "Methods", page 6 to 8.
Setting	5	The setting, location, relevant dates, including period of recruitment and data collection was included in the "Methods" section, page 6 and in the "Results" section, page 9.
Participants	6	(*a*) *Cohort study*— The eligibility criteria and the sources and methods of selection of participants are provided in detail in the "Methods" section, page 6.
		(*b*) *Cohort study*— No matching was performed in this study.
Variables	7	All outcomes, predictors, potential confounders and effect modifiers were defined, presented and discussed in the "Methods" section, page 6 to 8, "Results" section, page 9 to 12and "Discussion" section, page 13 to 16. Diagnostic criteria were provided in the "Methods" section, page 6, when applicable.
Data sources/ measurement	8*	Source of data and details of methods was provided in the "Methods" section, page 6 and 8.
Bias	9	Potential sources of bias were addressed in the "Discussion" section, page 13 to 16.
Study size	10	An explanation how the study size was arrived at was provided in the "Method" section, page 6.
Quantitative variables	11	An explanation how quantitative variables were handled in the analyses was provided in the "Methods" section, page 8.
Statistical methods	12	(*a*) All statistical methods were described in the "Methods" section, page 8.
		(*b*) All methods for subgroup analyses of the presented study were described in the "Methods" section, page 8.
		(*c*) An explanation how missing data was addressed was provided in the "Methods" section, page 8, "Results" section, pages 9 to 12 and "Discussion" section, pages 13 to 16.
		(*d*) *Cohort study*—No loss to follow-up occurred due to the study design.
		(*e*) No sensitivity analyses were performed in the study.

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Results		
Participants	13*	(a) Numbers of individuals at each stage of the study was reported in the "Results" section, page 9 and the "Discussion" section, page 13.
		(b) No non-participation occurred due to the study design.
		(c) No flow diagram was used for this study.
Descriptive data	14*	(a) Clinical characteristics of study participants were provided in table 1, page 9.
		(b) Number of participants with missing data for each variable of interest was provided in the "Results" section, page 9 and in table 1, page 9.
		(c) *Cohort study*—Follow-up time was summarised as time between diagnostic computed tomography scan and planning computed tomography scan in this study provided in the "Results" section, page 9.
Outcome data	15*	*Cohort study*— Numbers of outcome events was provided in the "Results" section, page 9 to 12.
Main results	16	(*a*) Means, medians, standard deviations, 95% confidence intervals and 25th and 75th percentiles were provided in the "Results" section, page 9 to 12, if applicable.
		(*b*) Category boundaries for continuous variables were specified in the "Methods" section, page 8 and "Results" section, pages 9 to 12.
		(*c*) No relative risk estimations were performed in this study.
Other analyses	17	All additional other analyses performed in the study were outlined in the "Methods" section, page 8 and "Results" section, pages 9 to 12.
Discussion		
Key results	18	Key results with reference to previously outlined study objectives were summarised in the "Discussion" section, pages 13 to 16.
Limitations	19	Limitations of the study, taking into account sources of potential bias and imprecision were outlined in the "Discussion" section, pages 13 to 16.
Interpretation	20	A cautious overall interpretation of the results was given in the "Discussion", pages 13 to 16and the "Conclusion" section, page 17.
Generalisability	21	The external validity of the study results was outlined in the "Discussion" section, pages 13 to 16 and table 4, page 15.
Other information		
Funding	22	No financial support for any of the work presented in the present manuscript was obtained, provided on page 19.

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BMJ Open

Specific growth rates calculated from computed tomographies in patients with head and neck squamous cell carcinoma: a retrospective study performed in Austria

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-025359.R2
Article Type:	Research
Date Submitted by the Author:	19-Dec-2018
Complete List of Authors:	Dejaco, Daniel; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Steinbichler, Teresa; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Schartinger, Volker; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Fischer, Natalie; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Anegg, Maria; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Dudas, Joszef; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery Posch, Andrea; Medical University of Innsbruck, Department of Radiationoncology Widmann, Gerlig; Medizinische Universitat Innsbruck, Department of Radiology Riechelmann, Herbert; Medical University of Innsbruck, Department of Otorhinolaryngology – Head and Neck Surgery
Primary Subject Heading:	Oncology
Secondary Subject Heading:	Ear, nose and throat/otolaryngology, Diagnostics, Radiology and imaging
Keywords:	head and neck squamous cell carcinoma, tumour volume, tumour growth rate, EGFR, Ki67, CD44

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Specific growth rates calculated from computed tomographies in patients with head and neck squamous cell carcinoma: a retrospective study performed in Austria

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Word count: 3848
Abstract: 238
References: 32
Figures: 4
Tables: 4

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ABSTRACT

OBJECTIVE: To provide data on specific growth rates (SGR) of primary tumours (PT-SGR) and largest pathological cervical lymph nodes (LN-SGR) for head and neck squamous cell carcinoma (HNSCC). To explore PT-SGR's and LN-SGR's correlation with selected biomarkers EGFR, Ki67 and CD44.

DESIGN AND SETTING: Retrospective study performed at a tertiary oncologic referral centre in Innsbruck, Austria.

PARTICIPANTS: Adult patients with incident HNSCC treated with primary radiotherapy (RT) or radiochemotherapy (RCT).

OUTCOME MEASURES: Volumes of the primary tumour (PT-volume) and largest pathological cervical lymph node (LN-volume) were measured in computed tomography (CT) scans obtained at time of diagnosis and subsequent planning CTs immediately prior to RT or RCT. SGRs were calculated assuming an exponential growth function. PT-SGR's and LN-SGR's correlation with EGFR, Ki67 and CD44 were explored.

RESULTS: In 123 patients, mean interval between diagnostic and planning CT was 29±21 days. PT-SGR was 1.8%±1.8% (mean±SD) per day and was positively correlated with EGFR, Ki67 and CD44 expression (p=0.02; p=0.02; p=0.03). LN-SGR was 1.7%±2.0% per day and increased with larger initial LN-volume, was lower in laryngeal cancer (p=0.003) and slowed down with time. LN-SGR was not correlated with EGFR, Ki67 or CD44 expression in primary tumours (p>0.12). New cartilage or bone infiltration occurred in 10 patients and new central lymph node necrosis in 8 patients.

