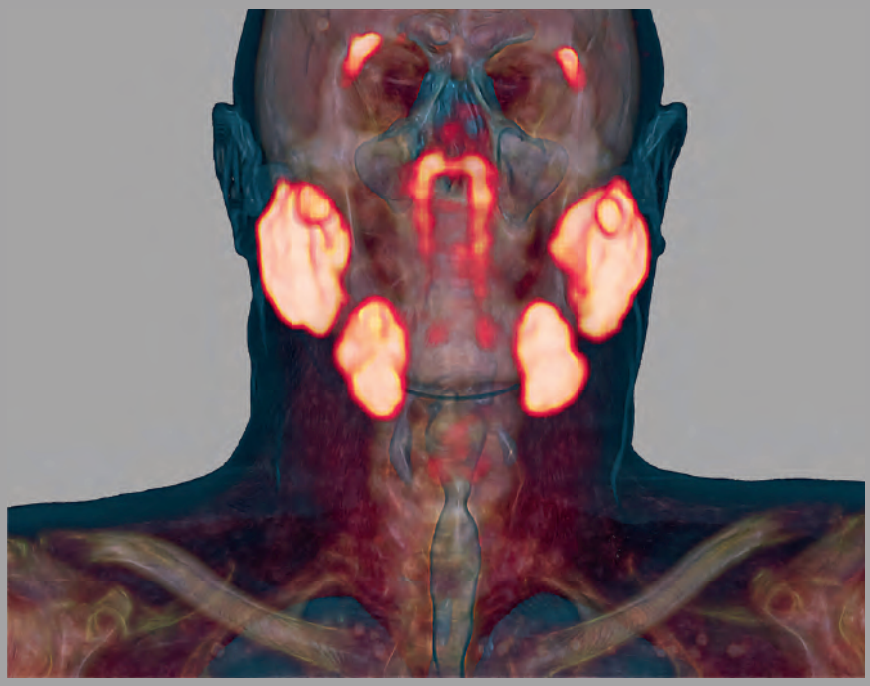
New epidemiological and PSMA-expression based insights in salivary gland tumors



Matthijs H. Valstar

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New epidemiological and PSMA-expression based insights in salivary gland tumors

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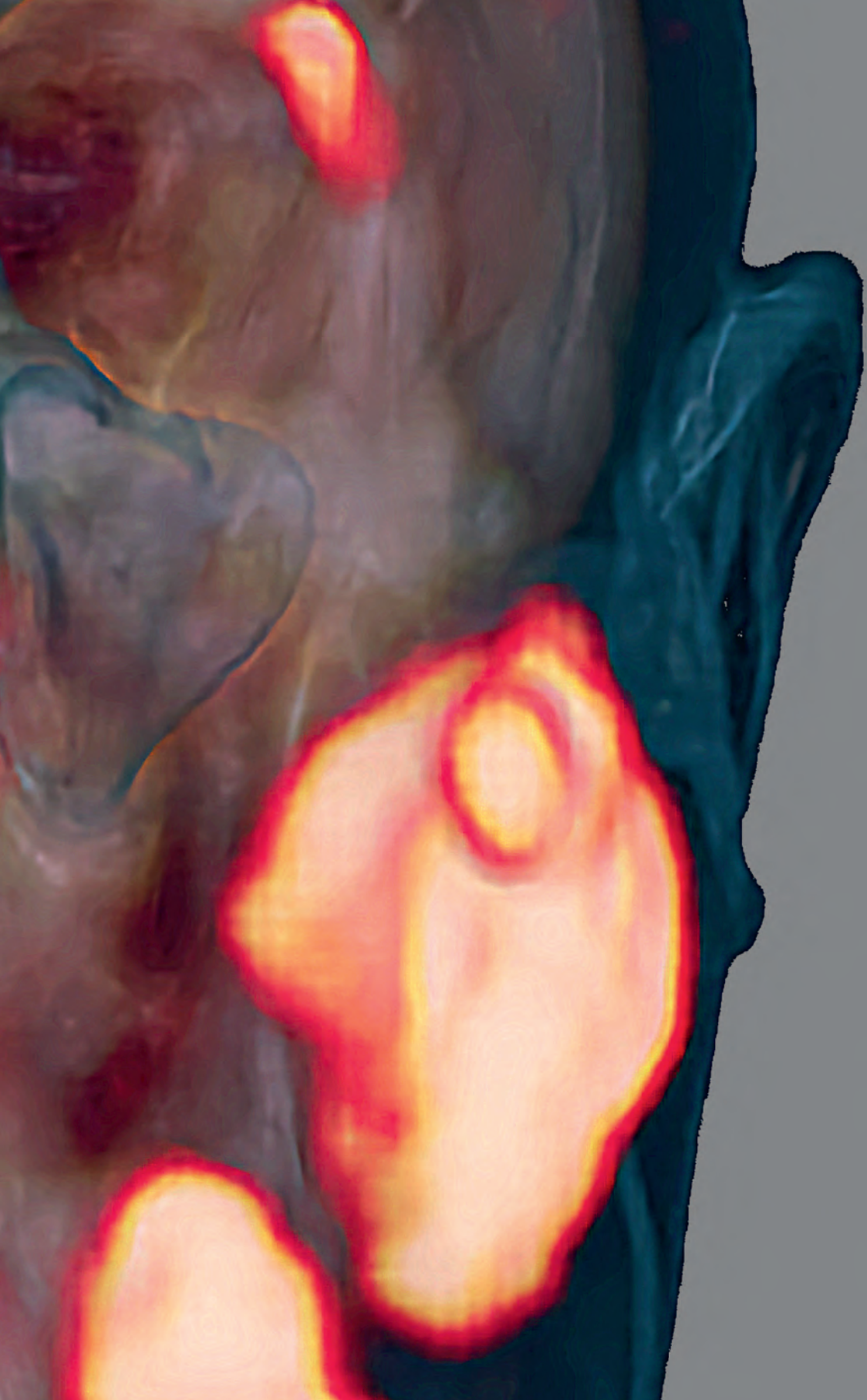
Contents



Chapter 1

General introduction and outline of this thesis

Anatomy and function

Humans are known to have three pairs of major salivary glands and about a thousand minor glands spread out in the airways and digestive tract mucosa and submucosa. These glands produce saliva which is important for several biological and mechanical functions. Saliva helps in digestion (it contains e.g. the enzyme amylase), moistening and lubrication of food, prevention of dental decay and wear, killing of germs such as bacteria, yeasts and viruses, protection against chemical agents and dehydration. It also plays a role in hemostasis.

The major salivary glands are located in front of and below the ears (parotid glands), under the lower jaw (submandibular glands) and under the tongue (sublingual glands) (Figure 1). The parotid and the submandibular glands have relatively long ducts and the sublingual glands have shorter ducts that are connected with the submandibular ducts. About 700-1000 of minor glands are known to be spread out all over the mucosa and submucosa of the oral cavity, the nose and the throat (nasopharynx, oropharynx and hypopharynx).

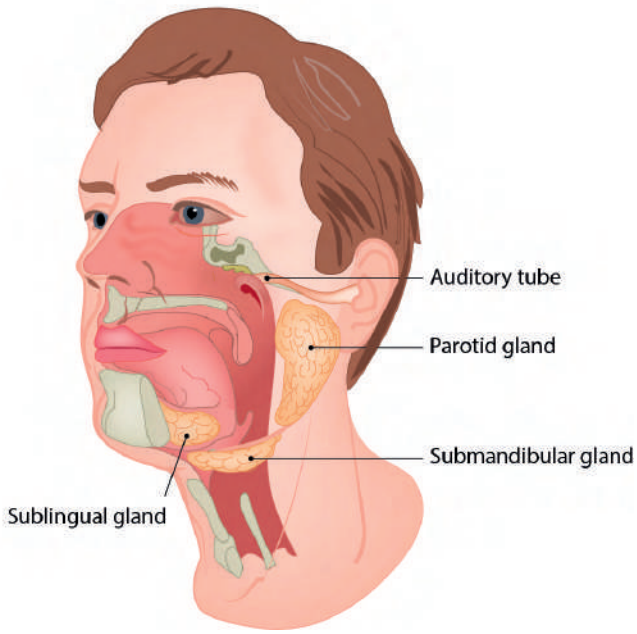


Figure 1: The position of the major salivary glands: the parotid, the sublingual and the submandibular gland (Illustration by R. Slagter, 2021).

The salivary glands contain many other anatomical structures such as lymph nodes and lymph vessels, blood vessels and nerves. The facial nerve, which controls the muscles of facial expression, runs through the parotid gland. It can therefore make surgery of particularly this gland challenging (Figure 2).

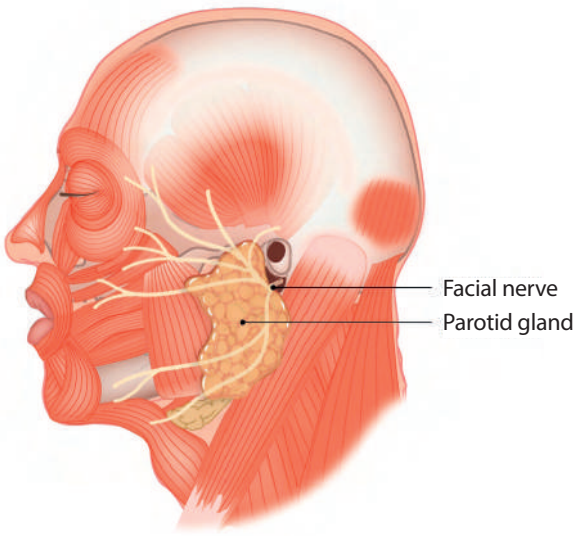


Figure 2: Lateral view of the head, showing the facial nerve running through the parotid and branching into 5 major branches: temporal/frontal, zygomatic, buccal, mandibular and cervical (Illustration by R. Slagter, 2021).

Embryology, histology and physiology

Embryologically, the first glands are formed in the 4-6th week of gestation, out of different layers of tissue, the ectoderm and surrounding mesenchyme (major salivary glands) and in case of the minor glands, also the endoderm [1]. These layers form ducts (tubuli) and units of serous, mucous or seromucous cells (acini) that make watery (serous) or slimy (mucous) saliva (Figure 3). Every day about 1-1.5 liter of saliva is produced, mostly by the parotids when stimulated and by the submandibular gland when not. The saliva contains dissolved salts in the form of ions such as Na^+, Cl^-, K^+, HCO_3- that have a role in the excretion process. It also carries enzymes such as amylase, which helps to break down starch. Saliva also contains proteins that give it the slimy properties such as mucines. The mucines help in the clearance of the oral cavity of germs. These

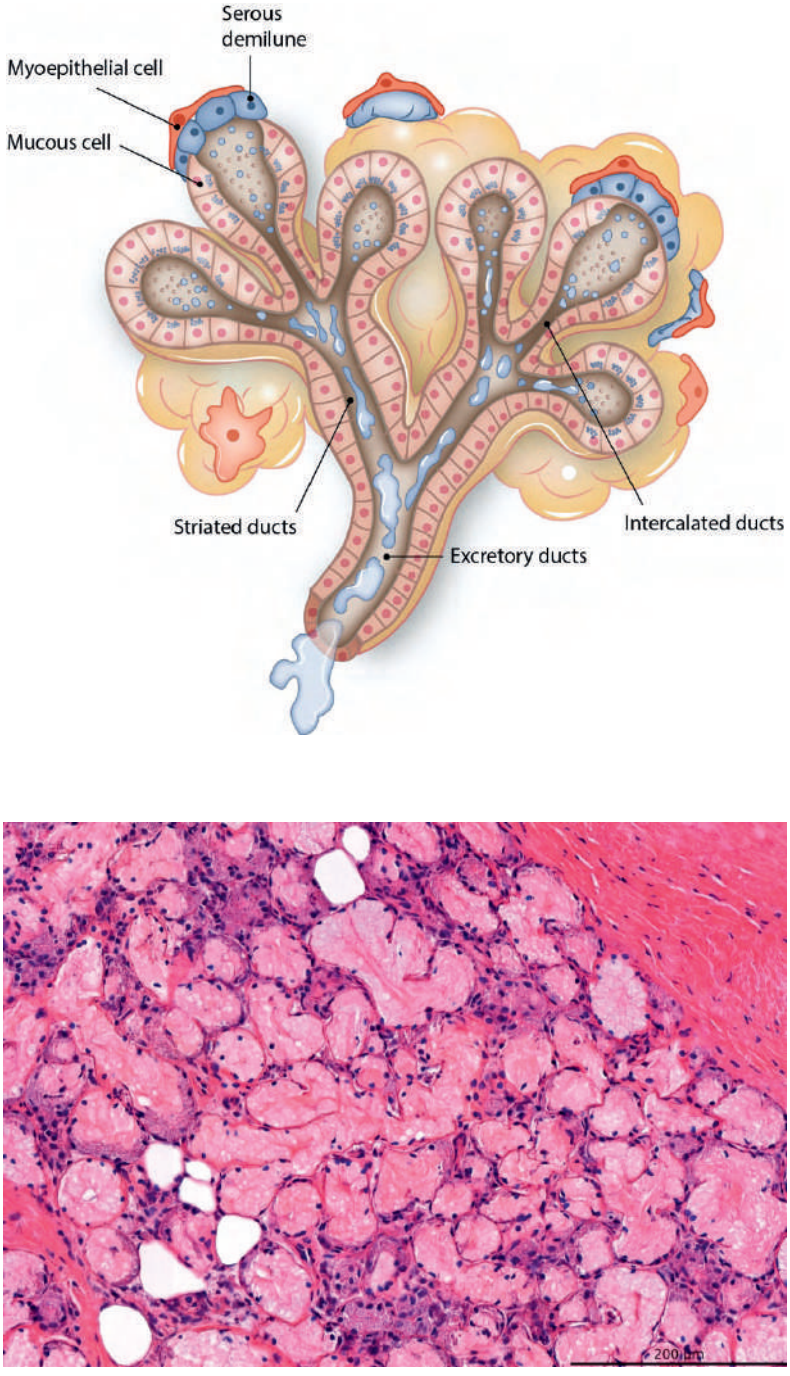


Figure 3: Upper panel showing the acinus, the smallest united of a salivary gland, with mucous and myoepithelial cell, serous demilunes and the excretory duct (Illustration: R. Slagter, 2021). Lower panel showing a HE-slide with mostly mucous glands.

proteins also help in keeping the surfaces of the mucosa and teeth smooth, which prevents mechanical wear. The salivary gland function is regulated by the autonomous nervous system, via parasympathetic and sympathetic fibers that run in several nerves in the neck and face to the glands, such as the auriculotemporal nerve for the parotid.

Function is not only regulated by the nerves, but can also be affected by hormones, medication (e.g. antihypertensive medication) and diseases such as the rheumatic autoimmune disorder Sjogren's syndrome.

Surgical pathology of salivary gland tumors

Surgery is sometimes needed to cure pathology such as salivary stones, infections, cysts, but also in case of neoplasms. Salivary gland tumors are rare tumors. Around 85% of these tumors are benign, but malignant salivary tumors also occur [2]. The most frequent salivary gland tumor is the salivary gland pleomorphic adenoma (SGPA) (Figure 4).

By definition, national cancer registries do not register benign tumors. Therefore, the SGPA incidence on a nationwide scale is difficult to determine. This tumor has a diversity of morphological presentations with a varying combination of different cell types (epithelial, myoepithelial and mesenchymal). It has two notorious characteristics that make it extra interesting. In case of damage to the tumor capsule during surgery, many small cell collections can be spilled that can cause multinodular recurrences. Solving this problem is a surgical challenge (also because of the location of the facial nerve) and in selected cases radiotherapy is thought to be of help, despite the fact that optimal proof of its effect is not yet available.

The SGPA sometimes turns into a malignant tumor, which is called a "carcinoma ex pleomorphic adenoma", despite is benign background. The available evidence has shown presence of malignant cells and behavior, as well as genetic mutations in parts of the benign SGPA. However, the complete molecular cascade from a benign SGPA without genetic mutations to a subsequent malignant recurrence with presence of carcinogenic mutations, has never been reported. The malignant

salivary gland tumors have a surprisingly wide range of histological presentations. The 2017 classification of the world health organization (WHO) mentions 22 different primary salivary gland cancers [3]. These tumors have malignant characteristics such as invasive growth, mitotic figures, necrosis and genetic mutations [4].

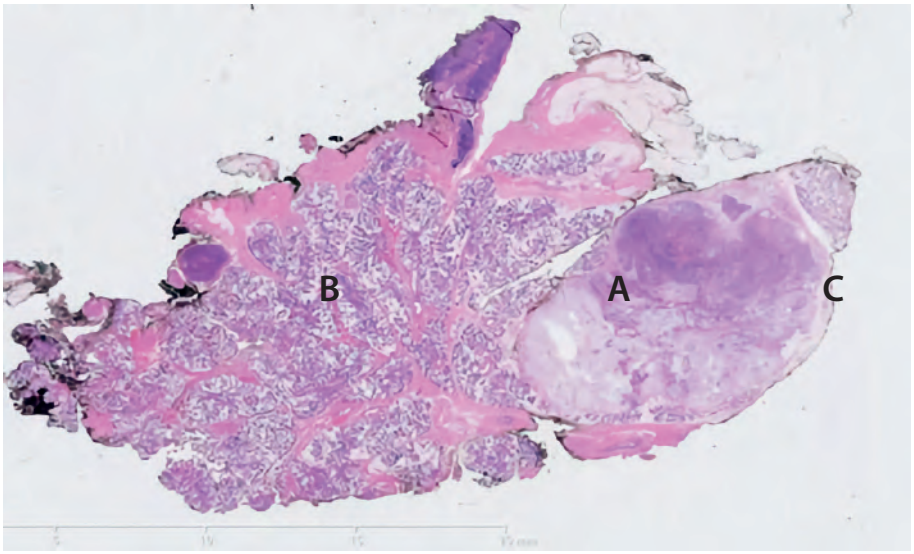


Figure 4: Salivary gland pleomorphic adenoma (SGPA) (A), partly covered by healthy salivary gland tissue (B), but on the right it is covered by only a thin tumor capsule (C) (Courtesy of dr. M.F. van Velthuysen).

Epidemiology

Despite of the large number of different salivary glands cancers, their overall incidence is very low, with a reported incidence of 0.9 per 100,000 persons per year in the Netherlands and similar numbers in Denmark [5,6] (Figure 5). Men and women were affected equally and patients were treated with surgery and adjuvant radiotherapy in most cases. The relative 5-year survival in the Netherlands was 69%. The benign tumors have a larger share in salivary gland tumor incidence. In a defined regional population in the UK, there were roughly 7 benign tumors and 1 malignant per 100,000 persons per year [2]. Spiro et al. reported the pleomorphic adenoma as histological diagnosis in 45% of the salivary gland tumors in a large series in the US [7].