CONCLUSIONS: HNSCC are fast growing tumours for which treatment must not be delayed. Clinical tumour growth rates are influences by EGFR, KI67 and CD44 expression.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- In patients with incident head and neck squamous cell carcinoma (HNSCC), specific growth rates (SGR) for primary tumours (PT) and largest pathological cervical lymph nodes (LN) were retrospectively calculated.
- SGR in percentage growth per day was calculated from two CT scans obtained at time of diagnosis and subsequent planning CTs immediately prior to radiochemotherapy as previously described (SGR=ln[1stvolume*2ndvolume]/[t2-t1]).
- Volumes in millilitres (mL) for PT and LN were calculated from maximum orthogonal diameters in all three planes applying an ellipsoid formula as previously described (volume=(π*[x*y*z/1000])/6).
- To explore the impact of SGR of PT and LN on overall survival, Kaplan Maier and Cox regression models were used, to explore the correlation of SGR with EGFR, Ki67 and CD44, Jonckheere-Terpstra tests were used.
- Limitations include retrospective study design, small number of patients, small interval of observation, lack of more modern imaging- and segmentation techniques.

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KEYWORDS

head and neck squamous cell carcinoma; tumour volume; tumour growth rate; EGFR; Ki67; CD44;

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INTRODUCTION

In patients with head and neck squamous cell carcinoma (HNSCC), the median treatment waiting time in the US almost doubled from 19 to 30 days between 1998 and 2011. In a recent cancer registry based study, Murphy and co-authors analysed almost 275.000 patients with HNSCC of the most common cancer sites. The authors observed an independent effect of increased treatment waiting time on overall survival (OS)[1] and calculated 46 to 52 days as threshold for decreased OS[2]. Similar observations were reported for oral cancer in a recent review including 18 studies[3].

A likely reason for the association of treatment waiting time and decreased OS is meantime tumour growth. Mathematical models to approximate tumour growth from imaging data are available since the 1960s[4]. Originally, direct curve fitting to calculate tumour volume doubling time (DT) was the standard method to assess tumour growth[4]. Recently, calculation of specific growth rate (SGR), defined as relative volume increase per unit of time, was proposed instead. It was reported more reliable for short time intervals and minor tumour volume differences[5].

Data on SGR of HNSCC is limited[6-7]. A median SGR for primary tumours (PT-SGR) of 0.74% per day in patients with oropharyngeal HNSCC waiting for RCT was reported by Murphy and colleagues. The authors assessed the PT-SGR in 85 patients between diagnostic CTs and planning CTs and concluded that rapid PT-SGR may predict treatment failure in these patients[6]. Van Bockel and co-authors reported a significant association between high PT-SGR and decreased OS (p=0.013) in 131 patients with laryngeal HNSCC[7].

In this retrospective study, we calculated SGR[5] of the primary tumour (PT-SGR) and largest pathological cervical lymph node (LN-SGR) of patients with incident HNSCC from CTs obtained at diagnosis and from planning CTs obtained directly before RT/RCT. We investigated the influence of various factors including several biomarkers on PT-SGR and LN-SGR, which were previously observed to be associated with tumour proliferation[8-12]. We were further interested in the influence of SGR on OS and on the development of new lymph node necrosis and bone or cartilage infiltration.

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MATERIALS AND METHODS

Tumour registry population

Patients referred to the Department of Otorhinolaryngology – Head and Neck Surgery, Medical University of Innsbruck, Austria, between 2008 and 2016 with incident histologically confirmed HNSCC were recorded in the clinical tumour registry. Disease was staged according to the seventh edition of the UICC TNM staging system[13] by an interdisciplinary tumour board. Inclusion criteria comprised histologically proven incident HNSCC at any site of the head and neck region including cancer of unknown primary (CUP), any UICC Stage, RT or RCT as primary treatment and availability of both a diagnostic CT and a planning CT. The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants.

Diagnostic CT and planning CT

At the time of clinical diagnosis, diagnostic CT was performed following the standardized CT head & neck imaging protocols at the Department of Radiology, Medical University of Innsbruck. A GE-Medical Systems Light Speed VCT® or Light speed 16 CT scanner® (GE Medical, Vienna, Austria) was used. The scan area ranged from the frontal sinus to the upper mediastinum with a resolution of 512x512 pixels. Slices were calculated from raw data with 2 millimetres (mm) thickness, collimation of 24x1.2mm and 0.45 pitch. Additional sagittal and coronal images were reconstructed. As contrast medium, Jopamiro 370® (Bracco Austria GmbH, Vienna, Austria) was administered intravenously adjusted to the patient's bodyweight. Planning CT scans were later performed at the Department of Radiation Oncology, Medical University of Innsbruck. Imaging protocols were followed as described above with the same CT scanners, contrast medium, scanning areas, resolutions and calculation protocols. Images from both CT scans were exported in Digital Imaging and Communications in Medicine (DICOM) format using IMPAX EE® (Agfa HealthCare, Bonn, Germany) Picture Archiving and Communication System (PACS®, Cerner, Kansas City USA). LN with a minimal axial diameter >10mm, a central necrosis >3mm or if present in neck levels close to the primary tumour in groups of >3 were classified pathological[14].

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Volume approximation, specific growth rate and tumour volume doubling time

Volumes were calculated as previously described[15]. In short, maximum orthogonal diameters in mm were measured for the primary tumour (PT) and the largest pathological cervical lymph (LN) in all three planes in axial and coronal sections (Figure 1). Volumes in millilitres (mL) were approximated employing an ellipsoid formula (volume=(π*[x*y*z/1000])/6). The largest cervical LN instead of all pathological cervical lymph nodes was considered sufficient for evaluation based on a high correlation previously observed[15]. Central lymph node necrosis and/or cartilage or bone infiltration of the primary tumour was recorded for additional analysis.

SGRs were assumed to be exponential and defined as the relative volume increase given in percent per day. For calculation of SGR, the equation described by Mehrara and co-authors was applied (SGR=ln[1stvolume*2ndvolume]/[t2-t1])[5]. For comparison with earlier studies, doubling times (DT) for primary tumours (PT-DT) and for largest pathological cervical lymph nodes (LN-DT) were calculated as the time difference*LN divided by the logarithm of the volume ratio of the two observations[16].

Analysis of EGFR, Ki67 and CD44 expression

Tumour biopsies were collected in 4% buffered formalin, fixed overnight and embedded using the ethanol – isopropanol – wax quick 4mm protocol of Histos 5 embedding processor® (Milestone, Bergamo, Italy). Five-micrometre thick paraffin sections were dewaxed and antigen retrieval was performed in a Discovery automated staining system® (Ventana, Tucson, AZ, USA). Primary antibodies were added to the sections by automatic dispensing either as ready-to-use, pre-diluted, stabilized solutions provided by the manufacturers: cyclin dependent kinase inhibitor 2A (p16) INK4® (Ventana, Cat. Nr. 6595294001), Ki67 antigen (Ki67; Linaris E059®, clone MIB-1®, Dossenheim, Germany), CD44 antigen (CD44; Diagnostic Biosystems /Antibodies Online, ABIN1020059, Aachen, Germany) and Epidermal Growth Factor Receptor (EGFR; Invitrogen, Vienna, Austria). Selection of these three biomarkers was based on which were previous observation about their possible associated with tumour proliferation[8-12]: 1) EGFR is a cell surface receptor which promotes proliferation, invasion, angiogenesis and metastatic spread in HNSCC, if overexpressed[8]. Thus, high expression of EGRF might suggest higher PT-SGR. 2) Ki67 is a nuclear protein express on cells in all phases of the cell cycle except in G0-phase.

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Thus, its expression marks the total fraction of proliferation cells in a tumour[9-11], suggestive of a possible positive correlation with PT-SGR. 3) Positive correlations between CD44 expression and advanced T categories were previously reported in a meta-analysis including thirty studies with 2102 patients[12]. Since T category is primarily based on maximal tumour diameter, a possible positive correlation between CD44 expression and PT-SGR might be suggested.

Immunohistochemical staining was completed by the Discovery automated staining system® (Ventana, Tucson, AZ, USA) using universal secondary antibody solution, haematoxilin counterstaining and the DAB MAP Kit (all Ventana products, Tucson, AZ, USA) as published previously[17]. All sections were stained with control mouse and rabbit immunoglobulins, using the same highest concentration as for the primary antibodies, and these controls were not reactive[18]. The immunohistochemical reactions were observed independently by two blinded observers, who collected 10 representative tumour cell nests from each specimen[19]. These regions were analysed on an Olympus BX50 microscope® (Olympus, Tokyo, Japan) and the staining intensity and representation of tumour cell nests were scored as previously described[20]. The cut-off for p16-positivity was 70% or more positive tumour cells[21].

Data analysis

Frequency data were presented in tabular form. For continuous data (volumes and growth rates), means and standard deviations (SD) as well as medians and 25th ($p25^{th}$) and 75th percentiles ($p75^{th}$) were provided. The median follow-up time was calculated as described by Schemper and Smith[22]. Logarithmic transformation was used to analyse volumetric data in regression models. Kruskal-Wallis and Jonckheere-Terpstra tests were used to evaluate the univariate influence of ordinal factors on growth rates. For survival analyses, Kaplan Maier and Cox regression models were used. For Kaplan Meier plots, growth rates were categorized in groups with slow, intermediate and rapid growth rates. All calculations were performed with SPSS 23.0 (IBM Corp., Armonk, NY).

Patient and Public Involvement

The development of the research question was based on previous publications exploring tumor volume in HNSCC[15] and its prognostic value if treated primarily with surgery[23]. Neither patients

nor the public were involved in the design of the study, the recruitment of the study or the conduct of the study.

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RESULTS

Study population

Between 2008 and 2016, 802 patients with incident HNSCC were recorded in the clinical cancer registry. Of 123 patients treated with primary RT or RCT, PT-volumes and LN-volumes were calculated from diagnostic and planning-CTs. Tumour sites included oral cavity, oropharyngeal, hypopharyngeal and laryngeal HNSCC. No patients with tumours of the nasopharynx, the paranasal sinuses or salivary glands were included. In five patients with CUP-syndrome, no PT-volume and in 27 patients with N0 stage neck, no LN-volume could be measured. Of the 123 patients, 32 were female. The mean±SD age was 63±10 years ranging from 38 to 87 years. Median follow up time was 45 months (95% confidence interval 42 to 48 months). Additional clinical data of the 123 included patients is provided in table 1.

Table 1: Clinical data of included 123 HNSCC patients.

		No. of patients
Sex	male	89
	female	32
Age	≤ 50 years	11
	51 – 60 years	42
	61 – 70 years	39
	71 – 80 years	26
	≥ 80 years	5
p16	negative	83
	positive	38
Tumour site	oral cavity	16
	oropharynx	55
	hypopharynx	24
	larynx	21
	carcinoma of unknown primary	5
Clinical UICC stage	stage I	4
	stage II	7
	stage III	17
	stage IVa	75
	stage IVb	16
	stage IVc	2

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Time interval, tumour and lymph node volumes

Mean±SD time interval between diagnostic CTs and planning CTs was 29±21 days ranging from 6 to 146 days. For PT, a total of 119 volume sets from diagnostic CT and planning CT were available (Figure 2). Mean PT-volume from diagnostic CTs was 16.3±20.4mL ranging from 0 (CUP-syndrome) to 100.8mL. Mean PT-volume from planning CTs was 24.5±26.8mL ranging from 0 to 160.3mL. The mean PT-volume increase of 6.7±17.2mL during the period between 1st and 2nd CT scans was highly significant (p<0.001).

For LN, a total of 96 volume sets were available (Figure 2). Mean LN-volume from diagnostic CTs was 9.2±22.2mL ranging from 0 (N0) to 156.2mL. Mean LN-volume from planning CTs was 15.2±31.5mL ranging from 0 to 233.5mL. The mean LN-volume increase of 5.3±17.3mL during the observation period was highly significant (p<0.001). Distributions of these parameters were right skewed. Medians and quartiles for PT-volume and LN-volume are provided in table 2.

Table 2: Volumes of primary tumours and largest pathological cervical lymph nodes in diagnostic and planning CTs in 123 patients with HNSCC. Median time interval between 1st and 2nd CT scans was 24 days. Specific growth rates were calculated as suggested by Mehrara and co-authors[5], tumour doubling-times were calculated as proposed by Schwartz[16].