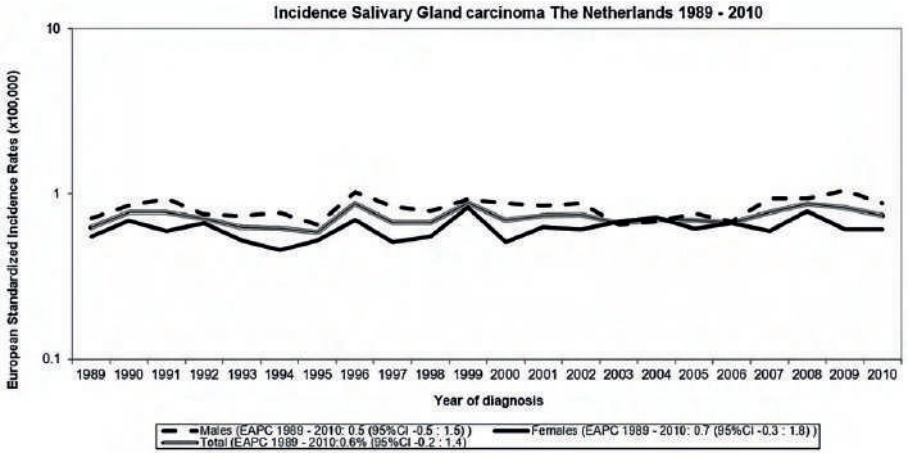


Figure 5: Incidence of salivary gland cancer in the Netherlands, showing an incidence of just under 1 case per 100,000 persons per year (dashed line, males; black, females; grey, total). Courtesy of dr. M. de Ridder [5].

Imaging of the salivary glands

Conventional imaging modalities (ultrasound, CT, MRI) show the major salivary glands in various detail. In tumor diagnostics, MRI depicts salivary gland tumors best. However, these modalities have never allowed visualization of minor salivary glands. Imaging of radioactive substances (ligands) that bind to specific molecules, is called molecular imaging. It allowed for new imaging possibilities for salivary glands and their function some years ago [8]. But recently a new type of molecule for depicting salivary glands has emerged. It can visualize the salivary glands on nuclear scans by showing prostate specific membrane antigen expression by gland tissues. It was originally named and indicated for patients with prostate cancer (PSMA PET/CT) (Figure 6). Possible new perspectives the technique provides for head and neck cancer have until recently not received any attention.

Aims and Outline of this thesis

Because of the interesting nature of salivary glands and salivary gland tumors, this thesis will explore a main topic in both fields: epidemiology of salivary gland tumors and imaging of the salivary glands by PSMA PET/CT.

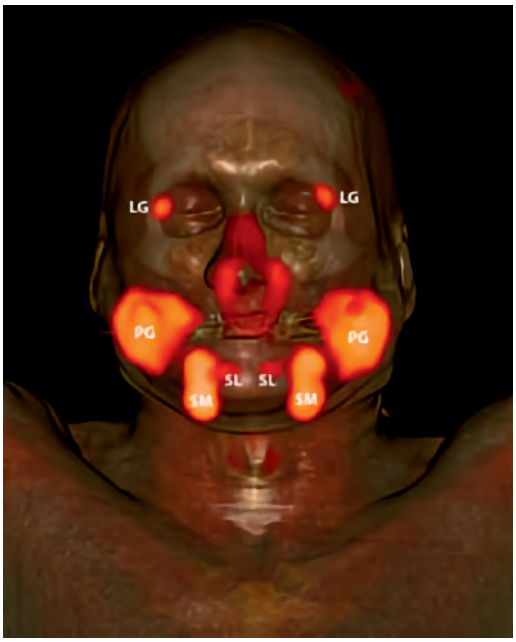


Figure 6: A PSMA PET/CT scan showing the major salivary glands (Parotid, PG; Sublingual, SL; Submandibular, SM), minor glands in the nose / nasopharynx and the lacrimal glands (LG), as published by Klein Nulent, Valstar, et al. in Chapter 7 [9].

Despite the fact that the SGPA is by far the most frequent salivary gland tumor and has a malignant potential, its epidemiology had never been investigated on a nationwide scale. It meant that there was no optimal insight in the risk of recurrence and malignant transformation. We aimed to provide such an insight in **Chapter 2,** by presenting the results of a nationwide study on the incidence of salivary gland pleomorphic adenoma, risk of recurrence and malignant transformation and risk factors for recurrence in the Netherlands. The findings in our study and a similar study that was conducted almost parallelly in Denmark, made us wonder whether a pattern could be discerned in behavior of recurrent pleomorphic adenomas in the published literature. **Chapter 3** presents the natural history of the recurrent pleomorphic adenoma, described in a systematic review and meta-analysis of the Danish and Dutch nationwide studies in an international collaboration. By combining data from these studies, a more detailed picture from rare events such as malignant transformation could be provided.

The concept of this process of a benign tumor turning malignant has been described by various reports showing small and large pieces of the puzzle. As an

example, mutations have been shown in parts of SGPAs that showed malignant behavior. But the complete process of malignant transformation at a genetic level, had never been shown yet in a single patient. In **Chapter 4** we present the first case showing this process as a proof of principle of malignant transformation of a pleomorphic adenoma.
Since literature suggests that women have a higher risk of developing SGPA than men, a hormonal role was suspected. Other arguments add to this thought, such as the presence of hormone receptors on SGPA and salivary gland malignancies. In patient care, clinicians in our hospital had earlier noted a sequence of events where women had bot a salivary gland tumor and breast cancer at some point in time. A local study suggested a correlation between the tumors and we wondered whether our nationwide cohorts of patients with either SGPA of salivary gland cancer would show an increased risk of breast cancer. **Chapter 5** aims to provide the answer to that question.

The developments in imaging of a particular molecule in a field in medicine can sometimes provide a new paradigm regarding a process in another specialty. It is as if a curtain is opened and one can see the view outside the window. Molecular imaging of the prostate specific membrane antigen (PSMA) is used for detection and follow-up of prostate cancer. Other tissues show up on these prostate patient scans as well, such as salivary glands, but these had not been systematically described yet. In order to be able to distinguish metastasized prostate cancer in the neck region from salivary gland tissue, we aimed to systematically describe uptake of the radioactive tracer (PSMA-ligand) for imaging with positron emission tomogram (PET) / computed tomography (CT) in the glands in the head and neck region in **Chapter 6.**
The technique of depicting salivary and seromucous glands can theoretically be used to assess and guide head and neck irradiation in head and neck cancer patients. We aimed to present cases to illustrate and discuss this idea in **Chapter 7.**
The images of the PSMA PET/CT scans consistently suggested a collection of (salivary) gland tissue in the dorsolateral nasopharynx, near the auditory tube entrance. Although a higher density of microscopic minor glands had been described before, these large glandular tissue collections did not seem to fit in the existing picture of up to a thousand mainly spread out glands in the aerodigestive tract. Could it be that these are highly concentrated collections are present in all humans, both male and female? And if so, could they be of clinical relevance

in radiotherapy for head and neck cancer? **Chapter 8** aims to answer all these questions in a histological study using a new 3-dimensional reconstruction technique, followed by an evaluation of imaging findings in 100 patients. The clinical relevance for head and neck radiotherapy is investigated in the last part of the chapter in a large clinical cohort of 723 patients, by looking at this region with an abundance of gland tissue as a potential organ at risk.

Comments and suggestions in letters-to-the-editor regarding this paper will addressed in a reply with support of additional investigations and results in **Chapter 9. Chapter 10** summarizes the research projects reported in this thesis and **Chapter 11** aims to look beyond the published papers and provides future perspectives for salivary gland (tumor) research.

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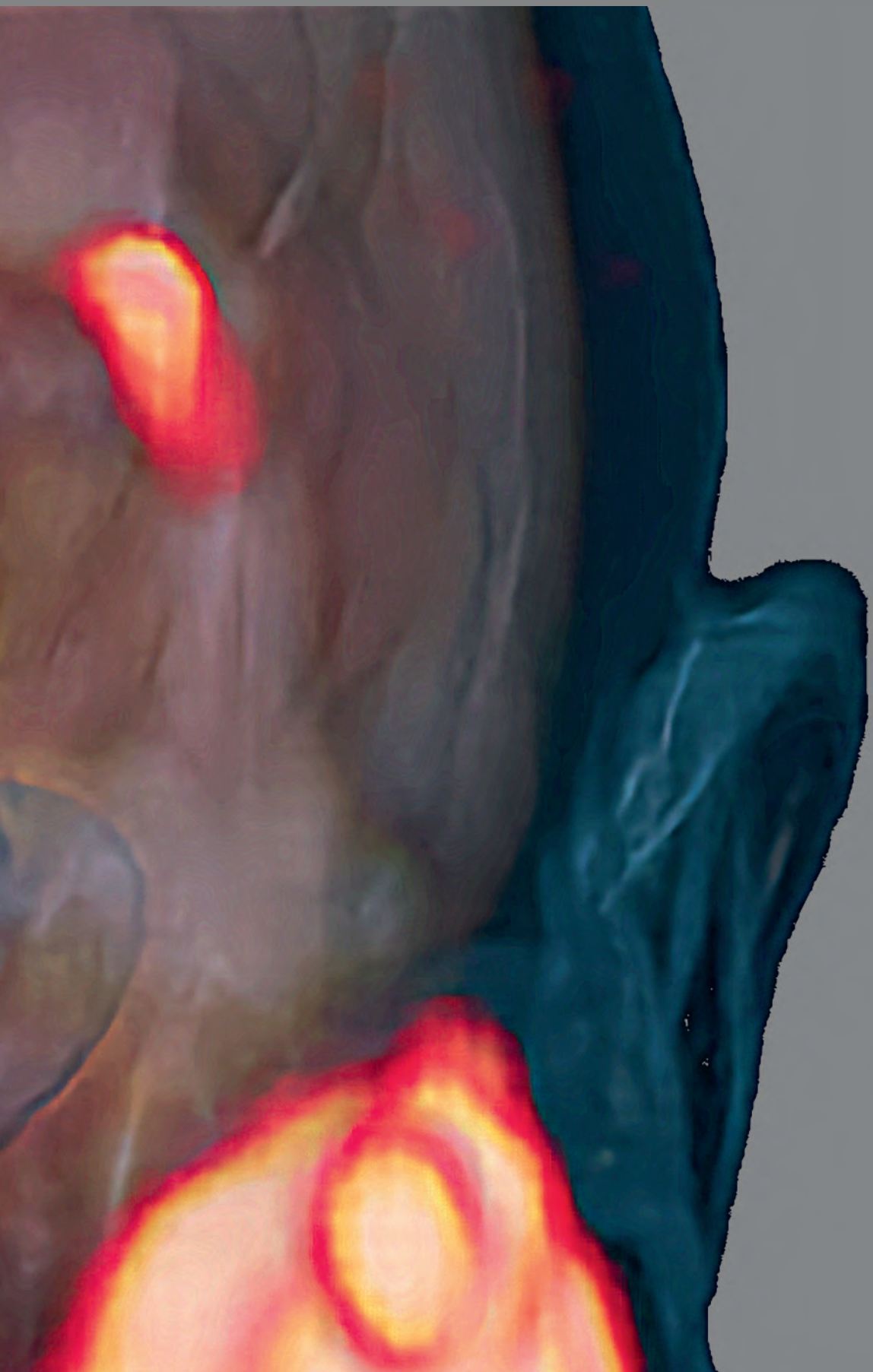
PART 1

Epidemiology of salivary gland tumors



Chapter 2

Salivary gland pleomorphic adenoma in the Netherlands: A nationwide observational study of tumor incidence, malignant transformation, recurrence, and risk factors for recurrence.

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E.C. van den Broek
M.M. Stuiver
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Presented at the Twelfth Congress of the European Association for Cranio-Maxillo-Facial Surgery in Prague, Czech Republic, 2014.

Oral Oncology 2017; 66:93-99

Abstract

Introduction: Whereas salivary gland pleomorphic adenoma (SGPA) is the most common type of salivary gland tumor, little is known about its epidemiology because national cancer registries do not register this disease.

Objectives: To establish SGPA incidence trends, rates of secondary malignant transformation and recurrence and associated factors in the Netherlands.

Materials and Methods: Data on incidence, epidemiology, secondary malignant transformation and recurrence were retrieved from the Dutch pathology registry (PALGA) for the years 1992, 1997, 2002, 2007, and 2012. Multivariate analysis was performed to discover the risk factors for recurrence.

Results: 3 506 cases of SGPA were recorded implying an overall European standardized rate of 4.2-4.9 per 100 000 person-years. Our figures showed a female preponderance (1:1.43) with an annual 1% rise in female incidence (95% confidence interval [CI]: 0.2-1.8) and a bimodal age distribution in women ($p<0.0001$). The overall 20-year recurrence rate was 6.7%, and median time to first recurrence was 7 years.
Positive and uncertain resection margins and younger age at diagnosis were risk factors for recurrence, with odds ratios (ORs) of 4.62 (95%CI 2.84-7.51), 4.08 (95%CI 2.24-7.43), and 0.42 (95%CI 0.29-0.63) respectively. Tumor locations in the minor salivary glands had lower odds of recurrence than tumors in the parotid (OR 0.24; 95% CI: 0.07-0.77; $p<0.016$). Malignant transformation occurred in 0.15% of SGPAs (3.2% of recurrences).

Conclusion: This first nationwide study clearly showed sex differences in SGPA epidemiology, possibly suggesting some underlying hormonal mechanism. Long-term recurrence risks were low, and secondary malignant transformation risks were very low.

Introduction

Most salivary gland tumors are benign, with malignancy found in roughly 14% of lesions [1,2]. The most common tumor type is salivary gland pleomorphic adenoma (SGPA), which accounts for more than 70% of benign epithelial tumors. These well-circumscribed tumors with ductal and myoepithelial elements are found in both the major and minor salivary glands with most occurring in the parotid gland. They are more common in women and age at diagnosis is mostly between 40-59 years old [2,3].

The standard of treatment is nerve-conserving, superficial parotidectomy (or extracapsular dissection in well trained hands). Recurrence is reported in 0-3% of patients [4,5]. Historically, enucleation was performed but this was associated with unacceptably high recurrence rates of up to 45% [6,7]. Results of postoperative radiotherapy for recurrent SGPA, show better local control (up to 94% after 20 years follow-up) than surgery only, in retrospective series [6,8,9].

In 1.8-6.2% of cases, SGPA transforms into carcinoma ex pleomorphic adenoma[4,10]. These cases make up 7.7-11.6% of all malignant salivary gland tumors [10,11]. In recurrent SGPA, de novo malignant transformation is reported in 0-23% [6].

As common a tumor as SGPA may be, its epidemiology has long remained uncertain for lack of national registration [4,12,13]. The literature reports research focused on benign salivary gland tumors in general or subgroups of SGPA [2,14–17]. Others have looked at regional incidence of SGPA or national incidence of parotid SGPA [1,4], but to our knowledge, national incidence of all-location SGPA and trends over time have not been investigated.

Of course, without any national data, no rates can be calculated for all-location SGPA incidence, recurrence, and secondary malignant transformation without a strong possibility of referral bias. We therefore decided to use the Dutch nationwide registry of pathology reports (PALGA). This registry is not restricted to any specific type of finding or disease, thus making a suitable database for studying SGPA epidemiology features, including trends over time.

Our primary aim was to accurately establish SGPA incidence rates and trends over time, as well as any age and sex differences. We further aimed to establish recurrence rates and risks of secondary malignant transformation and to explore risk factors. This knowledge will help physicians to measure treatment results and express population-based prognoses.

Materials and Methods

Database: Set up in 1991, the PALGA registry automatically receives anonymized pathology reports from all Dutch laboratories, which include age, sex, date, and diagnosis. Excerpts are available for research purposes.

Patient selection: We searched the PALGA registry for codes of pleomorphic adenoma or mixed tumor and manually checked all excerpts thus created for SGPA. Then, we included all patients who had a first histology diagnosis in 1992, 1997, 2002, 2007, or 2012. We excluded 442 patients (11%) for reasons mentioned in Additional Table A. Likewise, we analyzed histology and cytology data for recurrences up to September 1, 2013, defining recurrence as a secondary tumor occurring in the same tumor site at a minimum of six months post surgery.

Incidence: We calculated SGPA incidence in the Netherlands from mid-year population size figures provided by Statistics Netherlands (CBS) [18], and worked out the male to female incidence ratio by looking at average male and female incidence data. To cancel out changes in age structure of the Dutch population over time, we computed European standardized incidence rates (ESRs), basing our calculations on the "2013 reference population" [19,20].

Patient, tumor, and treatment characteristics: To further analyze our primary tumor data, we recorded sex, age at diagnosis, salivary gland of origin, side of the body, surgical procedure, and margin status. In case of ambiguity, we checked with the author pathologist to decide on interpretation.

Recurrence rates and malignant transformation: In the subgroup of patients with at least five years of follow-up, we calculated first-recurrence rates at 5, 10, and 15 years, as well as median time to first and subsequent recurrences. We

excluded primary carcinomas ex pleomorphic adenoma from our database, and counted secondary carcinomas ex pleomorphic adenoma (SGPAs that recurred as malignant tumors) both as malignant transformations and as recurrences. Carcinomas in situ ex pleomorphic adenoma were not considered malignant transformations.

Risk factors for recurrence: We investigated sex, age, tumor site, and margin status. As the type of surgery was not always specified, and reporting practices varied, we decided to exclude this factor for our study.

Statistical analysis: We analyzed our data with SPSS (version 21.0; SPSS Inc., Chicago, III) and R [21,22], taking a p-value of <0.05 to be statistically significant for all purposes. Using linear regression and the natural log rhythm of ESR, we computed annual percent changes (APCs) by sex and overall, and we applied finite mixture models to investigate distribution patterns for age at diagnosis [23]. With the Kaplan-Meier method, we calculated times to recurrence and identified potential predictors for recurrence using multivariate logistic regression analysis. In addition to our analysis of complete cases, we performed missing data analysis and multiple-imputation analysis, imputing missing data by letting the R MICE package generate five imputed datasets and comparing the pooled results to our analysis of complete cases.

Results

***Incidence*:** After data cleaning, 3 504 unique patients remained of a total of 3 948 diagnosed with pleomorphic adenoma (Table 1). Two patients developed a second primary SGPA at a different anatomical site. Overall crude incidence varied from 3.9 to 4.7 per 100 000 person-years (Tables 2a and 2b). ESR ranged between 4.2 and 4.9 per 100 000 person-years. After stratifying for sex, we found a statistically significant annual rise of ESR in women (APC= 1.0% per year; 95% CI: 0.2-1.8), but not in men (APC= 0% per year; 95% CI: -1.0 to 0.9) (Figure 1).

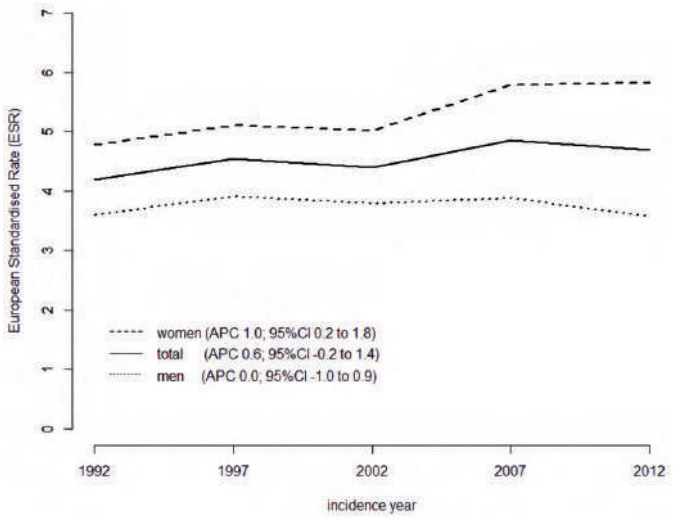

Figure 1. European Standardized Rate (ESR) in the five investigated years, with interpolation in the periods in between. The annual percent change (APC), calculated from the five years, shows an increase in female SGPA incidence.

Table 1. Population characteristics primary SGPA and 1st recurrence

		Primary (%)			1st Recurrence (%)
		Overall (n=3 506)	Male (n=1 421)	Female (n=2 085)	Overall (n=125)
Patients					
Age	Mean (range)	49 (8-94)	48 (9-92)	50 (8-94)	39 (8-89)
Age group	0-19	112 (3)	54 (4)	58 (3)	12 (10)
	20-39	959 (27)	393 (28)	566 (27)	60 (48)
	40-59	1 417 (40)	600 (42)	817 (39)	33 (26)
	60-79	919 (26)	343 (24)	576 (28)	18 (14)
	≥ 80	99 (3)	31 (2)	68 (3)	2 (2)
Location	Parotid gland	2 733 (78)	1 112 (78)	1 621 (78)	110 (88)
	Superficial lobe	*2603 (74)*	*1 066 (75)*	*1 537 (74)*	*102 (82)*
	Deep lobe	*130 (4)*	*46 (3)*	*84 (4)*	*8 (6)*
	Submandibular gland	310 (9)	93 (7)	217 (10)	9 (7)
	Sublingual gland	6 (<1)	4 (<1)	2 (<1)	0
	Minor salivary glands	377 (11)	187 (13)	190 (9)	6 (5)
	Unknown	38 (1)	13 (<1)	25 (1)	6 (5)
	Missing	42 (1)	12 (<1)	30 (1)	0

Table 1. continued

		Primary (%)			1st Recurrence (%)
		Overall (n=3 506)	Male (n=1 421)	Female (n=2 085)	Overall (n=125)
Side	Left	1 423 (41)	571 (40)	852 (41)	64 (51)
	Right	1 399 (40)	560 (39)	839 (40)	53 (42)
	Unknown	684 (19)	290 (20)	394 (19)	8 (6)
Treatment					
Procedure	Local excision	297 (8)			
	Partial parotidectomy	1 214 (35)			
	Total parotidectomy / Submandib. gl.resection	227 (6)			
	Subtotal parotidectomy	67 (2)			
	Excision deep lobe parotid	103 (3)			
	Biopsy	114 (3)			
	Unknown type of excision	1 449 (41)			
	Missing	35 (1)			
	Negative	2 028 (58)			
Clear margins	Positive	491 (14)			
	Uncertain	261 (7)			
	Unknown	726 (21)			

Table 2a. Number of SGPAs in the cohort in relation to the Dutch population

	SGPAs (n)			Dutch population (n)		
	M	F	Total	M	F	Total
1992	253	343	596	7 480 422	7 648 728	15 129 150
1997	280	384	664	7 696 803	7 870 304	15 567 107
2002	288	401	689	7 971 967	8 133 318	16 105 285
2007	304	466	770	8 088 514	8 269 478	16 357 992
2012	296	491	787	8 282 871	8 447 477	16 730 348
Total	1 421	2 085	3 506			

Abbreviations: SGPA salivary gland pleomorphic adenoma; M male; F female

Table 2b. Incidence of SGPAs in the Dutch population

	Crude incidence (per 100 000 per year)			ESR (per 100 000 per year)		
	M	F	Total	M	F	Total
1992	3.38	4.48	3.94	3.60	4.78	4.19
1997	3.64	4.88	4.27	3.91	5.11	4.54
2002	3.61	4.93	4.28	3.78	5.02	4.39
2007	3.76	5.64	4.71	3.88	5.79	4.85
2012	3.57	5.81	4.70	3.57	5.81	4.69

Abbreviations: ESR European Standardized Rate; SGPA Salivary Gland Pleomorphic Adenoma; M Male; F Female

Patient, tumor, and treatment characteristics: Primary SGPAs occurred more often in women (59.5%) than in men (40.5%) (Table 1), showing a female to male ratio of 1.43:1. The mean age at primary diagnosis was 48.0 in men, and 49.6 in women. Seventy-eight patients (2%) were under 18 when diagnosed. Around 40% of cases occurred in the age group of 40-59. In women, a bimodal age distribution was found, with peaks around the ages of 38 and 64 ($p<0.0001$). Age in men showed a normal distribution (Figure 2).

The most common tumor site by far was the parotid gland (78%), followed by the minor salivary glands (11%) and the submandibular glands (9%). Only six SGPAs occurred in sublingual glands (<1%).

Submandibular SGPA was more common in women than in men, but minor-gland SGPA was more common in men than women (Table 1). In patients under 18, the minor and submandibular glands were affected more often than in adults (Additional Table B).

Surgical technique was reported as partial parotidectomy (35%), local excision (8%) and complete gland removal (6%). In 41% of cases the excerpts did not specify the surgical technique and in 1%, there was no mention of type of procedure at all. Histological margins were negative in 58%, positive in 14%, uncertain in 7%, and not reported in 21%.

***Recurrence rates, characteristics, and malignant transformation*:** The disease recurred in 125 (4.6%) of the 2 719 patients who had at least five years of follow-up. Twenty (16%) also had a second recurrence, and two (10%) had a third. In 4 patients (0.15%), the disease recurred as carcinoma ex pleomorphic adenoma, which means that 3.2% of all recurrences (4/125) showed malignant transformation. First-recurrence rates were 2.3% at five years, 4.0% at 10 years, 5.6% at 15 years, and 6.7% at 20 years of follow-up, with a 7 years' median time to first recurrence (range 0.6-20.7, 95% CI 5.9-8.1) (Figure 3). Second-recurrence rates were 12% at five years and 14% at ten years of follow-up. The median time to second recurrence was 2 years (95% CI: 0.9-3.1). Sex distribution patterns were similar in both recurrences and primary tumors (58% females versus 42% males). The mean age at primary diagnosis was 40 in patients who later developed

recurrent disease and 49.3 in patients who did not develop recurrent disease. This 10-year age difference appeared in both sexes.

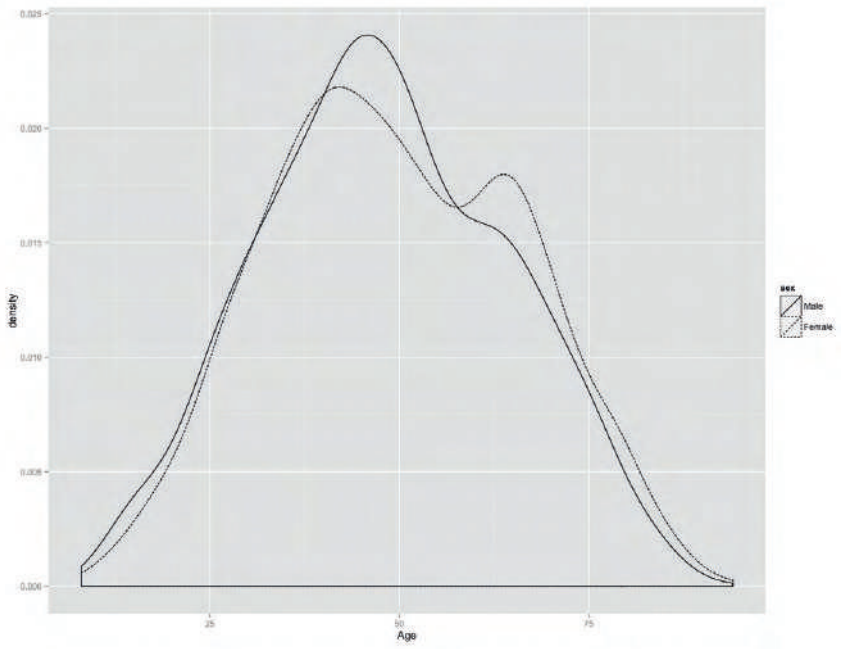

Figure 2. Age distribution, showing a bimodal curve in women.

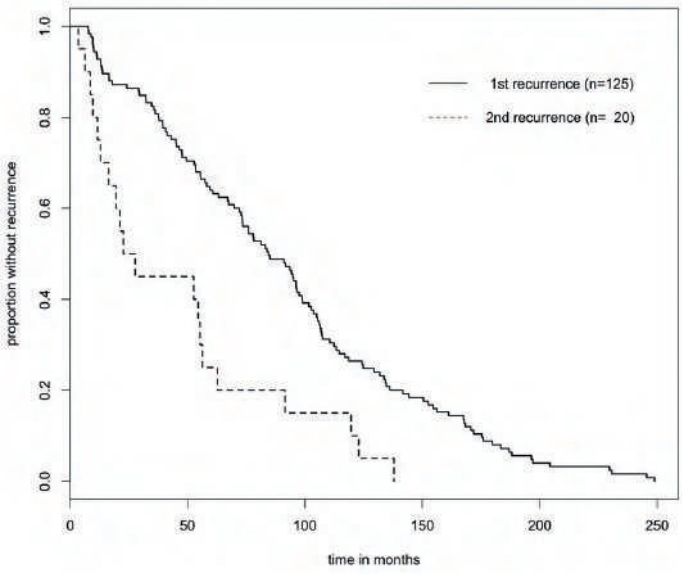

Figure 3: Recurrence-free survival in patients who developed a recurrence, reflecting a decrease in median time to recurrence: 7 years to 1ˢ recurrence; 2 years between 1st and 2nd.

Risk factors for recurrence: Margin status, age at diagnosis, and tumor location were all associated with risk of recurrence (Table 3). In patients with a reported margin status (complete cases, n=1 663), positive resection margins had an odds ratio for recurrence of 4.62 (95% CI 2.84-7.51), and 4.08 for uncertain margins (95% CI 2.24-7.43) compared to clear margins. For young age at diagnosis, the odds ratio was 0.42% (per IQR [25y]; 95% CI 0.29-0.63). Primary tumor location showed an odds ratio of 0.24 for minor salivary gland disease when compared to parotid disease (95% CI 0.07-0.77). Risk factors for malignant transformation of recurrent SGPA could not be determined, due to the low event rate (0.15%).

Missing data and imputation: Type of surgery performed and margin status were not mentioned in 42% and 21% of excerpts, respectively. There were 1 663 patients with complete information. Missing data on resection margins showed a significant association with recurrence (OR 1.5; 95% CI 1.00-2.23; p = 0.04). Taking this association into account, our analysis of imputed data with multiple-imputation models revealed the same risk factors as our analysis of complete data (Table 3).

Discussion

We report a large cohort of 3 506 patients with extended periods of follow-up and investigated SGPA incidence, recurrence, and secondary malignant transformation. Novel findings were a rising female incidence, a bimodal age distribution in women, and an overall 20-year recurrence risk of 6.7%. Positive or uncertain margins and younger age at diagnosis showed an increased overall risk of recurrence, whereas primary tumor locations in minor salivary glands showed lower recurrence.

Incidence: Direct comparisons with previous research on SGPA incidence are hard to make. In the past 50 years, crude incidence figures between 1.5 and 7.2 per 100 000 person-years [1,2,4,14–17] (Additional Table C) have been reported. However, most authors had not categorized tumors by anatomical site, and only one paper discussed national figures, which related solely to parotid SGPAs and did not standardize for age [4].

Table 3. Multivariate analysis of factors possibly associated with recurrence

	Complete-case analysis			
	β-Coefficient	SE (of β)	OR (95% CI)	p-value
Resection margins				
Negative	Reference			
Positive	1.53	0.25	4.62 (2.84 to 7.51)	<0.001
Uncertain	1.41	0.31	4.08 (2.24 to 7.43)	<0.001
Female	-0.15	0.23	1.16 (0.75 to 1.81)	0.501
Age *	-0.86	0.18	0.42 (0.29 to 0.63)	<0.001
Location				
Parotid gland	Reference			
Submandibular gland	-1.02	0.60	0.36 (0.11 to 1.16)	0.087
Minor gland	-1.44	0.60	0.24 (0.07 to 0.77)	0.016
Deep lobe of parotid gland	0.13	0.45	1.13 (0.47 to 2.73)	0.778
	Imputed analysis			
	β-Estimate	SE	OR (95% CI)	p-value
Resection margins				
Negative	Reference			
Positive	1.47	0.24	4.35 (2.75 to 6.96)	<0.001
Uncertain	1.38	0.29	3.98 (2.23 to 7.10)	<0.001
Female	-0.07	0.19	0.93 (0.63 to 1.35)	0.711
Age	-0.04	0.01	0.96 (0.95 to 0.97)	<0.001
Location				
Parotid gland	Reference			
Submandibular gland	-0.34	0.38	0.71 (0.34 to 1.51)	0.374
Minor gland	-0.86	0.38	0.42 (0.20 to 0.89)	0.024
Deep lobe of parotid gland	0.24	0.39	1.28 (0.59 to 2.75)	0.535

Abbreviations: OR odds ratio ; SE standard error; CI confidence interval, * β and OR for 1 interquartile range (25 years) of change.

Interestingly, SGPA ESR in 2012 was 4.7 per 100 000 person-years, whereas salivary-gland cancer ESR in 2010 was 0.74 [11]. These figures indicate that any salivary gland lump is 6.5 times more likely to be SGPA than carcinoma.

The 1% annual increase of SGPA ESR in women was a remarkable finding, as was the female preponderance of SGPA. Possibly, women are more aware of their appearance than men and more willing to seek medical attention for any lumps they find [24–26]. On the other hand, there may also be an influence of gonadal hormones, as in breast cancer, since SGPA is known to express estrogen and progesterone receptors [27,28]. Salivary gland neoplasms have been associated with breast cancer before [29]. One risk factor for breast cancer is advanced maternal age at first childbirth [30–32]. In the Netherlands, this age rose from 28.0 to 29.4 in the period we investigated [18]. A link with the increase we found in female SGPA incidence is not inconceivable.

Patient, tumor, and treatment characteristics: The bimodal age distribution in female SGPA incidence remains unexplained. Further research is needed to explore any hormone influences.

According to the literature, salivary gland tumors affect the parotid, submandibular, and minor glands in a ratio of 10:1:1 [1,33]. The ratio we found was 12:1:2, possibly because of an absence of selection bias in our data.

In our cohort, submandibular SGPAs were more common in women than in men, whereas minor salivary gland SGPAs were more common in men than in women. Since we found no previous mention of any sex differences in SGPA location, further research is needed to confirm and explain this finding.

As the PALGA database focuses on pathology, information on the type of surgery performed was often missing (42%). Recently, new insights about the benefits of standardized structured pathology reporting [34] have led to improved reporting practices for high-incidence cancers in Dutch laboratories. Hopefully, this systematic approach will be adopted for other diseases, too, including for SGPA.

Resection margins had not been recorded in 21% of cases. In a posthoc analysis, these cases turned out to have a 1.5-fold higher likelihood of recurrence, even after adjustment for gender, age, location and type of treatment. There may be several reasons why margin data are often missing. First, SGPAs are usually removed without complete margins of normal salivary gland tissue, for instance when they are close to the facial nerve. Second, covering (pseudo) capsules may be very thin, and multinodular growth patterns make it hard to determine whether any nodules have been left behind. Third, SGPAs are benign, so there is little priority in describing their margins, unless the pathology order holds a specific request to do so, along with sufficient clinical information.

Recurrence rates and malignant transformation: Whereas the 4.6% first-recurrence rate we found in patients with at least five years of follow-up (n=2 719) replicates previous findings [6], our 12% second-recurrence rate at five years is lower than the 14% stated in most papers (Additional Table D). However, some caution is needed here, as populations and follow-up periods vary between cohorts, and none of the figures have taken any clinical or mortality data into account.

For this present research project, we excluded malignant transformations of primary SGPA, diagnosed as carcinoma ex pleomorphic adenoma at first presentation without a history of SGPA. In earlier research, however, we found 34 cases of salivary gland carcinoma ex pleomorphic adenoma in the same period of investigation. [11]. Four occurred in recurrent SGPA and were added to our database, leaving 30 cases to account for a 1.1% risk of de novo malignant transformation of primary SGPA (30 in 2 749). This is a similar percentage as the 1.7% that could be calculated from the population in Denmark[4]. Earlier publications reported a mean 6.2% risk, but their figures relate to single-center data and may reflect a referral bias [10,35].

The 0.15% secondary malignant transformation rate we found (carcinoma ex pleomorphic adenoma in recurrent SGPA in our SGPA cohort; 3.2% of all recurrences) is in the lower range of earlier findings [6]. These numbers are also lower than in Denmark, reported at 0.35% and 12.6% respectively. To some extent, the differences may be explained by different inclusion criteria, but more importantly, compared to smaller studies, we ruled out referral bias by compiling a nationwide cohort, rather than using single-center data.

Our results confirm that at a population level, complete surgical removal of SGPA can be difficult, leading to a 4.6% first-recurrence rate and a 16% second-recurrence rate (median times to recurrence 7 and 2 years, respectively). Recurrences are often multinodular, with a mean number of 26 nodules (range 1-266) found in the primary resection bed [36]. These figures provide a strong argument for MRI follow-up after all first recurrences, to avoid a need for more extensive surgery at some later point in time.