	Diagnostic CT volume[1)] [ml]	Planning CT volume[1)] [ml]	SGR[2)] [%/day]	DT[3)]
Primary tumour	9.4 (3.3; 21.4)	16.7 (5.4; 31.6)	1.4 (0.6; 2.7)	43 (24; 85)
Lymph node	2.0 (0.2; 9.5;)	3.7 (0.6; 19.4)	1.2 (0.3; 2.5)	41 (25; 80)

1) Median (25th and 75th percentile)

2) Specific growth rate (median, 25th and 75th percentile) percent per day

3) Tumour doubling time in days (median, 25th and 75th percentile)

Specific growth rates (SGR) and tumour doubling times (DT)

Mean±SD PT-SGR was 1.8%±1.8%/day ranging from minus 2.6%/day (volume decrease) to 8.6%/day. Mean LN-SGR was 1.7%±2.0%/day ranging from minus 1.5% to 11.0%/day. PT-SGR and LN-SGR were right-skewed. For medians and quartiles see table 2. Mean PT-SGR of tumours

of the oral cavity, oropharynx, hypopharynx and larynx were 3.1%±1.5%/day, 1.8%±1.7%/day, 2.0%±2.0%/day and 1.5%±2.1%/day, respectively. Mean LN-SGR for the primary tumour sites oral cavity, oropharynx, hypopharynx and larynx were 3.4%±3.9%/day, 1.9%±2.2%/day, 1.9%±1.4%/day and 0.8%±1.1%/day, respectively. For tumour sites, medians and quartiles are provided in table 3.

Table 3: Specific growth rates (SGR) for primary tumours (PT) and largest pathological cervical lymph nodes (LN) for common tumour sites of 123 patients with incident HNSCC.

Tumour site	n	PT-SGR[1)]	LN-SGR[1)]
Oral cavity	15	2.4 (1.0; 3.9)	0.8 (0.0; 1.5)
Oropharynx	55	1.4 (0.7; 2.6)	2.5 (0.4; 2.6)
Hypopharynx	25	1.7 (0.6; 2.7)	2.1 (0.6; 2.9)
Larynx	21	1.0 (0.3; 3.1)	0.8 (0.0; 1.4)
Carcinoma of unknown primary	5	0.0 (0.0; 0.0)	1.4 (1.1; 5.2)

1) Median (25th and 75th percentile)

Median PT-DT was 43 days. The 25th percentile was 24 and the 75th percentile 85 days. Median LN-DT was 41 days. The 25th percentile was 25 and the 75th percentile 80 days (Tab. 2). PT-DT and LN-DT were considerably right skewed.

Factors influencing specific growth rates

PT-SGR was independent of the initial PT-volume in diagnostic CT (p=0.19). PT-SGR did also not depend on the interval between 1st and 2nd CT (p=0.14). Moreover, tumour site had no significant impact on SGR (p=0.58; Tab. 3). Interestingly, PT-SGR positively correlated with the expression of EGFR, Ki67 and CD44 (p=0.02; p=0.02 and p=0.03, respectively; Figure 3). The expression of p16 had no influence on PT-SGR (p=0.21).

In contrast, LN-SGR depended on the initial LN-volume measured in diagnostic CT (p=0.003) with higher LN-SGRs in lymph nodes with larger LN-volumes in the 1st CT. Also, the interval between the diagnostic CT and planning CT significantly influenced LN-SGR (p=0.003). The longer the interval, the lower the observed LN-SGR. Moreover, LN-SGR was significantly

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influenced by tumour site (p=0.032; Tab. 3) with smallest growth rates in lymph nodes from laryngeal HNSCC (p=0.003). In contrast to PT-SGR, neither EGFR, Ki67, CD44 nor p16 expression in PTs significantly correlated with LN-SGR (p=0.12; p=0.31; p=0.75 and p=0.81, respectively).

Specific-growth-rate and overall survival

PT-SGR nearly missed significant impact on OS when used as a single covariate in a Cox regression model (log rank p=0.054). For Kaplan Meier analyses, PT-SGR were categorized in 3 groups: slow (< 0.3%/day; n=22); intermediate (0.3% ≤ 3%/day; n=73) and rapid (>3%/day; n=26). Survival curves of these 3 SGR groups did not differ significantly (log rank p=0.45; Figure 4). Likewise, LN-SGR had no significant impact on OS as a covariate in Cox regression (log rank p=0.83) nor in Kaplan Meier analyses (log rank p=0.97; data not shown).

Cartilage or bone infiltration and central lymph node necrosis

Cartilage or bone infiltration was observed in diagnostic CTs of 40/123 (33%) patients and in planning CTs of 50/123 (41%) patients. Thus, during the median 24 days interval between the two CTs, new cartilage or bone infiltration occurred in 10 patients, which was not influenced by PT-SGR (p=0.918). However, cartilage or bone infiltration had a significant negative impact on survival (p=0.003) and was significantly more frequent in p16 negative tumours (p=0.02).

Lymph node necrosis was observed in diagnostic CTs of 74/97 (76%) patients. LN-SGR was significantly higher in patient with lymph nose necrosis (p<0.001), was associated with poorer survival (p=0.03) and did not depend on p16 status. Lymph nose necrosis was observed in planning CTs of 82/97 (85%) patients. Thus, during the median 24 days interval between the two CTs, new lymph node necrosis occurred in 8 patients, which was not influenced by LN-SGR (p=0.818). Central lymph node necrosis had a significant negative impact on survival (p<0.05).

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DISCUSSION

Long waiting times for treatment of head and neck cancer result in decreased overall survival[1-3]. One reason might be meantime tumour growth. We examined tumour growth during the time between the initial diagnostic CT scan and the planning CT for RT or RCT in 123 patients with incident HNSCC. The majority of the patients suffered from incident stage Iva oropharyngeal HNSCC.