Risk factors for recurrence: We found margin status to be the primary risk factor for recurrence. However, our margin data were based on microscopy, whereas in practice, margin status is often determined macroscopically by the surgeon. In many resections, sufficient margins cannot be taken because of adjacent facial nerve branches, and the pathologist will only have a very thin capsule to examine. This problem may raise doubt as to the reliability of microscopy data for multivariate analysis. Still, if margins are positive or uncertain, it is highly plausible to expect higher recurrence, since positive microscopic margins are accepted as a primary cause for tumors to recur, as are rupture and spillage[5,6].

A second recurrence risk factor we found was age. Mean age at primary SGPA diagnosis was 49 in patients who did not develop a recurrence later on, and 40 in patients who did. Although there may be an age bias here (higher age suggesting shorter survival, with death as a competing event), our findings are in line with literature [33,37–39]. Some researchers have explained the age difference by suggesting that surgeons tend to take a less radical approach and make smaller incisions in younger patients, for esthetical reasons [36]. Our multivariate analysis, however, did not show any correlation between age and margin status. Wittekindt et al. observed a further age difference. In their study population, mean age at primary diagnosis turned out to be lower in single-recurrence patients than in multiple-recurrence patients (30.2 versus 40.3) [36]. Possibly, tumor biology is somehow different in younger patients, because of hormonal aspects, genetic background, or some other factor as yet unknown.

A third risk factor for recurrence in our cohort was tumor location, which to our knowledge is a novel finding. SGPA in minor salivary glands was found to recur less frequently than SGPA in larger glands. Lumps in the minor glands are possibly more likely to be noted at an earlier stage. Moreover, complete excision of these lumps is easier to achieve, although margin status may be hard to assess for lack of capsule formation [40].

Female gender was not found to be a recurrence risk factor, which is in line with Maran et al. [41] in smaller series, but in contrast to other publications [36,42,43].

Limitations: There are some limitations to our study. First, there is a slight information bias. Given the suboptimal diagnostic accuracy of cytology (84-99%)% [44], we included histology-confirmed SGPA, only. With only 98 cytology diagnoses, however, and no data on non-pathology-proven recurrences, the 4.6% recurrence rate we found may be something of an underestimate, although hardly a gross one.

A second limitation is the lack of radiotherapy data, because literature suggests there is a role for radiotherapy in the adjuvant treatment of recurrent SGPA.

Third, since we retrieved all our information from non-standardized pathology reports, there may be an interpretation bias concerning the description of margins by pathologists and the information supplied by surgeons.

Conclusions

Nationwide pathology data regarding SGPA in the Netherlands in the period 1992-2012 reflect some remarkable incidence trends: female incidence was on the rise, there was a bimodal age distribution in women, and women were affected more often than men. These findings may suggest some underlying hormonal mechanism.
Overall figures for this period showed an ESR ranging between 4.2 and 4.9 per 100 000 person-years, a 4.6% first-recurrence rate after at least five years of follow-up, and a 6.7% recurrence rate at 20 years of follow-up. Malignant transformation had occurred in 1.1% of primary, and 0.15% of secondary SGPAs at 5 years of follow-up (3.2% of all recurrences).

Risk factors for recurrence were positive or uncertain surgical margins, younger age at primary diagnosis, and primary tumor location, with lower odds for minor-gland primaries to recur, when compared to parotid SGPAs. Where margin data were missing, the odds of recurrence were higher, which emphasizes the need for improved, possibly standardized reporting in a joint effort by both surgeons and pathologists alike.

Appendix A: Supplemental material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2017.01.004

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Chapter 3

Natural history of Recurrent Pleomorphic Adenoma: Implications on management.

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One by one
Only the Good die young
They're only flyin' too close to the sun
We'll remember

Queen 1997

Presented at the annual British Association of Head and Neck Oncologists (BAHNO) meeting, and Dutch Association of Oral and Maxillofacial Surgery (NVMKA) meeting in 2018.

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Abstract

Background: Treating recurrent pleomorphic adenoma (RPA) aims to reduce risk of malignant transformation (MT) while avoiding facial nerve injury. Our objective was to systematically investigate this natural history of RPA and address the current rational for its treatment.

Methods: The follow-up data of two nationwide series of pleomorphic adenoma was pooled with a focus on risk of MT and analyzed against the literature.

Results: The combined nationwide data (n=9,003 PA patients) showed 3.1% with 1st recurrence of which 6.2% were malignant. In the literature 1st RPA rate was >7% at 20 years follow-up. MT occurred in 0-7%, and facial nerve damage increased from with each surgery 3-16% at 1st RPA to 18-30% at 2nd RPA.

Conclusions: RPA showed a characteristic course with surgery being unreliable and damage to the facial nerve. The risk of MT was low. This might give flexibility towards a more conservative approach of management.

Introduction

Pleomorphic adenoma (PA) is the most frequent tumor of the major salivary glands, mainly affecting the parotid and has a reputation for recurrence and malignant transformation (MT). These two features along with the threat of facial nerve injury are the main concerns in the clinical management of patients with recurrent pleomorphic adenoma (RPA). In spite of improvements in treatment and outcome during the last 4 decades, the reputation for recurrence and malignant transformation lingers and is reinforced when a seemingly uneventful surgical procedure results in RPA. The fact that recurrent disease is now uncommon means data on the topic of RPA is sparse and the clinical picture obscure. This has led to different opinions on management and a general empathy towards, and low threshold for, repeated surgery whenever a recurrence is identified.

Information on RPA treatment outcome is available in two forms. The most common form is small single/two center studies that detail treatment results with varying inclusion criteria and endpoints, providing a confusing cloud of information. Another more structured form of reporting is based on nationwide databases pertaining to benign pleomorphic adenoma. These are rare because of a lack of national registries of the benign PA. Two large population-based studies have recently thrown light on the subject of RPA and MT incidence in a more comprehensive way [1,2]. These large series focused on primary tumor incidence, recurrence rate, MT and risk factors for recurrence. They are unique in scale, have a long follow-up after primary tumor treatment and do not suffer from the selection bias influencing the conclusions drawn from previous institutional series.

The intention of this study was to form a comprehensive picture of RPA behavior and subsequently present an updated view on its management. In this context, the two national data sets were used as a framework in which to fit the complimentary but fragmented information from the institutional series.

Materials and methods

The follow-up data of the only two previously published nationwide series of PA was pooled with a focus on risk of MT and analyzed against the background of

smaller institutional series. A recurrence was defined as a histological or cytological diagnosis of a PA after previous PA surgery in the same site. A MT was defined as a histological diagnosis of a carcinoma arising in or in the same location as a previous PA. A systematic literature search was undertaken in Pubmed, Embase (Ovid) and Scopus. Studies published in the English literature during the 40-year period between 1978 and 2017 that reported on treatment and outcome of series of ≥20 RPAs were identified, not limited to the parotid (Fig. 1: PRISM flow diagram). Search terms were "Adenoma, Pleomorphic"[Mesh] OR Pleomorphic Adenoma* [tiab] OR (mixed salivary [tiab] AND gland tumor* [tiab])) AND ("Neoplasm Recurrence, Local"[Mesh] OR recurren* [ti] OR residual [ti] OR relaps* [ti] OR return* [ti] OR recrudescence* [ti]. The content of the articles was evaluated in duplo systematically by completing a predesigned form including the following aspects: study details, epidemiology (incidence, gender, age at diagnosis of primary PA and RPA), treatment of PA and RPA (surgery/radiotherapy), pathology (multinodularity of specimen) and outcome (recurrence rate/ MT rate/ facial nerve function). Case files in the national series were available for patients who had RPA and subsequently developed MT, under the strict national database privacy regulations. Their clinical details and outcome data were retrieved and evaluated in conjunction with the pathology data.

Results

The Dutch and Danish studies reported on 3,506 (1992-2012, with 5-year intervals, major and minor salivary glands) and 5,497 (1990-2010, parotid only) cases of primary PAs, respectively, and their subsequent 125 and 151 RPAs [1,2]. The literature search identified 622 articles which were reduced to 76 after review. Of these, 28 (including the two national series) met the inclusion criteria for this study, but two Danish series described patients from the same national population (Table 1 [1-28]) [1,24]. The 25 institutional articles reported on case series of 20-126 RPA patients (total N= 1,187) treated between 1950-2016 in 23 single and two dual center reports. Six series reported only treatment details of 1st occurrence of RPA, whereas the others reported mixed series of 1st and subsequent RPAs. The two recent studies of the Danish and Dutch national pathology databases focused on primary tumor incidence, recurrence, malignant transformation

and (in the latter) risk factors for recurrence. It should however be noted that the recurrence risk according to the type of treatment could not be determined, due to the type of data in these national registries. In contrast, the aim of most single/two center studies was to report treatment results with recurrence rate as primary endpoint. Other endpoints were the role of radiotherapy, risk factors for secondary recurrence and the incidence of facial nerve damage.

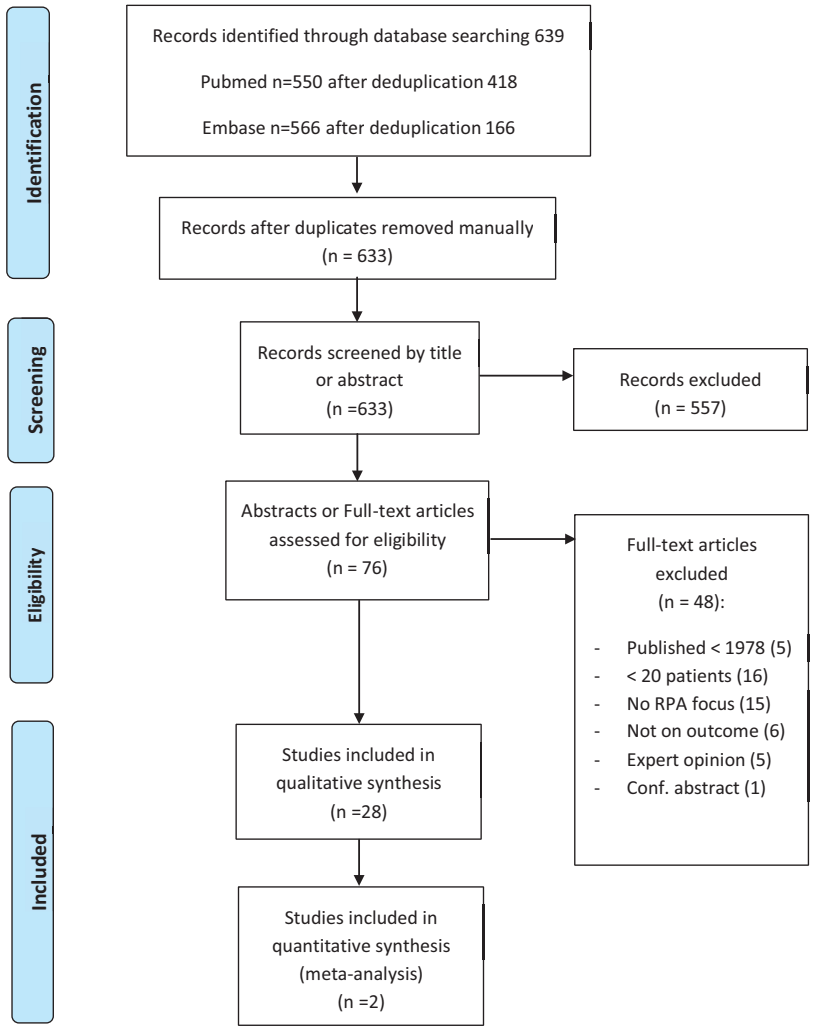

Figure 1: Prism flow diagram of search and study inclusion

Table 1: Included studies

Author, ref	Period	RPA	Cohort selection criteria	Study aim
Andreasen[1]	1985-2010	151	PA, 1st, 2nd RPA, PAR	Incidence, rec. rate, MT
Valstar[2]	1992-2012	125	PA, 1st, 2nd RPA, all SG*	Incidence, rec. rate, MT
Niparko[3]	1935-1975	48	1st + later RPAs, PAR	Analysis of treated cases
Dawson[4]	1950-1971	31	1st and later RPAs	Role of RT in RPA
Myssiorek[5]	1950-1983	27	unclear	Rec. patterns, causes, treatment
Renehan[6]	1952-1992	114	1st RPA, PAR, excl. residual tumor/ spill	Treatment, outcome
Fee[7]	1955-1973	26	unclear, PAR, excl. <3mo	Results of surgical therapy
Chen[8]	1960-2004	34	1st + later RPA, excl. CEPA / insuff follow-up	Role of RT in RPA
Jackson[9]	1962-1992	38	1st and later RPAs	Treatment results
Phillips[10]	1965-1985	126	1st + later RPAs	Tumor behavior+ outcome
Yugueros[11]	1965-1993	39	1st + later RPAs, PAR	Limited resection + RT vs parotidectomy
Leverstein[12]	1974-1995	29	1st + later RPAs, PAR	Treatment results
Wittekindt[13]	1974-2004	108	1st + later RPAs	Histopath., risk factors RPA
Laskawi[14]	1975-1991	94	1st + later RPAs	Treatment, outcome
Glas[15]	1976-1992	52	1st + later RPA, PAR, excl<6mo	Treatment, outcome
Samson[16]	1979-1988	21	Unclear RPA; all subtot. excision + RT	Outcome FN preservation + RT
Makeieff[17]	1981-2003	32	unclear, PAR, excl. FND / RT / MT	FN palsy, recovery with/ without monitoring
Maxwell[18]	1982-1997	35	1st RPA, no prior RT	Treatment results
Makeieff[19]	1982-2008	62	1st + later RPAs	Outcome, riskfactors RPA
Zbaren[20]	1983-2001	33	1st RPA, PAR, excl. residual tumor	Effect initial treatment, outcome
Redaelli deZinis[21]	1983-2004	33	1st + later RPAs	Management and progn. factors
Carew[22]	1984-1993	31	1st and later RPAs	Control rate and role of RT
Suh[23]	1984-2004	20	1st RPA, incl. MT, PAR	Charact. RPA, risk fact. MT
Nohr[24]	1985-2012	198	1st + later RPAs and FND	Distribution + sympt. in FND
Malard[25]	1988-2008	32	1st + later RPA, intra-PAR	Results, progn. factors
Leonetti[26]	1989-2002	42	1st + later RPAs, PAR	Diagnosis, treatment, result
AbuGhanem[27]	1991-2013	22	1st + later RPAs, PAR	Treatment, outcome
Liu[28]	2004-2012	58	unclear, excl.FND, MT, RT	Benefit intraop. FN monitor

PA=pleomorphic adenoma; RPA=recurrent pleomorphic adenoma; PAR=parotid; SG=salivary glands; RT=radiotherapy; FND=facial nerve dysfunction; CEPA=carcinoma ex pleomorphic adenoma; MT=malignant transformation

Initial presentation

The national data sets provided a crude and European Standardized Rate for primary PA of 4-5/100,000/year [1,2]. The crude 1st RPA rate depended on follow-up time. Although contemporary series reported this to be <2%, it increased from 2% at 5 years to 7% at 20 years in data not corrected for death as a competing event

[1,2,29]. PA was more common in females, with a female to male ratio in Dutch and Danish series of 1.43:1 and 1.70:1 respectively (overall ratio 1.6:1). Similarly, in all series, a female preponderance was found among RPA patients (Supplementary Table A), but female gender was not a risk factor for recurrence [2]. The median age at presentation of the primary PA was ~50 years in the national series. In patients who later developed RPA, the age at presentation of the initial PA was 10-15 years younger than patients who did not develop recurrence [1,2,29,20]. The median age at 1st RPA was 40-49 (range 13-87) [1,6,18]. In at least 44% of cases, multiple nodules were clinically apparent and this was nearly 100% when the tissues were examined histologically, demonstrating that RPA is usually a multifocal disorder (Supplementary Table B) [12,19,26].

Treatment of recurrent pleomorphic adenoma

The surgical technique used initially to treat the primary tumor in the cohort of patients with tumor recurrence varied widely, with local excision/enucleation in up to 87% of patients in the older studies and 9% in more recent studies (Supplementary Table A) [6,27]. The risks of recurrence and facial nerve injury depended on the extent of previous PA- or RPA surgery [6,21]. This literature review does not provide adequate data to draw conclusions on the role of initial surgical technique regarding the risk of developing RPA and is therefore not explored further (Supplementary Table A and B). The extent of initial surgery, however, could be expected to impact on subsequent surgical options as a comprehensive form of salvage parotidectomy following a minimalist initial procedure would still be an option without undue risk to the facial nerve. In contrast, an initial parotidectomy followed by multinodular recurrence could be likely to be managed by either local excision of nodules, or an extended/radical parotidectomy [13]. There was too much heterogeneity in the literature to draw general conclusions on optimal surgical RPA treatment. Postoperative RT, however, seems to reduce the risk of recurrence. Renehan et al. showed a significant decrease in recurrence rate in the cohort of patients with multinodular recurrence that received postoperative vs no RT: 4% vs 43% at 15 years follow-up [6]. The control rate in other series similarly showed reduced recurrence rates following RT, and Chen et al. reported a control rate of 94% after a median follow-up of 17 years in patients with postoperative RT [4,8,30]. Recurrence after surgery with gross macroscopic residual disease and RT, was higher [16].

Outcome after recurrent pleomorphic adenoma treatment

Recurrence: In the combined Danish and Dutch cohorts with a mean overall follow-up of 12.5 years, 276/9,003 (3.1%) of patients developed 1st recurrent disease in whom further re-recurrence occurred at a rate of 70/151 (46%, mean follow-up of 14.1 years) and 20/125 (16%, mean follow-up of 9.9 years), respectively (Fig. 2) [1,2]. Re-recurrence specifically after 1st RPA treatment is described in only a few institutional studies [3,6,18,20,23]. These single center series similarly showed an overall 2nd RPA rate of 15-50% and even up to 60% if further recurrences were included (Supplementary Table C). The time interval to recurrence decreased from (median) 7 years for a 1st RPA to 2 years for a 2nd RPA in the Dutch national series [2]. Similarly, a decrease in interval is reported in numerous single center series [6,7,10,14,19-23,25]. Roughly, each surgical intervention halved the time to recurrence.

Malignant Transformation: The Danish/Dutch studies seem to have the most comprehensive data on this topic. In the combined PA data set (N=9,003) with a collective mean follow-up of 12.5 years, 276 cases (3.1%) had recurrent disease, including 23 malignancies (0.3%) (*after* the original publication that is used in this review, one additional patient had MT in the Dutch series). At 1st recurrence, malignant salivary gland tumor was present in 17/276 cases (6.2%). In the remaining 259 benign cases, 90 patients had 1-8 further episodes of recurrence, and in this population six developed MT (6/90, 6.6%), at a mean of 15 years following primary PA surgery (Table 2 and 3) (range 2.1-27 years). Treatment of the latter group was by surgery and RT in all cases and local recurrence occurred in 4/6. One patient died of disease. Overall, 23 of 276 recurring tumors (8.3%) were diagnosed with malignant salivary disease. These figures can be interpreted in different ways (see discussion).