The mean interval between 1st and 2nd CT was 29 days ranging from 6 to 146 days. In this time, diagnostic work up, interdisciplinary tumour board presentation and pre-treatment procedures including dental treatments and application of percutaneous gastrostomies were carried out. PT- and LN-volumes in diagnostic and planning CTs were assessed using identical protocols. PT-SGR and LN-SGR were calculated as proposed by Mehrara and co-authors[5]. This mean interval is comparable to previously reports[1]. Moreover, it is considerably shorter than intervals of 46 to 52 days, which were reported as threshold for decreased OS[2]. From a clinical perspective, this is considered positive. However, in terms of exploring SGR's in advanced stage HNSCC, this interval may not be sufficient to accurately determine great changes in PT-volume or LN-volume.

Due to the skewness of volumetric data, the median was considered an appropriate measure of central tendency, but the mean is also provided for comparison with previous publications. Mean PT-volume in diagnostic CTs was approximately 16mL (median≈9mL). In planning CTs of the same patients 3-4 weeks later, mean PT-volume was approximately 25mL (median≈17mL; Tab. 2). These PT-volumes are in line with previous publications reporting mean volumes of 11-37mL[23-26]. Mean LN-volume measured in diagnostic CTs was 9mL (median≈2mL) and approximately 15mL (median≈4mL) in planning CTs (Tab 2). Previously reported mean LN-volumes were considerably larger with 22-25mL[27, 28]. However, in these studies the volume of all pathological cervical lymph nodes was measured instead of only the largest one as in this study.

Tumour and pathological lymph node growth can be reported as doubling time (DT) or specific growth rates (SGR). DT is the number of days the tumour needs to double its volume[4]. The

lower the DT, the faster the tumour growth. Since DT was frequently reported in earlier studies, it was provided for comparison. SGR is defined as relative increase of volume in percent per day. The resulting variable is constant, linear and independent of the initially measured volumes[5]. SGR is considered less affected by measurement uncertainties of short time intervals and minor volume differences than DT[5].

For both, DT and SGR, tumour volumes are required. A volume approximation method from CT scans, which reflects frequently available diagnostics, was used here. This approximation was based on measurements of maximum orthogonal diameters, similar to a formula described in 1990 by MacDonald and co-workers to approximate volumes of brain tumours[29]. The method employed an ellipsoid formula, which was applicable over a wide range of PT and LN sizes and resulted only in a slight underestimation of 8% of the PT-volume measured with the reference method[15]. A similar approximation method has also been used by Jensen and co-authors[30].
More sophisticated segmentation- and more modern imaging techniques, may have allowed for better tumour margin delineation. However, the first require specific workstations with limited availability[15] (i.e. semi-automated or automated slice-by-slice segmentation) and large inter-observer variations may remain[31]. The latter may allow better visualisation of oropharyngeal and oral HNSCC (i.e. magnetic resonance imaging MRI) or better visualisation of locally advanced tumours (i.e. fluordesoxyglucose positron emission tomography FDG-PET). However, both imaging techniques are not as frequently available as CT scans and other limitations apply. For MRIs blurred tumour margins may be observed if patients swallow or breathe, for FDG-PET a lack of spatial resolution may be disadvantageous in smaller HNSCC tumours[31].

Mean primary tumour specific growth rates (PT-SGR) in incident HNSCC was 1.8%/day (Tab. 2), unimodally distributed, slightly right skewed and independent of the initial PT-volume, which supports the basic concept underlying SGR calculation. The interval between diagnostic CT and planning CT did not influence PT-SGR (p=0.1), suggesting that no marked growth deceleration occurred with longer waiting times. Interestingly, PT-SGRs did not significantly differ by tumour site (p=0.6; Tab 3). Murphy and colleagues observed a lower median SGR of 0.74%/day in patients with oropharyngeal cancer[6]. However, the authors did not include sites with faster

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tumour growth such as the oral cavity (median SGR 2.4%/day) and hypopharynx (median SGR 2.0%/day). Moreover, most of the patients reported by Murphy and colleagues had T1 or T2 HNSCC and were p16 positive.

The median PT-DT in this study was 43 days. The 25th percentile was 24 and the 75th percentile 85 days. Median LN-DT was 41 days. The 25th percentile was 25 and the 75th percentile 80 days (Tab. 2). Jensen and co-authors reported a slower growth rate in 61 patients with HNSCC with a median PT-DT of 99 days. The authors however additionally stated that half of the patients showed a faster growth rate with a PT-DT of 30 days[30]. When compared with DT of other solid tumours, this means that HNSCC reveal rapid tumour growth. For breast cancer, Ingebly and co-authors reported a median DT of 285 days[4]. For lung cancer, DTs vary depending on histology between median 42 days for metastases as reported by Loeffler and colleagues and 181 for non-small-cellular lung cancer as reported by Winer-Muram and co-workers[4,32]. For pancreatic adenocarcinoma, Furkawa and colleagues reported a mean PT-DT of 144 days[32]. For Sarcomas median PT-DTs of 35 days were reported by Blomqvist and co-authors[32]. Additional median PT-DTs for additional solid tumours are provided in table 4.

Table 4: Reported tumour volume doubling times for selected solid tumours*.

Tumour	**Median DT [days]**	**n**
Present study	47	123
HNSCC[30]	99**	61
Breast cancer[4]	285	16
Lung bronchioalveolarcarcinoma[32]	181	9
Non small-cell lung carcinoma[32]	181	6
Lung metastasis[4]	42	24
Pancreatic adenocarcinoma[32]	144	9
Sarcoma[32]	35	21

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*modified after Mehrara and co-authors[32]

**Jensen and co-workers also stated tumour volume doubling times for the faster growing half of the patients with a median PT-DT of 30[30].

PT-SGRs significantly correlated with the expression of EGFR, Ki67 and CD44 (p=0.021; p=0.018 and p=0.031, respectively) with higher PT-SGRs in patients with higher expression of the three biomarkers (Figure 3). These correlations appear biologically sound. A correlation of clinical tumour growth rates and expression of these biomarkers within the tumour of the same patients was not yet reported. EGFR is a cell surface receptor which promotes proliferation, invasion, angiogenesis and metastatic spread in HNSCC, if overexpressed[8]. Ki-67 is a nuclear protein expressed on cells in all phases of the cell cycle except in G0-phase. Thus, its expression marks the total fraction of proliferating cells in a tumour[9]. In laryngeal and hypopharyngeal SCC, an association between Ki67 expression and advanced tumour stages has been reported[10-11]. In line with our observation of a correlation of CD44 expression and PT-SGR, an association between advanced T categories and high CD44 expressions has been reported in a meta-analysis including thirty studies with 2102 patients[12]. No significant correlation between PT-SGR and p16 expression was observed (p=0.81). This observation differs from previous observations made by Murphy and colleagues. The authors observed that p16 expression correlated well with PT-SGR with faster growth rates in p16 negative HNSCCs. However, the authors included oropharyngeal HNSCC only and the majority (79%) were p16 positive tumours[6]. When used as a covariate in a Cox model, PT-SGR had no significant effect on OS (log rank p=0.054). No significant differences of survival curves were also observed, if PT-SGR were categorized in groups of low, medium and high growth rates (log rank p=0.45; Figure 4). In contrast, van Bockel and co-worker observed a significant association between PT-SGR and OS in laryngeal cancer[7].