In the literature, the three single institution series describing >100 treated RPAs showed a MT rate of 0-7%, occurring at different stages of recurrence [6,10,13]. A pattern of higher MT rates following RT could not be discerned. The reported rates of MT in RPA with surgery alone as well as with adjuvant RT were 0-9% (Supplementary Table D).

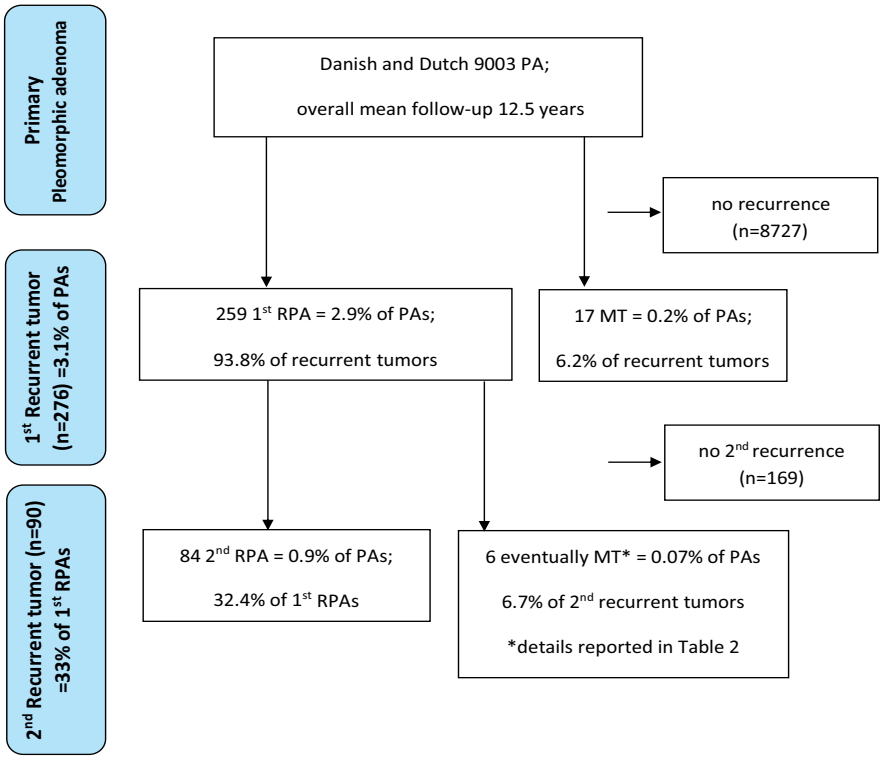

Figure 2: Flow diagram of the combined Danish-Dutch (C) PA population, with recurrences and MT, showing how proportions are calculated.[1,2] (For flow diagrams of separate Danish and Dutch populations, see Supplemental figures A and B.)

Table 2: Clinical course of Dutch and Danish patients (A-F) with a malignant transformation (MT) after an earlier treated RPA (also see figure 2C).

	Recurrences (n)									
Case	1	2	3	4	5	6	7	8	9	Time to MT (months)
A	53	12	8	MT12	83LR					85
B	56	MT114								170
C	48	13	5	18	6	6	11	11	MT35	153
D	6	12	50	32	34	25	56	60	MT49	324
E	25	24	6	12	17	183	27	MT29		323
F	13	7	MT5							25

MT=malignant transformation; LR=local recurrence

Table 3: Clinicopathologic characteristics of Dutch and Danish patients (A-F) with a malignant transformation (MT) after a treated RPA (see also figure 2c).

	Histology	Treatment	Clinical course (months)
Case			
A	Myoepithelial carcinoma	Surgery + RT	LR 83 post MT; DOC 296 post PA (212 post MT)
B	Adenocarcinoma NOS, grade III	Surgery + RT	NED 238
C	Myoepithelial carcinoma	Surgery + RT	LR 35; NED 153
D	Salivary duct carcinoma	Surgery + RT	DOC 41
E	Salivary duct carcinoma	Surgery + RT	LR 21; DOD 29
F	Myoepithelial carcinoma	Surgery + RT	LR 10, LR 23; DOC 61

NOS= not otherwise specified; NED= no evidence of disease; LR= local recurrence; DOD= dead of disease; DM=distant metastases; DOC=dead of other causes

Facial nerve injury: This is the principal measure of surgical morbidity, and the risk of permanent facial nerve damage increases with number of surgeries. At the first RPA, facial nerve damage was 3-16% rising to 15-21% for 2nd recurrence to 18-30% for 3rd RPA (i.e. 4th operation) (Supplementary Table E) [6,15,20,22,26,27].

Discussion

The objective of this study was to provide a comprehensive overview of RPA behavior and address the current rational for its treatment. Namely, the focus lies on parotid RPA, as the risk of recurrence in other major as well as minor salivary glands is lower and does not confer a risk of facial nerve damage [2]. Historically, the management of patients with RPA has been directed largely by the fear of MT with incidences quoted from 0-24% and consequently a low threshold for repeated surgical intervention [13,31,32]. RPA poses an inherent problem for analysis, since it is uncommon but also because long follow-up is needed to trace the natural history of this disease. Consequently, most publications involve small series, and in many cases stretch back in time to the 1950/60's where standards of reporting were different. The focus of the articles therefore varied, and the reporting of events was not uniform. The method adopted to provide insight into RPA behavior therefor was to pool data regarding patient, tumor, treatment and outcome characteristics from the most comprehensive epidemiological datasets and present it with the information obtained by a systematic review of the literature. Taking this approach, a natural history for RPA emerged from the previous opaque cloud of information and is sufficiently distinct to inform current thinking regarding the management of this disease.

Recurrent disease occurs in younger patients and females are afflicted 1.6 times as often as males [1,2]. The majority of recurrent lesions are multifocal in nature, and surgical elimination is understandably unreliable even with radical surgery [13]. The peculiar aspect of surgical intervention is that the time to recurrence decreases with repeated surgery. Time to the first recurrence is 7-10 years, then 2-5 years to the second recurrence and even shorter intervals to the 3rd and 4th recurrences [2,33]. The biological background for this is unknown. Repeat surgery carries another penalty in the form of injury to the facial nerve. This risk is difficult to gauge from the literature because it depends on the extent of the initial surgery, administration of adjuvant RT, and how aggressively the second operation is pursued which is known to vary between institutions. The first surgery for primary tumor carries ~1-4% risk of permanent injury to one or more branches of the facial nerve, and this increases to 3-16% at the next procedure. But if a traditional superficial parotidectomy has been performed previously, then the patient should be counselled about injury rates as high as 30% [6,13]. Recent reviews and meta-analyses indicate that modern minimally invasive parotid techniques have a similar recurrence rate to traditional superficial parotidectomy, although this should be consolidated by longer follow-up [34-36]. Their advantage in this context is that a definitive parotid dissection is still possible.

The incidences of RPA after surgery for the first recurrence in the two populations studies are quite different with the Danish being 46% (mean follow-up 14.5 years) and 14% in the Dutch group (mean follow-up 9.9 years). A possible explanation for this difference is that the Danish study related only to parotid PA whereas the Dutch included also minor salivary glands. These are reported to have a lower risk of recurrence [2]. Also, recurrences occurring within 6 months after primary PA surgery were excluded in the Dutch series, as they were regarded as residual tumor. Another contributing factor could be the option of adjuvant RT which was available in the Netherlands but not in Denmark. A fourth reason is the longer follow-up in the Danish material and finally the theoretical but unlikely possibility of differences in the quality of surgical approach between the two nations.

Although there seems to be a clear advantage of adjuvant RT from earlier mentioned case series, no randomized controlled trials (RCTs) have been carried out and enthusiasm is tempered by known side-effects in a target population that is relatively young. There is not only radiation toxicity, but a theoretical risk of radiation-induced tumors or MT of the RPA [37]. The limited data in the current

literature review was not sufficient to substantiate or refute consequences of RT for the risk of MT development [8,11,22]. However, a recent systematic review by McLoughlin et al found a benefit of RT in RPA and no increased risk of MT in the available evidence, that (similar to our review) lacks RCTs [38]. Overall, RT administered to especially a young patient population is known to carry inherent side-effects and a theoretical, but yet unobserved MT-risk which have to be balanced against treatment benefits. The most important benefit seems to be a lower rate of re-recurrence, with the corollary of less surgery and reduced risk to the facial nerve [13,30,31,32,39].

Historically, the threat of MT has had an important bearing on the approach to management of RPA. The reported risk of MT in PA in the literature has depended on a number of factors including patient selection, anatomical sites included, sample size, duration of follow-up and institutional selection bias which have hampered the ability to draw firm conclusions [1,2,6,18,20,23]. The present study does not provide a definitive answer as it also suffers from some of these limitations. However, from the national Dutch and Danish data it can be concluded that MT in RPA is an uncommon event occurring in at most only 0.3% (23/9,003) of patients who present with a primary PA, and around 6% in both 1st and subsequent RPA's.

But care should be taken in accepting these figures at face value. Based on clinical experience, there is the impression that patients with MT at first recurrence and patients with MT at a later recurrence could at least partly form two different populations. Salivary gland tumor histology is notoriously difficult to interpret and it would be astonishing if there were no diagnostic discrepancies in a large series of 9,003 tumors. It is conceivable that a number of low-grade malignant tumors masqueraded as benign lumps were initially recorded as PA and their true nature became apparent at 1st recurrence (N=17). The suggestion is that (part of) these may have been malignant from the start. If correct, this would mean that the MT rate in RPA is even lower and this is the subject of a separate study currently in progress.

For the clinician managing patients with a histologically proven benign 1st RPA, the salient information is that MT is low at 2,3% (6/259) and in the present study only occurred in 6 of the 90 patients (6.6%) who failed further surgery (2-8 episodes) at mean 12.5 years follow up after the initial treatment. This is the information the patient and clinician need in order to agree a management plan suited to the individual patient.

In conclusion, the traditional perceived risk of MT after RPA (up to 24%), which has been at the center of the clinical management of RPA, has now been more clearly defined and quantified as relatively low by the pooled Danish/Dutch national data [1,2]. This new picture of RPA raises the question whether the current low threshold for repeated surgery is optimal, especially with the risk of facial nerve injury in mind. Informed discussion with the patient can now be based on more scientifically valid information on risk of MT. The population at risk of RPA are young adults and the majority are females in the formative years of their life. Facial disfigurement with or without facial nerve palsy at this juncture in one's life can transform life opportunities. In contrast, nodules of RPA can be monitored easily, inexpensively and accurately by ultrasound examination and suspicious nodules can be sampled by FNAC [40]. It is acknowledged that it is unlikely that a young adult with RPA can avoid further surgical treatment, but if disfiguring surgery and possibly RT can be delayed a decade or more by judicious surveillance with patient collaboration and "lump picking", it may have less impact on life opportunities and the risk of radiation-induced complications. This more conservative treatment strategy can be adopted in other patient categories as well, based on well informed patient preferences as part of a personalized treatment plan.

Supplemental information can be accessed via a link to the journal website.
https://onlinelibrary.wiley.com/doi/10.1002/hed.26137

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Chapter 4

Malignant transformation of salivary gland pleomorphic adenoma: Proof of principle

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Abstract

Supposed risk of malignant transformation of salivary gland pleomorphic adenoma is an important reason for aggressive retreatment in recurrent pleomorphic adenoma. However, although the diagnostic category "carcinoma ex-pleomorphic adenoma" suggests that malignant transformation of a pleomorphic adenoma is a regular event, this has to date not been shown to occur in sequential lesions of one patient. Here we show the molecular events in transformation to malignancy of a pleomorphic adenoma of the parotid gland. Detailed molecular analysis revealed a *LIFR*/*PLAG1* translocation characteristic for pleomorphic adenoma and, next to this, a *PIK3R1* frameshift mutation and several allelic imbalances. In subsequent malignant recurrences the same *LIFR*/*PLAG1* translocation, *PIK3R1* frameshift mutation and allelic imbalances were present in addition to *TP53* mutations. This case thus not only shows malignant transformation of salivary gland pleomorphic adenoma but also demonstrates that molecular analysis can be of help in recognizing malignancy in the rare instance of recurrent pleomorphic adenoma.

Introduction

The risk of malignant transformation from the most common salivary gland tumor, pleomorphic adenoma (SGPA), to the 5th most frequent salivary gland carcinoma (carcinoma ex pleomorphic adenoma; CEPA), is notorious [1,2]. It can lead to dilemmas in both pathological diagnosis and surgical/adjuvant radiation treatment of primary and recurrent pleomorphic adenoma (RPA). This risk of malignant transformation is, however, rare as only 3% of SGPAs recur at 12.5 year follow-up, of which 6% seem to show malignant transformation [1].

The hypothesis that malignant transformation of SGPA occurs is mainly based on the recognition of a pleomorphic adenoma component in malignant salivary gland tumors that are therefore classified as "carcinoma ex pleomorphic adenoma". In these tumors, morphological as well as molecular transitions are seen from the benign pleomorphic adenoma component to the malignant carcinoma component [3–13]. At the molecular level, the pleomorphic adenoma component is recognized by the presence of *PLAG1* and *HMGA2* gene fusions[6,7,14], while the malignant component harbours additional mutations such as *TP53*, *c-MYC*, *RAS*, and *P21*[8,15–19]. These events have been shown within CEPA as well as in studies comparing SGPA and CEPA cases but not in sequential lesions from one patient [8,15–18].

CEPA can be diagnosed as a primary malignant tumor or as a recurrence after a benign earlier resected SGPA [9,20,21]. In both cases, malignant transformation is thought to result from progression of a SGPA, due to accumulation of genetic changes. SGPA itself is characterized by *PLAG1* gene overexpression frequently due to a chromosomal translocation resulting in a gene fusion with several candidate genes [10–12]. Progression to CEPA due to *HMGIC* and possibly *MDM2* amplification has been suggested [3,13].

This report shows for the first time that malignant transformation is accompanied by the accumulation of mutations in tumor driver genes in sequentially occurring parotid tumors, by means of targeted next generation sequencing (NGS) including copy number variation detection analysis using single nucleotide polymorphisms (SNPs) on 12 chromosomes and RNA-based gene-fusion analysis.

Material and Methods

Case: A 35-year old female presented with a painful parotid tumor in the deep lobe without facial nerve palsy. Fine needle aspiration cytology (FNAC) revealed a salivary gland tumor, possibly SGPA, uncertainly benign (Milan classification 4b). After a superficial parotidectomy, the tumor was removed with (where possible) surrounding gland. Histology showed a SGPA (Figure 1A, D), 4 cm in diameter, with a large epithelial component, completely removed without margins. Infiltrative growth, mitotic figures or necrosis were not observed.

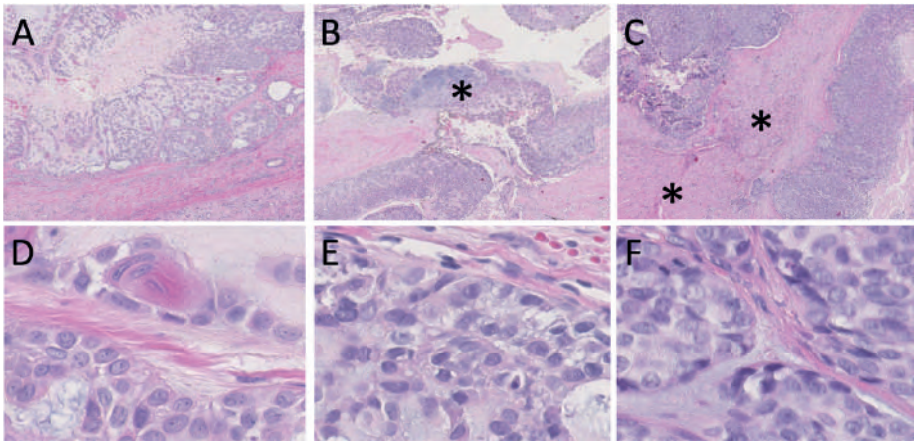

Figure 1. H&E histology (2x and 60x magnification) of the primary, first and second recurrent tumors (A and D, B and E, C and F respectively) showing progression of the pleomorphic adenoma without cytonuclear atypia to a carcinoma with more nuclear atypia and mitoses (mitosis in left upper quadrant of F). Pre-existent pleomorphic adenoma is seen at the asterisk in B. Infiltrative growth in skeletal muscle (asterisks) is present in C.

Three years later the patient presented with a painful recurrent tumor and paresthesia of the tongue. FNAC was unsuccessful due to extreme pain. An MRI showed a local recurrence, 5.7 cm in diameter. The tumor and remnant of the deep lobe were resected after a lateral mandibulotomy. The facial nerve was sacrificed and reconstructed. At pathology, a cellular salivary gland tumor was seen, without obvious mitosis or necrosis and with areas clearly classifying as pleomorphic adenoma (Figure 1B, E). Infiltrative growth could not be evaluated as the lesion was excised without margins. The lesion was classified as a benign recurrence reaching into the resection surface. Postoperative radiotherapy (RT, 66 Gray) was administered.

A year later, a lump medial to the sternocleidomastoid muscle was biopsied because the patient refused FNA, and this lesion was classified as suspect for adenocarcinoma. A modified radical neck dissection was performed (levels 1-5) with reconstruction of the accessory nerve and the wound defect. Pathology showed a multinodular basal cell adenocarcinoma (Figure 1C, F), reaching into the resection surface. All 24 lymph nodes were free of tumor. Postoperative RT to the neck (60 Gray) was administered.

In order to understand the clinical behaviour, all previous slides were reviewed and molecular analysis was performed. DNA and RNA from all three lesions and normal tissue was isolated. Targeted next generation sequencing (NGS) was performed on an Ion Torrent S5XL prime system using a custom-made panel for mutation and copy number variation detection using single nucleotide polymorphisms (SNPs) on 12 chromosomes (panel information supplementary material, Table S1)[22,23]. In addition, RNA-based gene fusion detection using the Archer FusionPlex Sarcoma panel (ArcherDx, Boulder, CO, USA) was performed. Immunohistochemistry for Ki-67 and p53 was performed. The medical ethical committee of the Erasmus Medial Center Rotterdam, the Netherlands approved this study (MEC-2020-0270).

Results

All three lesions showed the same *LIFR/PLAG1* gene fusion (supplementary material, Figure S1), an identical somatic frameshift mutation in the *PIK3R1* gene (supplementary material, Figure S2) and identical patterns of allelic imbalance on chromosomes 5, 7 and 8 (Figure 4 and supplementary material, Figure S3), confirming their clonal relation.
Additionally, the first recurrent tumor showed a *TP53* p.R248Q (c.743G>A) mutation with a variant allele frequency (VAF) of 46% (supplementary material, Figure S2). The second recurrent tumor showed a different *TP53* mutation: *TP53* p.Y220C (c.659A>G) with a VAF of 51% (supplementary material, Figure S2).

The low-level/subclonal presence of both *TP53* mutations found in the two recurrences was investigated in the three tumor samples with a very sensitive NGS approach using unique molecular identifiers (ThermoFisher Oncomine™ Lung

cfDNA Assay V1 , Thermo Fisher Scientific, Waltham, MA, USA). With a limit of detection (LOD) down to 0.3% no indication was obtained for subclonal presence of these *TP53* mutations in the three tumor samples.

Additional immunohistochemistry (IHC) on the three tumors showed a Ki-67 index of 2%, 15% and 40%, respectively (Figure 2). p53 IHC was scored as described by Köbel et al. [24] and showed wild type expression in the first lesion (Figure 3A) and mutant expression in the second recurrence (Figure 3C), but p53 immunohistochemistry was equivocal in the first recurrence (Figure 3B). Resected tumor 11 months after the second recurrence showed the known *LIFR/PLAG1* and *PIK3R1* clonal fingerprint and the *TP53* p.R248Q (c.743G>A) mutation of the first recurrence.

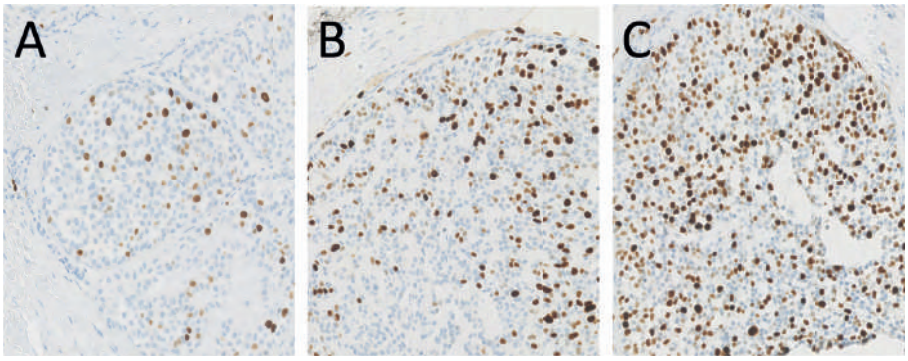

Figure 2. Ki-67 immunohistochemistry (14x magnification of digital image) showing increasing expression of Ki-67 over time (A primary tumor, B first recurrence, C second recurrence).

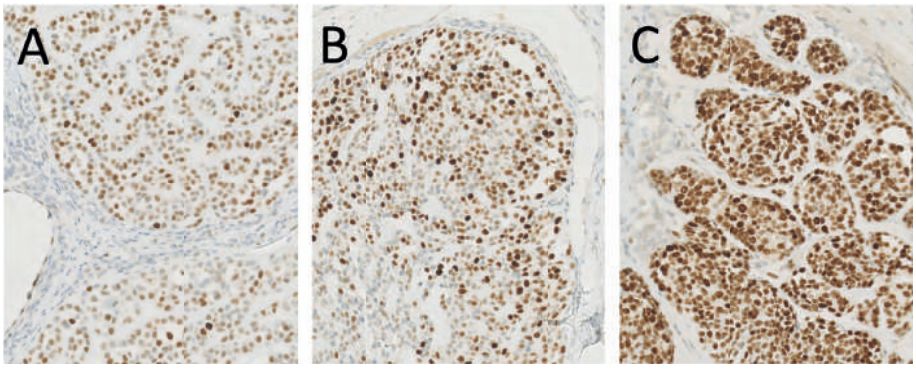

Figure 3. p53 immunohistochemistry (14x magnification of digital image) (Ventana BP53/11) showing (A) wild type, (B) equivocal and (C) mutant expression of p53 in the primary tumor, first recurrence and second recurrence respectively.

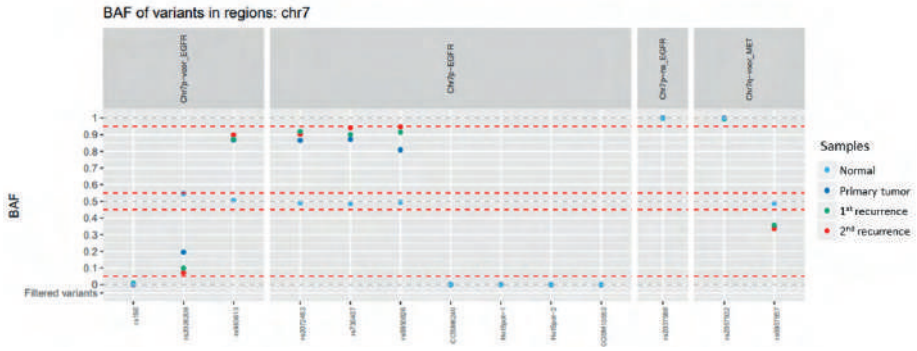

Figure 4. An identical pattern of allelic imbalance was found on chromosomes 5, 7 (shown in this figure) and 8. The imbalances are present in the primary pleomorphic adenoma, the first and the second recurrence. BAF = B-Allele Frequency.

Discussion

This report shows for the first time chronological genetic steps of progression of SGPA to a malignant salivary gland tumor, accompanied by the occurrence of *TP53* mutations. The two lesions with different clonal *TP53* mutations probably represent two different recurrences from the same tumor, as multinodular recurrence is common. *TP53* mutations have been reported as the most frequent mutations in salivary gland carcinomas in general, followed by abnormalities in the cyclin and PI3K pathways (including PIK3R1)[25].

All lesions showed the same *LIFR/PLAG1* gene fusion, *PIK3R1* mutation and allelic imbalance pattern, revealing their origin from the primary SGPA. It is tempting to speculate that the combination of the *LIFR/PLAG1* gene fusion and the *PIK3R1* mutation with loss of the other allele were drivers for proliferation whereas the additional *TP53* mutations initiated the malignant transformation. Additional support for this comes from a mouse model with overexpression of *Plag1* targeted to salivary glands and hyperactivation of the PI3K pathway by a conditional salivary gland *Pten* knockout mouse model which both resulted in pleomorphic adenoma formation[26,27].

The occurrence of new *TP53* mutations in the recurrences does not confirm a causal role for these mutations in malignant transformation since the specificity of *TP53* mutation for this process is unknown. However, newly present *TP53*

mutations in genetically related subsequent recurrent tumors were accompanied histologically by malignancy; thus a driver role seems likely, as *TP53* is a well-known tumor suppressor gene [28]. Loss of *TP53* function is the most common molecular aberration in human malignancies and is involved in initiation and progression of many malignant disorders. Based on results from DNA mutation, single nucleotide polymorphism (SNP) and DNA amplicon coverage analyses, all three lesions are probably bi-allelic for the *TP53* locus (supplementary material, Figure S3), and therefore have one functional intact *TP53* allele. The identified *TP53* mutations p.R248Q and p.Y220C, however, are known to exert a dominant-negative effect leading to complete inactivation of *TP53* without a second hit mutation (e.g. loss of the wild type *TP53* allele)[29].

In this case, the first recurrence was not originally recognized as malignant although the proliferation rate of 15% was relatively high [30]. In the second recurrence, morphology and proliferation rate were obvious clues for malignancy. In retrospect, p53 and Ki-67 IHC might have raised the suspicion of malignancy, warranting molecular analysis.

Several aspects complicate salivary gland tumor diagnosis in general, like the rare nature and the wide morphological spectrum. Although SGPA is the most frequent primary salivary gland tumor, it is still a rare tumor (European Standardized Rate: 4.5/100,000; as opposed to 62/100,000 for breast cancer)[31]. Some of its characteristics can make correct diagnosis challenging, like the morphological overlap with features of some of the 22 salivary gland carcinomas[20,32]. Furthermore, malignant transformation of SGPA occurs only occasionally, at a frequency of 6% of first RPAs [1]. And as shown in this case, malignant transformation can be difficult to recognize as the first recurrence already harboured a *TP53* mutation but was not identified as malignant morphologically. This shows that, in the rare case of a RPA, molecular analysis can be of value in recognizing early malignant transformation.

Clinically, the risks of infiltrative growth and malignant transformation have been important arguments for aggressive surgical treatment of RPA, which warrants the sacrifice of vital structures in selected cases. This case for the first time illustrates that malignant transformation of SGPA occurs and that molecular analysis can help to recognize malignancy. The combination of histology and molecular analysis

can make a more solid diagnosis possible, which is imperative for appropriate clinical decision making.

Acknowledgements
None. This work was not supported by internal or external funding.

Supplementary Material

Figure S1. (see below) Analysis with the Archer Fusionplex Sarcoma panel
Figure S2. (see below) NGS analysis of *TP53* and *PIK3R1* mutations in the primary tumor and the first and second recurrences
Figure S3. (see below) NGS analysis of allelic imbalances in the primary tumor and the first and second recurrences
Table S1. Custom-made NGS panel information. Data are available at the journal website: https://onlinelibrary.wiley.com/doi/10.1002/cjp2.216

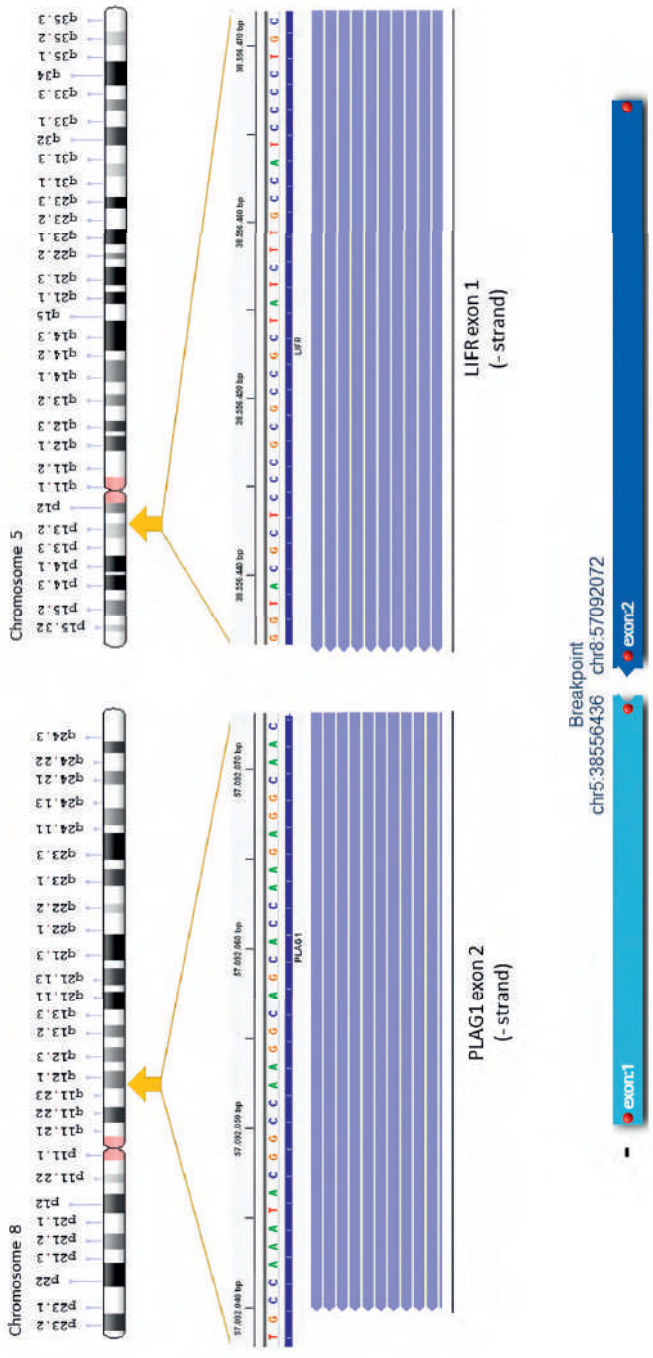

Figure S1. Analysis with the Archer Fusionplex Sarcoma panel showed the identical *LIFR/PLAG1* gene fusion present in three subsequent tumors.

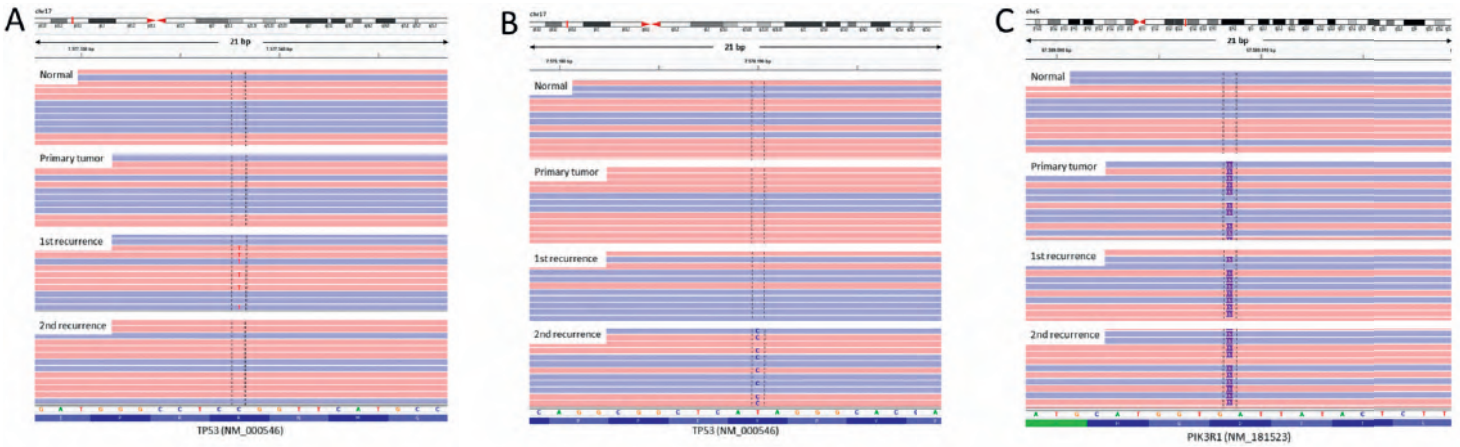

Figure S2. NGS analysis of *TP53* and *PIL3R1* mutations in the primary tumor and the first and second recurrences showing that (A) the first recurrence harboured a *TP53* p.R248Q; c.743G>A mutation in exon 7 (VAF: 46%). (B) In the second recurrence a different *TP53* mutation was present in exon 6: *TP53* p.Y220C; c.659A>G (VAF: 51%). Both mutations were absent in the primary tumor. (C) The *PIK3R1* mutation was present in the primary tumor, and both the first and second recurrences.

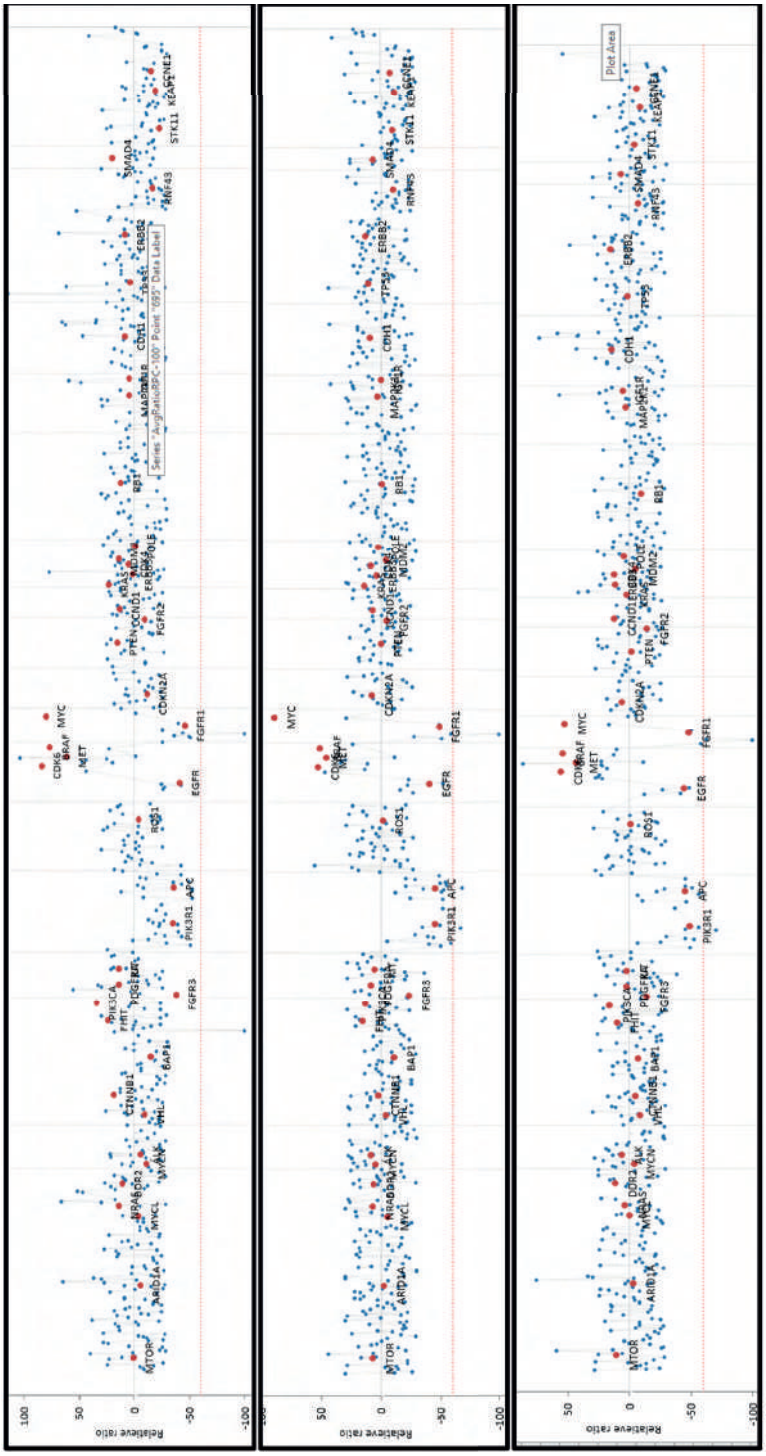

Figure S3. NGS analysis of imbalances in (A) the primary tumor and (B) the first and (C) the second recurrences. The relative coverage ratio (compared to normal DNA) for each DNA amplicon is shown (Y-axis) as a blue dot for chromosomes 1-22 (X-axis). The figures indicate that all three tumors show a very similar imbalance pattern. Loss of PIK3R1 (#4), APC (#4), EGFR (#7) and FGFR1 (#7) are compatible with the mutation (PIK3R1 VAFs) and SNP results. Gain and amplification of CDK6 (#7), MET (#7), BRAF (#7) and MYC (#8) are compatible with the SNP results. TP53 (#17) shows no aberration, compatible with the SNP and mutation results (TP53 VAFs). Likely, all three lesions have two TP53 alleles (no imbalance); in the primary tumor, two wild-type TP53 alleles; in both the recurrent tumors, one mutant TP53 allele and one wild-type TP53 allele. The mutations in both recurrences are reported to have a dominant-negative effect (the inactive p53 protein, derived from the mutant TP53 allele, inactivates the wild-type p53 protein, derived from the wildtype TP53 allele).