To our knowledge, no data on lymph node specific growth rates (LN-SGR) in HNSCC have been previously reported. In the investigated patients, the mean LN-SGR was 1.7%/day, similar to mean PT-SGR (Tab. 2). In contrast to PT-SGR, LN-SGR did depend on initial LN-volumes in diagnostic CT scans (p=0.003) with higher LN-SGRs in lymph nodes with larger initial LN-volumes. Also, the interval between diagnostic CT and planning CT significantly influenced LN-

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SGR (p=0.003). The longer the interval, the lower the observed LN-SGR, suggesting growth slowdown. Moreover, LN-SGR was significantly influenced by tumour site (p=0.03; Tab. 3), with smallest growth rates in lymph nodes from laryngeal HNSCC. In contrast to PT-SGR, LN-SGR did not depend on expressions of EGFR, Ki67, CD44 nor p16 expression in primary tumours (p=0.115; p=0.311; p=0.746 and p=0.809 respectively). In LN, specific growth rates had no significant impact on OS either (p>0.05).

New cartilage or bone infiltrations during the observation period were observed in 10 patients and new central lymph node necroses were observed 8 patients. Specific growth rates had no significant impact on the development of either of them. Both, initial cartilage or bone infiltration and central lymph node necrosis were associated with worse survival. However, probably due to the low number of events, no significant impact of new cartilage or bone infiltration nor new central lymph node necrosis on survival was observed in Kaplan Meier analysis (both log rank p>0.05).

Certain limitation of the present study need to be addressed. Firstly, this small numbered retrospective study predominantly exploring patients with advanced stage oropharyngeal HNSCC should be supplemented by larger, prospective investigation, which also included patients with limited disease of all common HNSCC tumour sites. Secondly, the approximation method[15] proposed here may be easily performed from frequently available CT scans over a wide range of different PT- and LN-volumes. However, more sophisticated segmentation- and more modern imaging techniques may have allowed for better tumour margin delineation and hence form more accurate SGRs. Thirdly, the comparably short interval between diagnostic CT scans and planning CT scans may be considered positive from a clinical perspective. However, in terms of exploring SGRs in advanced stage HNSCC, this interval may not be sufficient to accurately determine great changes in PT- and LN-volume.

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CONCLUSION

Head and neck squamous cell carcinomas are rapidly growing malignancies. Primary tumours and lymph nodes grow more than 1% per day. Consequently, time matters, and treatment must not be delayed.

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ABBREVIATIONS

CD44	CD44 antigen
CT	computed tomography
DICOM	Digital Imaging and Communications in Medicine
DT	tumour volume doubling time
EGFR	epidermal growth factor receptor
FDG-PET	fluordesoxyglucose positron emission tomography
HNSCC	head and neck squamous cell carcinoma
Ki67	Ki67 antigen
LN	largest pathological cervical lymph node
LN-DT	largest pathological cervical lymph node doubling time
LN-SGR	largest pathological cervical lymph node specific growth rate
MRI	magnetic resonance imaging
p16	Cyclin-dependent kinase inhibitor 2A
PACS	Picture Archiving and Communication System
PT	primary tumour
PT-DT	primary tumour volume doubling time
PT-SGR	primary tumour specific growth rate
RCT	radiochemotherapy
RT	radiotherapy
SD	standard deviation
SGR	specific growth rate
TNM	tumour, node, metastasis
UICC	Union internationale contre le cancer

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REQUIRED STATEMTENTS

Acknowledgements

This work has not yet been previously presented at a conference or as a conference abstract.

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Ethics statement

The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Competing interest and funding statement

All authors have completed the Unified Competing Interest form and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

Contributorship statement

Daniel Dejaco has written major parts of the manuscript and performed the calculations of tumour and lymph node volumes. Teresa Steinbichler was involved in establishing the study concept, design and the internal review process of the manuscript. Schartinger Volker established the population based tumour registry from which patients were enrolled. He further obtained the ethics committee approval for the study. Natalie Fischer performed additional tumor and pathological cervical lymph node measurements and was involved in the internal

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review process of the manuscript. Maria Anegg has performed additional calculations and data base work in the population based tumour registry. Joszef Dudas performed the immunohistochemical staining of the tumour biopsies and was involved in establishing the study concept, design and the internal review process of the manuscript. Andrea Posch provided the radiotherapy-planning CT scans and performed performed additional tumor and pathological cervical lymph node measurements. Gerlig Widmann supervised the tumor and pathological cervical lymph node measurements as an experienced head and neck radiologist. Herbert Riechelmann supervised the writing process of the manuscript and performed all statistical calculations in the manuscript.

Transparency declaration

The Corresponding Author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study have been explained.

Details of ethical approval and patient consent

The review board of the Medical University of Innsbruck had approved the study (UN4590) and informed consent was obtained from all study participants.

Data sharing

No additional data from the study is available.

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FIGURE LEGENDS

Figure 1: Tumour growth assessment using maximal orthogonal tumour diameters from axial contrast enhanced diagnostic CT scans (A) and subsequent planning CT scans (B)

Axial contrast enhanced diagnostic CT scan (A) and subsequent planning CT scan (B) 46 days later of a cT4a cN2b cM0 squamous cell carcinoma of the oral cavity. The maximum anterior-posterior and medio-lateral tumour diameters (white lines) were measured from axial scans, the cranio-caudal tumour diameters were measured from corresponding coronal scans (not depicted). The tumour volume was assessed as previously described using an ellipsoid formula[15]. PT-volume from diagnostic CT was 14.8mL, PT-volume from planning CT was 51.0mL translating to a PT-SGR of 2.8%/day.