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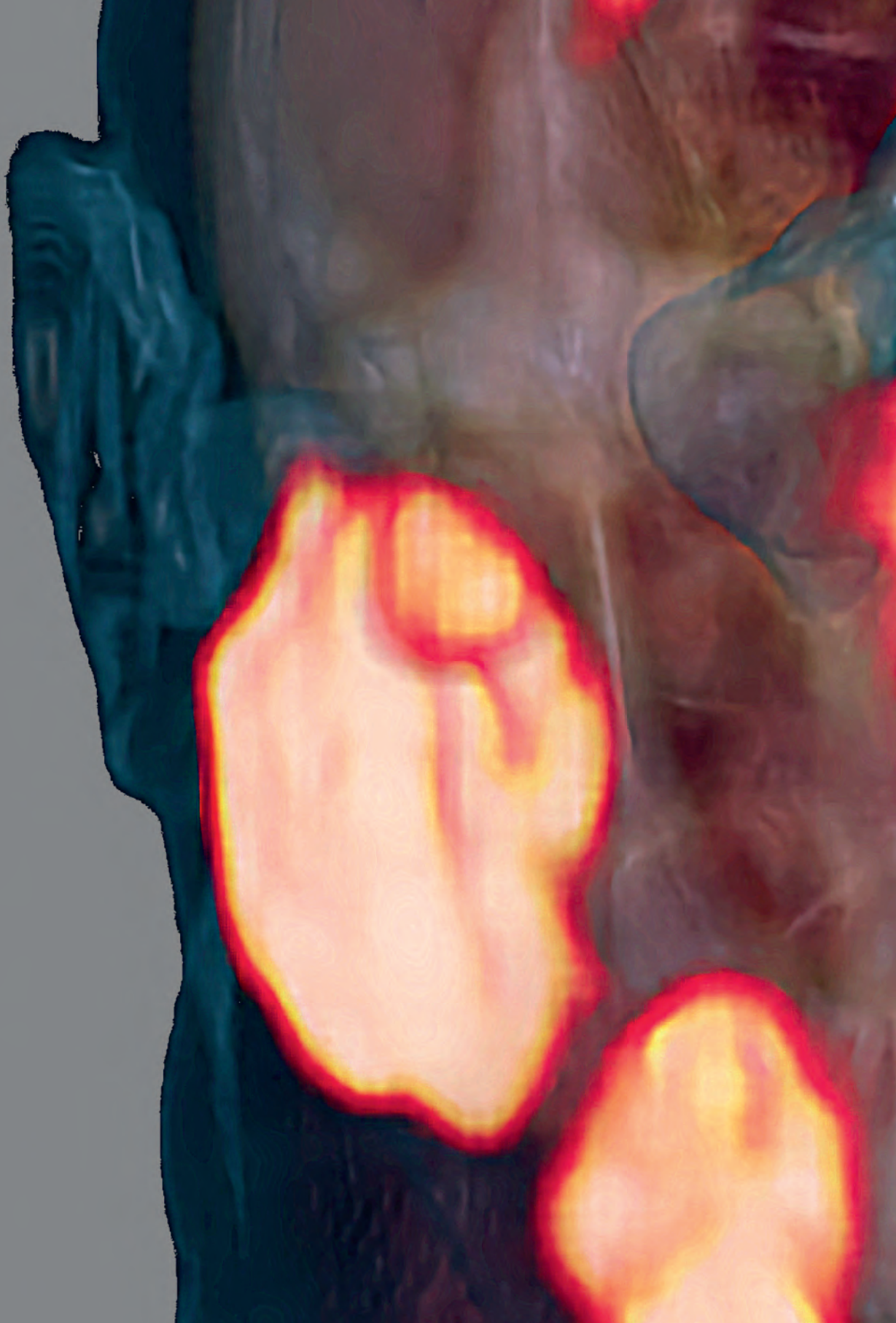

Chapter 5

Risk of breast cancer in women with a previous salivary gland carcinoma or pleomorphic adenoma in the Netherlands.

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Abstract

Introduction: Salivary and mammary gland tumors show morphological similarities and share various characteristics, including frequent overexpression of hormone receptors and female preponderance. Although this may suggest a common etiology, it remains unclear whether patients with a salivary gland tumor carry an increased risk of breast cancer (BC). Our purpose was to determine the risk of BC in women diagnosed with salivary gland carcinoma (SGC) or pleomorphic adenoma (SGPA).

Materials and Methods: BC incidence (invasive and in situ) was assessed in 2 nationwide cohorts: one comprising 1,567 women diagnosed with SGC and one with 2,083 women with SGPA. BC incidence was compared with general population rates using standardized incidence ratio (SIR). BC risk was assessed according to age at SGC/SGPA diagnosis, follow-up time and (for SGC patients) histological subtype.

Results: The mean follow-up was 7.0 years after SGC and 9.9 after SGPA diagnosis. During follow-up, 52 patients with SGC and 74 patients with SGPA developed BC. The median time to BC was 6 years after SGC and 7 after SGPA. The cumulative risk at 10 years of follow-up was 3.1% after SGC and 3.5% after SGPA (95% Confidence Interval (95%CI) 2.1-4.7% and 2.6%-4.6%, respectively). BC incidence was 1.59 times (95%CI 1.19-2.09) higher in the SGC-cohort than expected based on incidence rates in the general population. SGPA-patients showed a 1.48 times (95%CI 1.16-1.86) higher incidence.

Discussion: Women with SGC or SGPA have a slightly increased risk of BC. The magnitude of risk justifies raising awareness, but is no reason for BC screening.

Introduction

Major salivary gland cancer (SGC) and salivary gland pleomorphic adenoma (SGPA) together constitute almost three quarters of all salivary gland tumors, and have a yearly incidence of respectively 0.7 and 4.9/100,000 person-years in the Netherlands (European Standardized Rate)[1,2]. Benign salivary gland tumors occur 6-7 times more frequently than malignant tumors and the SGPA, which accounts for two thirds of all benign salivary gland tumors, may occasionally show malignant transformation[2–6].

Salivary and mammary gland tumors show morphological similarities and salivary gland-like type tumors have been described in the breast[7–11]. They also share other characteristics, including frequent overexpression of hormone receptors and in some histologies a female preponderance, most consistently in salivary gland adenoid cystic carcinoma and SGPA[1–3,12–16].

Whereas the larger amount of glandular breast tissue (next to hormonal, lifestyle, and genetic factors) puts women at higher risk of BC than men, gland volume does not explain the higher risk of a salivary gland tumor in women, since there are no gender differences in salivary gland size[17]. Differential attitudes towards physical appearance or towards medical attention seeking behaviour between males and females are unlikely to play a major role in explaining gender variation in incidence of SGC and SGPA, due to visibility of the tumor[18–20].

Although the similarities may suggest a common etiology, it remains uncertain whether patients with a salivary gland tumor carry an increased risk of breast cancer (BC). Literature is inconclusive regarding whether risk is increased, and if so, to what extent and in which patients. A previous salivary gland tumor as a risk factor for BC has been reported in the literature, but studies are ambiguous, possibly due to the variation in sample size and inclusion criteria[21–28]. Support for a hormonal component in salivary gland cancer risk was earlier reported in an epidemiological study, and a hormonal influence could also have played a role in the recently reported higher risk of SGC in women after BC[29,30].

The objective of this study was to determine whether women diagnosed with a SGC or SGPA have an increased risk of developing BC in two nationwide population-based cohorts with long term follow-up and complete cancer incidence data.

Materials and Methods

Cohorts: To assess the association between salivary gland tumors and BC risk in women, we used two nationwide Dutch registries. The Netherlands cancer registry (NCR) was used to establish a cohort including all incident malignant major salivary gland tumors (ICD-O-3 codes: C07: Parotid; C08: other and unspecified major salivary gland), diagnosed in the Netherlands between January 1st, 1989 and December 31st, 2014, without limitations regarding inclusion by age or previous other malignancies. All subsequent ductal in-situ as well as invasive first BCs, registered in the NCR, which occurred until December 31st, 2014 in this cohort were identified. The NCR receives its data mainly from the nationwide histopathology and cytopathology network and archive in The Netherlands (PALGA- in Dutch: Pathologisch Anatomisch Landelijk Geautomatiseerd Archief), but also from hospital discharge diagnosis registries (e.g. patients with a clinically or radiologically diagnosed SGC, who did not undergo biopsy or surgical treatment). Vital status and in-situ and invasive breast cancer incidence were complete until December 31st, 2014. Treatment details were reported and usually included surgery with post-operative radiotherapy in selected cases. The latter typically consists of external beam radiotherapy, which is usually up to 70 Gray (Gy) on the tumor and 50 Gy on the neck, and has a horizontal direction and stays above the clavicle. For the SGPA cohort, we selected all women from a previously established SGPA cohort that included all Dutch pathologically confirmed incident SGPA diagnoses in the PALGA registry between January 1st,1992 - December 31st 2012 with (for logistical reasons to reduce the size of the cohort) a 5-year interval [2]. Therefore, all included SGPA patients were from the years 1992, 1997, 2002, 2007 and 2012. PALGA records all cytological and histological diagnoses, including those of benign diseases like SGPA, and has complete coverage of the Netherlands since 1991. PALGA is one of only a few national registries worldwide that include benign tumors, which allowed establishing our nationwide SGPA cohort[2,3]. The Palga registry does not contain standardized information on therapy. However, all patients in this cohort had a histological diagnose, thus had surgery. Radiotherapy is typically applied in selected recurrent SGPA cases. All subsequent DCIS as well as all invasive first BCs, registered in PALGA, which occurred until December 31st, 2013 in this cohort were identified. Basal cell carcinoma and melanoma of the breast were excluded. Patients with BC before SGC or SGPA were excluded as well.

Statistical analysis: Time at risk started at date of SGC or SGPA diagnosis. For SGC follow-up ended at the date of BC diagnosis, death, emigration, or last follow-up, whichever came first. In SGPA patients, the expected number of cases and cumulative risk of BC were based on the cumulative follow-up time. Since vital status is not registered in the PALGA registry, mortality among SGPA patients was imputed using gender-, age-, and calendar-year specific life-tables for the Dutch population, generating 50 imputed datasets, under the assumption that SGPA patients, as SGPA is a benign disease, had a similar life expectancy as the Dutch general population. Estimated cumulative risks and standard errors in each imputed dataset were subsequently pooled using Rubin's rule.

Expected BC incidence in the SGC cohort was estimated using the Hakulinen method[31]. The Standardized Incidence Ratio (SIR) was calculated as the ratio between the observed and expected number of BC cases in both the SGC-cohort and the SGPA-cohort. In order to compare the observed BC incidence in our study population with the BC incidence among Dutch females from the general population, we used external reference rates. Using age-specific (5-year age groups) and calendar-year specific BC incidence rates for the Dutch female population, we calculated the number of BCs we could have expected if our cohort would have had the same age-specific BC incidence as the general population, based on the number of person-years of follow-up our women accrued in each 5-year age group during each year of follow-up. This method of using external data as reference data has been extensively used previously[32]. Thus, the number of BC cases in both the SGC-cohort and the SGPA-cohort are compared with BC cases in a contemporized follow-up period in contemporized age categories, in the Dutch population.

As a sensitivity analysis, the SIR was also determined for BCs occurring ≥ 3 months after the index salivary gland tumor, thereby excluding potential synchronous BC. In this analysis time at risk started at 93 days after SGC or SGPA diagnosis. Also, as a sensitivity analysis risk of invasive and in situ BC was evaluated separately. BC risk was also assessed and stratified for SGC/ SGPA age, follow-up time and (in SGC patients) histological subtype. For SGC patients the 5 and 10-year survival after BC was calculated, with a subgroup analysis for patients younger than 65 years. The 95% confidence intervals (95%CI) were calculated assuming a Poisson distribution for the observed number of events. Tests for homogeneity and trend of SIRs were performed using Poisson regression models based on collapsed

person-time data. Statistical analysis was performed using STATA 13 (StataCorp LP, College Station, USA).

Results

Our study included 3,650 women: 1,567 women with SGC and 2,083 women with SGPA, translating into a yearly mean of 60 and 417 women respectively (Table 1; Supplementary table 2). The overall median age at diagnosis of the salivary gland tumor was 56 years (IQR 45-68). The median follow-up was 7 years (IQR 2-12) after SGC. The median follow-up after an SGPA diagnosis was 6 years (IQR 2-16). During follow-up 52 SGC patients developed BC, of whom 46 (88%) had invasive and 6 (12%) in situ BC (Table 2; Supplementary table 3). Of the SGPA patients 74 developed BC, of whom 68 (92%) had invasive and 6 (8%) in situ BC. Overall, the median age at BC diagnosis was 63 years (IQR 51-74). The median interval between salivary gland tumor and BC diagnosis was 6 years in SGC (range 0-24; IQR 3-9) and 7 years (range 0-20; IQR 3-11) in SGPA. In SGC patients, the 5- and 10-year survival rate after invasive BC was respectively 59.8% (95%CI 42.7-73.4%) and 42.0% (95%CI 24.9-58.1%). For patients younger than 65 years, this was 78.9% (95%CI 52.4-91.7%) and 70.1% (95%CI 40.5-87.0%). The BC receptorstatus could be determined in most cases (Table 2).

Comparison with the general population: The cumulative risk of BC in SGC patients was 5.3% (95% CI 3.8-7.3%) at 10 and 8.2% (95% CI 5.8-11.5%) at 20 years, respectively (Table 3). The comparison to the expected risk based on the general population of 2.9% at 10 years and 6.0% at 20 years (dotted line) is made in figure 1A. The cumulative risk of BC among SGPA patients was 3.5% (95% CI 2.6-4.6%) at 10 years and 7.2% (95% CI 5.4-9.4%) at 20 years, respectively (Table 3). In comparison, the expected cumulative BC risk, based on age-matched incidence in the general population, was 2.2% at 10 and 5.2% at 20 years, respectively (Figure 1B). Overall, among SGC patients the incidence of BC was 1.59 times (95% CI 1.19-2.09) higher than expected based on general population rates. BC incidence was 1.48 times (95% CI 1.16-1.86) higher than expected among SGPA patients. The SIRs when risk of DCIS and invasive BC in the SGC-cohort were estimated separately were similar (SIR 1.86 for in situ BC; 95% CI 0.68-4.04 and SIR 1.57 for invasive BC; 95% CI 1.14-2.09, respectively). SIRs for BC (invasive or in situ)

did not vary with age at SGC diagnosis (SIR <50 years: 1.54, 95% CI 0.79-2.68; SIR 50-69 years: 1.69, 95% CI 1.10-2.48; SIR ≥70 years: 1.48, 95% CI 0.81-2.49, p-heterogeneity=0.91) nor did they change with follow-up duration (SIR <10 years: 1.76, 95% CI 1.28-2.38; SIR 10-19 years: 0.92, 95% CI 0.37-1.89; SIR ≥20 years: 2.70, 95% CI 0.56-7.90, p-heterogeneity=0.19) or histological SGC subtype (table 3). For SGPA patients, BC incidence did neither vary with age at SGPA diagnosis nor with follow-up duration. A sensitivity analysis, showed similar risks after exclusion of synchronous BC (n=4), defined as all BC diagnosed < 3 months after the salivary gland tumor diagnosis; the SIR was 1.57; (95% CI 1.14-2.09) for SGC and 1.33 (95% CI 1.04-1.64) for SGPA, respectively.

In the SGC-cohort, the absolute excess risk (AER) of DCIS and invasive BC combined was 17.7, in other words 17.7 excess BC for every 1,000 women, each followed for 10 years. In the SGPA-cohort the AER was 12.8 per 10,000 person-years.

Table 1. Population characteristics of the salivary gland carcinoma (SGC) or salivary gland pleomorphic adenoma (SGPA) with and without *subsequent* breast cancer (BC).

	SGC		SGPA	
	Subseq. BC (n=52)	No subseq. BC (n=1,515)	BC (n=74)	no BC (n=2,009)
Age at diagnosis, yrs† (IQR)	57 (49-69)	63 (47-75)	50 (42-66)	48 (37-63)
Year of salivary tumor diagnose				
1989-1994	13	282	22	321
1995-2004	25	557	37	746
2005-2013	14	676	15	942
Histology SG tumor*, N(%)				
Adenoid cystic ca.	9 (17.3)	305 (20.1)		
Muco-epidermoid ca.	9 (17.3)	227 (15)		
Acinic cell ca.	9 (17.3)	263 (17.4)		
Ca. ex Pleomorphic Adenoma	6 (11.5)	106 (7)		
Adenoca., NOS	7 (13.5)	204 (13.5)		
Squamous cell ca.	2 (3.8)	95 (6.3)		
Myo-epithelial ca.	4 (7.7)	95 (6.3)		
Salivary duct ca.	1 (1.9)	29 (1.9)		
Other salivary gland ca.	5 (9.6)	190 (12.5)		
Pleomorphic adenoma			74 (100)	2,009 (100)
Stage, N (%)			NA	NA
I	15 (28.8)	436 (28.8)		
II	10 (19.2)	226 (14.9)		
III	4 (7.7)	163 (10.8)		
IV	5 (9.6)	328 (21.7)		

Table 1. continued

	SGC		SGPA	
	Subseq. BC (n=52)	No subseq. BC (n=1,515)	BC (n=74)	no BC (n=2,009)
Age at diagnosis, yrs† (IQR)	57 (49-69)	63 (47-75)	50 (42-66)	48 (37-63)
Unknown/unavailable	18 (34.6)	362 (23.9)		
Treatment SG tumor N (%)				
Surgery with radiotherapy	27 (51.9)	881 (58.2)	74 (100.0)	2083 (100)
Surgery only	13 (25)	359 (23.7)		
Radiotherapy only	3 (5.8)	91 (6)		
No therapy	3 (5.8)	76 (5)		
Surgery+radiotherapy+other	5 (9.6)	41 (2.7)		
Other	0 (0)	42 (2.8)		
Unknown/unavailabe	1 (1.9)	25 (1.7)		

*Histology ICD-O 3.1 Codes are shown in Suppl. table 2; † = Median; NA= not applicable

Table 2. Breast cancer (BC) histology and receptor status of patients with a salivary gland carcinoma (SGC) or salivary gland pleomorphic adenoma (SGPA) and subsequent BC.