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Figure 2: Correlation of primary tumour volume (A) and largest pathological lymph node volume (B) measured from diagnostic CT scans and a planning CT scans

Scattergram of primary tumour volumes (A) and largest pathological lymph node volume (B) measured from diagnostic CT scans (x-axis) planning CT scans (y-axis). Both axes are on log scale. The diagonal line represents the line of identity. Dots above this line indicate volume increases.

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Figure 3: Specific growth rates of the primary tumours and percentage of Ki67 positive cells

Percentage of Ki67 positive immunohistochemistry in tumour samples of HNSCC patients and according primary tumour specific growth rates. The percentage of Ki67 positive cells was grouped in 0%, 1 to 30%, 31 to 60% and more than 60% of cancer cells (x-axis). Mean specific growth rates of HNSCC primary tumour (y-axis) were obtained 119 patients. Small bars represent standard deviation. PT-SGR positively correlated with the expression of Ki67 (Jonckheere-Terpstra p=0.02).

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Figure 4: Kaplan Maier plot for specific growth rates of primary tumours grouped by low, medium and high growth rate

Kaplan Maier plot of specific growth rates (SGR) of primary tumours grouped by low (black line; SGR< 0.3%/day; n=22); medium (dark grey line; 0.3% ≤ SGR < 3%/day; n=73) and high (pale grey line; SGR >3%/day; n=26) growth rates. X-axis represents time in months, Y-axis overall survival. The survival curves of the 3 specific growth rate groups did not differ significantly (log rank p=0.45).

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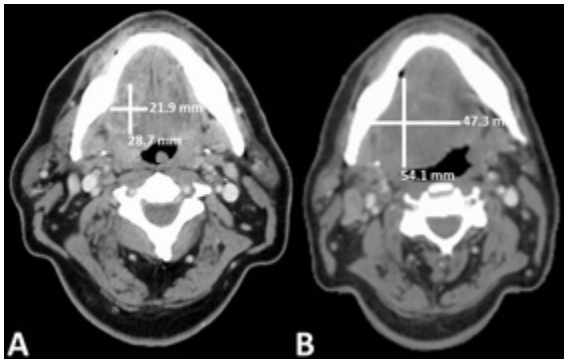

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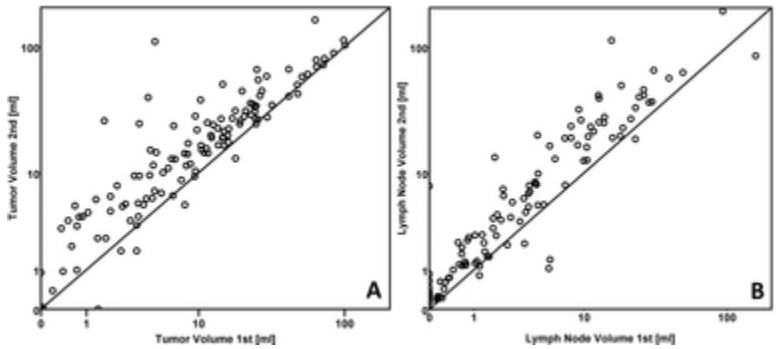

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Scattergram of primary tumour volumes (A) and largest pathological lymph node volume (B) measured from diagnostic CT scans (x-axis) planning CT scans (y-axis). Both axes are on log scale. The diagonal line represents the line of identity. Dots above this line indicate volume increases.

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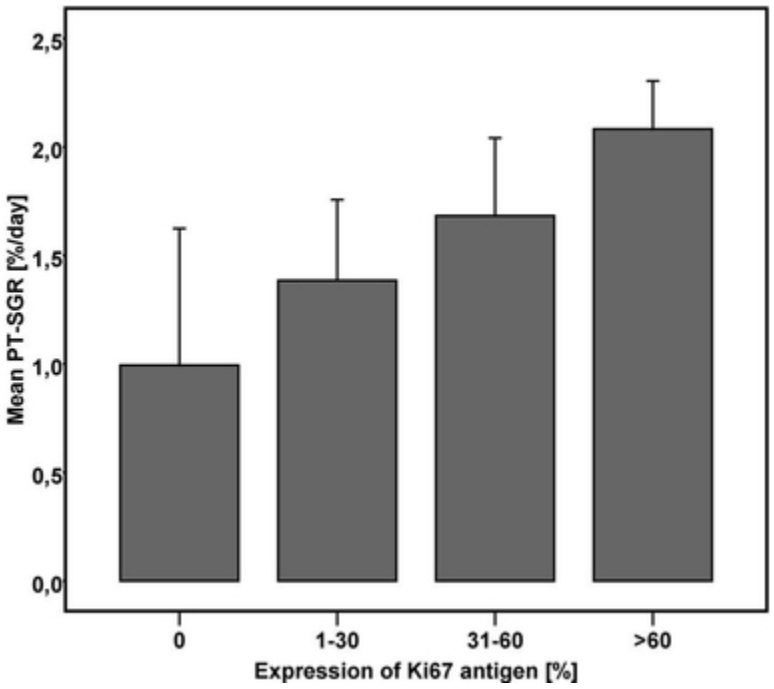

Figure 3: Specific growth rates of the primary tumours and percentage of Ki67 positive cells
Percentage of Ki67 positive immunohistochemistry in tumour samples of HNSCC patients and according primary tumour specific growth rates. The percentage of Ki67 positive cells was grouped in 0%, 1 to 30%, 31 to 60% and more than 60% of cancer cells (x-axis). Mean specific growth rates of HNSCC primary tumour (y-axis) were obtained 119 patients. Small bars represent standard deviation. PT-SGR positively correlated with the expression of Ki67 (Jonckheere-Terpstra p=0.02).

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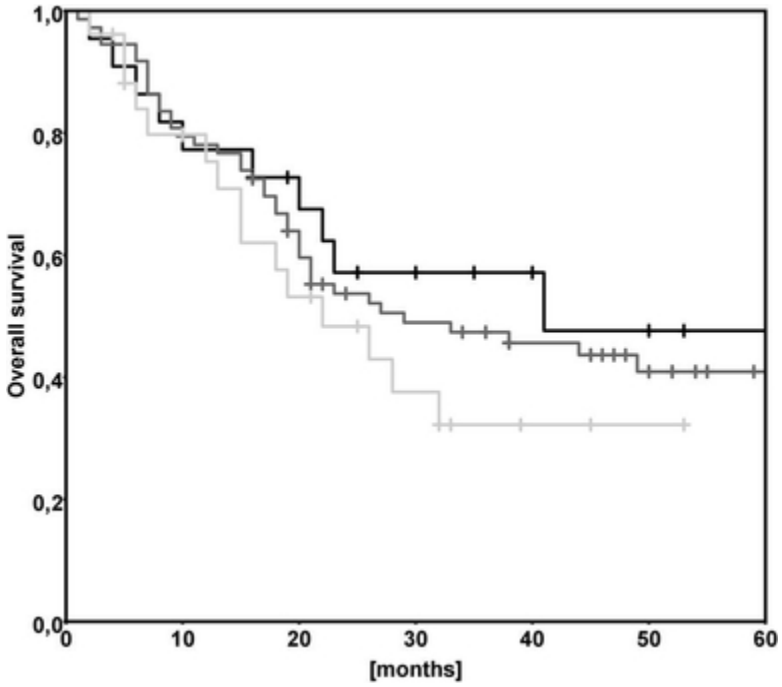

Figure 4: Kaplan Maier plot for specific growth rates of primary tumours grouped by low, medium and high growth rate

Kaplan Maier plot of specific growth rates (SGR) of primary tumours grouped by low (black line; SGR< 0.3%/day; n=22); medium (dark grey line; 0.3% ≤ SGR < 3%/day; n=73) and high (pale grey line; SGR >3%/day; n=26) growth rates. X-axis represents time in months, Y-axis overall survival. The survival curves of the 3 specific growth rate groups did not differ significantly (log rank p=0.45).

16x14mm (600 x 600 DPI)

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STROBE Statement: "Time matters – the fast growth of head and neck squamous cell carcinoma"

	Item No	Answer
Title and abstract	1	(*a*) The retrospective study design was described in the "Abstract" section, page 2, line 7, the "Strengths and Limitations of the Study" section, page 3 and the "Introduction" section, page 5.
		(*b*) An informative and balanced summary of what was done was provided in the "Abstract" section, page 2.
Introduction		
Background/rationale	2	The scientific background and rational for the investigation was reported in the "Introduction" section of the manuscript, page 5.
Objectives	3	The objective of the study was specified in the "Abstract" section, page 2 and at the end of the "Introduction" section, page 2.
Methods		
Study design	4	Key elements of the study design were mentioned early in the "Abstract" section, page 2 and the "Introduction" section, page 5 of the manuscript. Additional details of the study design were outlined in the "Methods", page 6 to 8.
Setting	5	The setting, location, relevant dates, including period of recruitment and data collection was included in the "Methods" section, page 6 and in the "Results" section, page 9.
Participants	6	(*a*) *Cohort study*— The eligibility criteria and the sources and methods of selection of participants are provided in detail in the "Methods" section, page 6.
		(*b*) *Cohort study*— No matching was performed in this study.
Variables	7	All outcomes, predictors, potential confounders and effect modifiers were defined, presented and discussed in the "Methods" section, page 6 to 8, "Results" section, page 9 to 12and "Discussion" section, page 13 to 16. Diagnostic criteria were provided in the "Methods" section, page 6, when applicable.
Data sources/ measurement	8*	Source of data and details of methods was provided in the "Methods" section, page 6 and 8.
Bias	9	Potential sources of bias were addressed in the "Discussion" section, page 13 to 16.
Study size	10	An explanation how the study size was arrived at was provided in the "Method" section, page 6.
Quantitative variables	11	An explanation how quantitative variables were handled in the analyses was provided in the "Methods" section, page 8.
Statistical methods	12	(*a*) All statistical methods were described in the "Methods" section, page 8.
		(*b*) All methods for subgroup analyses of the presented study were described in the "Methods" section, page 8.
		(*c*) An explanation how missing data was addressed was provided in the "Methods" section, page 8, "Results" section, pages 9 to 12 and "Discussion" section, pages 13 to 16.
		(*d*) *Cohort study*—No loss to follow-up occurred due to the study design.
		(*e*) No sensitivity analyses were performed in the study.

Continued on next page

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Results		
Participants	13*	(a) Numbers of individuals at each stage of the study was reported in the "Results" section, page 9 and the "Discussion" section, page 13.
		(b) No non-participation occurred due to the study design.
		(c) No flow diagram was used for this study.
Descriptive data	14*	(a) Clinical characteristics of study participants were provided in table 1, page 9.
		(b) Number of participants with missing data for each variable of interest was provided in the "Results" section, page 9 and in table 1, page 9.
		(c) *Cohort study*—Follow-up time was summarised as time between diagnostic computed tomography scan and planning computed tomography scan in this study provided in the "Results" section, page 9.
Outcome data	15*	*Cohort study*— Numbers of outcome events was provided in the "Results" section, page 9 to 12.
Main results	16	(*a*) Means, medians, standard deviations, 95% confidence intervals and 25th and 75th percentiles were provided in the "Results" section, page 9 to 12, if applicable.
		(*b*) Category boundaries for continuous variables were specified in the "Methods" section, page 8 and "Results" section, pages 9 to 12.
		(*c*) No relative risk estimations were performed in this study.
Other analyses	17	All additional other analyses performed in the study were outlined in the "Methods" section, page 8 and "Results" section, pages 9 to 12.
Discussion		
Key results	18	Key results with reference to previously outlined study objectives were summarised in the "Discussion" section, pages 13 to 16.
Limitations	19	Limitations of the study, taking into account sources of potential bias and imprecision were outlined in the "Discussion" section, pages 13 to 16.
Interpretation	20	A cautious overall interpretation of the results was given in the "Discussion", pages 13 to 16and the "Conclusion" section, page 17.
Generalisability	21	The external validity of the study results was outlined in the "Discussion" section, pages 13 to 16 and table 4, page 15.
Other information		
Funding	22	No financial support for any of the work presented in the present manuscript was obtained, provided on page 19.

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