	BC after SGC (n=52)	BC after SGPA (n=74)
Year of BC diagnose, N (%)		
1989-1994	2 (3.8)	23 (31.1)
1995-2004	11 (21.2)	37 (50)
2005-2014	39 (75)	14 (18.9)
Age at diagnosis		
Median, yrs (IQR)	64 (57-76)	61 (50-69)
<50	6 (11.5)	17 (23)
50-69	25 (48.1)	38 (51.4)
>70	21 (40.3)	19 (25.7)
Histology BC*, N (%)		
Invasive carcinoma		
Lobular carcinoma	6 (11.5)	5 (6.7)
Ductal/Adeno carcinoma	39 (75)	57 (77)
Other BC (e.g. undiff. / NOS)	1 (1.9)	6 (8.1)
In situ carcinoma		
DCIS	6 (11.5)	6 (8.1)
Receptor status, N (%)		
ER§ +	28 (53.8)	51 (68.9)
ER –	8 (15.4)	11 (14.9)
ER unknown	16 (30.8)	12 (16.2)
PR¥ +	22 (42.3)	39 (52.7)
PR –	11 (21.2)	19 (25.7)
PR unknown	19 (36.5)	16 (21.6)
Her2neu‡ +	6 (11.5)	8 (10.8)
Her2neu –	14 (26.9)	40 (54.1)
Her2neu unknown	32 (61.5)	26 (35.1)

Table 3. Standardized Incidence ratios (SIR), absolute excess risk (AER) and 20-year cumulative risk of breast cancer following diagnosis of a salivary gland carcinoma (SGC) or a salivary gland pleomorphic adenoma (SGPA). * Average number of person-years over 50 imputed datasets; # SIR, AER, cumulative risks and confidence intervals based on pooled estimates; NA = not applicable.

	SGC						SGPA					
	Events SGC	Person years	SIR	95%CI	AER	20-year cum risk	Events SGPA	Person years*	SIR#	95%CI#	AER#	20-year cum risk#
All	52	10945	1.59	1.19-2.09	17.7	8.2%	74	18852	1.48	1.16-1.86	12.8	7.2%
Age (years)												
<50	12	4369	1.54	0.79-2.68	9.6	3.2%	36	10797	1.85	1.30-2.56	15.3	6.7%
50-69	26	4056	1.69	1.10-2.48	26.2	12.4%	25	6222	1.06	0.69-1.57	2.4	6.4%
≥70	14	2520	1.48	0.81-2.49	18.1	16.2%	13	1833	1.87	1.00-3.20	33.1	11.5%
P-heterogeneity			*0.91*						*0.07*			
Follow-up (years)												
<10	42	8229	1.76	1.27-2.38	7.8	NA	53	13720	1.56	1.17-2.04	13.9	NA
10-19	7	2421	0.92	0.37-1.89	-2.6	NA	21	4896	1.39	0.86-2.12	12.0	NA
≥20	3	296	2.70	0.56-7.90	64.0	NA	0	235	0	0-4.31	-36.5	NA
P-trend			*0.48*						*0.37*			
Histology												
Adenoid cystic carcinoma	9	2659	1.18	0.54-2.23	5.0	6.1%						
Muco-epidermoid carcinoma	9	1868	1.96	0.90-3.72	23.6	7.0%						
Acinic cell carcinoma	9	2393	1.40	0.64-2.65	10.7	5.5%						
Carcinoma ex pleomorphic adenoma	6	751	2.33	0.86-5.08	45.7	8.9%						
Adenocarcinoma, nos	7	1246	1.66	0.67-3.43	22.4	13.2%						
Carcinoma, other	12	2028	1.68	0.87-2.93	23.9	13.1%						
P-heterogeneity			*0.81*									

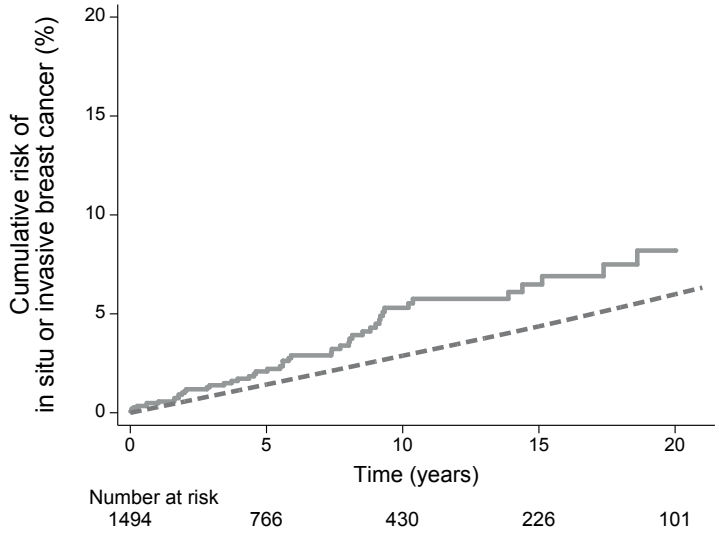

Figure 1A: Cumulative risk of invasive and in situ BC in the complete SGC-cohort (excluding patients with BC before SGC). The solid line represents observed and dashed line the population-expected cumulative risk (corrected for age of the SGC cohort).

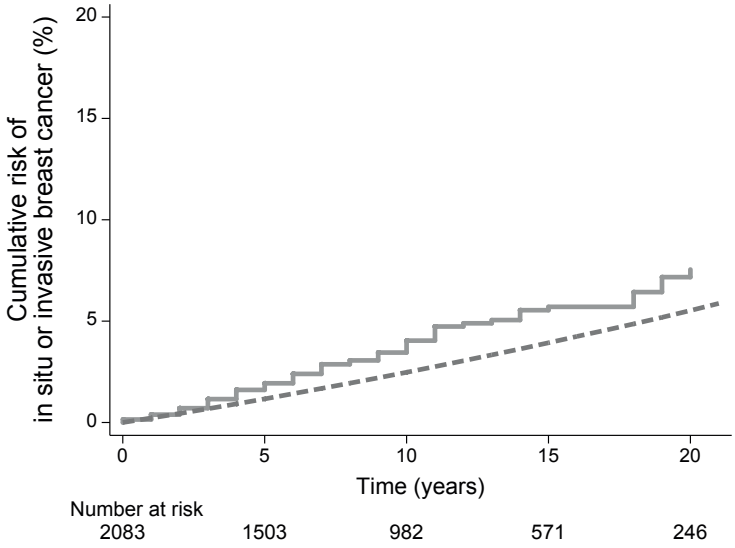

Figure 1B: Cumulative risk of invasive and in situ BC in the complete SGPA-cohort (including patients with BC before SGPA). Solid line represents observed and dashed line the population-expected cumulative risk.

Discussion

In this large and nationwide study with long-term complete follow-up, including 3,650 women diagnosed with SGC and SGPA, the risk of developing a subsequent BC is moderately increased. This roughly 50% higher relative risk of BC can be better interpreted by mentioning absolute numbers in an example. For a 50-year old woman, the risk to develop breast cancer in the next 10 years (until the age of 60) equals 2.9%[33]. After a previous diagnosis with SGC or SGPA, this risk would be 4.3%.

In contrast to many previous studies on BC risk after a salivary gland tumor, the nationwide cohorts used in our study either consisted solely of malignant or of benign tumors. The high number of patients allowed unbiased estimation of relative and absolute risk in both type of lesions and gave rise to the possibility of subgroup analysis.

The lower age at diagnosis of second tumors in the SGPA-cohort compared to the SGC-cohort (Table 2), was in line with the lower age of the SGPA patients and earlier publications[1,2]. The increased BC risk after SGC or SGPA did not vary much with age at index tumor diagnosis nor with SGC histological subtype. The finding of an increased risk in patients diagnosed with BC ≥3 months after the salivary gland tumor, showed that the increased BC incidence was not likely based on surveillance bias caused by diagnostic evaluation of the index tumor.

Results of previous studies varied from no risk increase upto 8-fold increased risks (Table 4). Many of these studies had methodological shortcomings which made comparison and generalization of these results difficult[21–28]. For instance, most studies did not provide a confidence interval for the risk estimates presented [23–26,28]. The completeness of follow-up may have been a problem in single institution cohorts[21,22,28]. Missing a diagnosis of even one patient with a second primary BC, would have had a substantial negative impact on the estimated risk in these cohorts which included between 4 to 15 BC cases each. Also, there could be a referral bias in comprehensive cancer centers, because of inclusion of a higher proportion of patients with second or multiple primaries. Variation in BC incidence between the USA and Europe unlikely explains the high risk in two American studies[21,26]. Additionally, BC incidence rates in the SEER registry are generally lower than in European populations[34]. A later conducted population based SEER study could not confirm the reported higher BC risk after an earlier

SGC (SIR 1.07; 95% CI 0.72-1.53)[27]. This absence of an increased risk could have been caused by differences in inclusion, by including lesions of undefined histology (overall 25%) as a first tumor. These could have been metastases of e.g. squamous cell carcinoma of the skin of the face or skull to the parotid, which could have attenuated a higher risk of BC.

Table 4. A comparison of publications investigating breast cancer (BC) incidence in women after diagnosis of a salivary gland tumor (SGT).

Ref, year	Women (n)	SGT (M/B)	Years at risk	Observed BC	Expected BC	O/E (SIR)	P value	95% CI
[21], 1968	396	M	1,652	7	0.9	7.8	0.00004	NR
[22], 1969	297	M	3,033	4	4.0	1.0	NA	NA
[23], 1972	349	M	2,443	8	4.2	1.9	0.6	NR
[24], 1977	453	M+B	2,315	6	2.6	2.3	<0.05	NR
[25], 1983	367	M	2,868	7	5.4	1.3	NR	0.5-2.7
[26], 1984	190	M+B	629	4	0.83	4.8	0.01	NR
[27], 1999	1718	M*	10,789	30	28	1.07	NR	0.72-1.53
[28], 2005	439	M+B	3,382	15	5.93	2.5	0.003	1.4-4.2
Current, 2019	2083	B	18,852	74	50	1.48	NR	1.16-1.86
	1567	M	10,995	52	32.6	1.59	NR	1.19-2.09

M = malignant; B = benign; NA = not applicable; NR = not reported; *25% undetermined / mixed histology and 13% squamous cell carcinoma

The increased risk of BC after a salivary gland tumor in our nationwide cohorts, is of similar magnitude as some of the known risk factors, such as alcohol intake or use oral contraceptives (Supplementary table 1) and is remarkable. It could theoretically be caused by several mechanisms.

Common endocrine mechanism and common environmental / lifestyle factors: A positive association between (high levels of) endogenous estrogens or estrogen exposure and risk of various female cancers (breast, ovarian and endometrial cancer) has been consistently described in the epidemiological literature[35–38]. Estrogens are thought to cause this increased cancer risk via cell proliferation, DNA-damage (as a result of estradiol metabolism to genotoxic metabolites) and inhibition of cell repair mechanisms[39]. Estrogen exposure may have similar effects on salivary glandular tissue, which also expresses estrogen receptors. It could therefore be that women with high endogenous estrogen levels (e.g. in post-menopausal high BMI) or exposure (e.g. in postmenopausal hormone replacement therapy or use of oral contraceptives) have an increased risk of SGC or SGPA[40–44]. If estrogen indeed has an effect on both risk of SGC and risk of SGPA,

this could also explain the reported female preponderance of SGC and SGPA. In the literature, generally only a small portion of salivary gland tumors show ER-overexpression based on immunohistochemistry, possibly because mainly ER-α and not ER-β expression status has been assessed[45–51]. Nevertheless, although ER-β expression in salivary gland tumors was only reported in a limited number of series, ER-β was overexpressed in 27 of 38 (71%) patients in a series of adenoid cystic carcinomas and in 57 of 80 (73%) in a series of salivary duct carcinomas[16,52].

In SGPA and adenoid cystic carcinoma, the slight, but relevant difference in incidence between males and females (female to male incidence ratio of 1.4:1 and 1.2:1, respectively) and the overexpression of estrogen β receptor (ER-β), compared to normal salivary gland, may suggest a role for the proliferative influence of estrogens in tumorigenesis[2,14,53]. Similar considerations have been mentioned in earlier reports of experimental anti-estrogen treatment for SGC in the literature [8,54,55]. From the above, it could be concluded it is a realistic possibility that estrogens play a role in the association between salivary gland tumors and BC.

Salivary gland tumor treatment or metastasis: It is unlikely that SGC of SGPA treatment contributes to an increased BC risk since locoregional treatment does not affect breast tissue. In external beam radiotherapy for SGC, the breast is not an organ at risk due to the direct dose, although scattered radiation could in theory add to the risk of primary breast cancer. This dose can be calculated. After radiotherapy for SGC in the earlier mentioned example of a 50-year old female patient, this estimated additional lifetime risk is 0.3%, *for all cancers.* So, scattering does not contribute substantially to the risk of BC.

An increased BC risk due to SGC chemotherapy (depending on histology, but mostly cisplatin based) is not very likely[56,57]. Solitary metastasis of SGC to the breast is not very likely. The SGPA is known to almost never metastasize at all. Also, if there were cases, these would probably have been recorded in the pathology database or cancer registry not as BC but as metastasized salivary gland tumor.
Common genetic susceptibility: Germline mutations that could account for the occurrence of both a salivary gland tumor and BC in the same woman have not been identified yet, and the literature on this topic is limited. One retrospective study including 5,754 proven or likely carriers from 187 *BRCA1 or BRCA2* positive

pedigrees, reported 3 SGCs[58]. Although the authors reported an increased SGC incidence, a 95% CI was not provided. Mersch and colleagues did not find any SGC in 1,072 patients with a *BRCA1* or *BRCA2* mutation, who had received genetic counselling[59]. In a study of 268 patients with SGPA, upon evaluation for *BRCA1*, one had a *BRCA1* mutation (who earlier also had BC)[60]. Other forms of genetic predisposition i.e. based on (multiple) single-nucleotide polymorphisms (SNPs) have not been reported. Although SGPA is characterized by a chromosomal translocation that causes activation of the pleomorphic adenoma gene 1 (PLAG1) on chromosome 8q12, germline mutations have not yet been identified[61–65].

Limitations: Although the results of this study suggest that salivary gland tumors and BC share a common (hormonal) etiological factor, further investigation will be needed to provide more insight in the underlying mechanism. It is still unclear whether affected women who develop both a salivary gland tumor and BC are e.g. more susceptible for the effect of estrogens, have higher blood levels or have had more estrogen exposure. Data on BC risk factors were unavailable as these are not routinely collected by the Dutch cancer registry.

Person time after an SGPA diagnosis was not available and imputed assuming these patients had a similar life expectancy as the general Dutch population. SGPA does carry a very small risk of malignant transformation of approximately 0.15%, which may lead to a slightly higher mortality than that of the general population[2]. We may thus have overestimated the follow-up in the SGPA cohort, which would have resulted in slight overestimation of the expected number of BC cases[2], and subsequently an underestimation of the SIR for BC. On the other hand, follow-up of SGC patients is very complete, with information on dates of death or migration provided by linkage with the Dutch nationwide population registry, and near complete information on BCs occurring in this cohort, available from the same source.

In summary, there are some indications that suggest an endocrine mechanism behind the increased incidence of BC in salivary gland tumors. Data on a possible genetic background are insufficient. Sequencing of the cohorts for possible germline mutations might provide further clues regarding genetic predisposition, but this would require very large cohorts.

Clinical implications: Women with a SGC or SGPA have a moderately increased risk of BC, compared to women in the general population. The relative risk for BC in patients with SGC or SGPA (together almost 75% of salivary gland tumors) is of similar magnitude as in patients who have one of various classical BC risk factors. However, the magnitude of the relative risk is no reason for recommending an intensified follow-up schedule. Nevertheless, our study should raise awareness of a slightly increased risk of developing BC among female patients diagnosed with SGC or SGPA and their treating physicians.

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PART 2

Molecular imaging of PSMA in salivary glands

Chapter 6

Physiologic distribution of PSMA-ligand in salivary glands and seromucous glands of the head and neck on PET/CT

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Abstract

Objectives: Prostate-specific membrane antigen (PSMA) positron emission tomography/computed tomography (PET/CT) is used for detection and (re) staging of prostate cancer. However, healthy salivary, seromucous, and lacrimal glands also have high PSMA-ligand uptake. This study aimed to describe physiologic PSMA-ligand uptake distribution characteristics in the head and neck to aid in PSMA PET/CT interpretation and to identify possible new clinical applications for PSMA-ligand imaging.

Study Design: Thirty consecutive patients who underwent PSMA PET/CT for prostate cancer were evaluated. Tracer maximum standardized uptake values (SUV_{max}) in the salivary, seromucous, and lacrimal glands were determined visually and quantitatively. Overall and intraindividual variations were reported.

Results: All gland locations had increased tracer uptake. The mean $SUV_{max} \pm$ standard deviation varied: parotid 12.3 ± 3.9; submandibular 11.7 ± 3.5; sublingual 4.5 ± 1.9; soft palate 2.4 ± 0.5; pharyngeal wall 4.3 ± 1.3; nasal mucosa 3.4 ± 0.9; supraglottic larynx 2.7 ± 0.7; and lacrimal 6.2 ± 2.2. The parotid had the largest overall variation in SUV_{max} (5.2-22.9), and the sublingual glands had the largest mean intraindividual difference (18.1%).

Conclusions: Major and minor salivary and seromucous glands consistently have high PSMA-ligand uptake. Minor gland locations can be selectively visualized by this technique for the first time. This provides potential new applications such as quantification of present salivary gland tissues and individualization of radiotherapy for head and neck cancer or lutetium-177-PSMA radionuclide treatment.

Introduction

Functional imaging of tissues expressing the prostate-specific membrane antigen (PSMA) with radiolabelled ligand Gallium-68 [^{68}Ga]-HBED-CC-Glu-NH-CO-NH-Lys(Ahx) and positron emission tomography combined with computed tomography (PSMA PET/CT), is primarily used for the detection and (re)staging of prostate cancer [1].

However, binding of PSMA-ligands is not limited to prostate cancer cells. Clinical experience with PSMA PET/CT for prostate cancer has revealed consistent and significant uptake in other tissues, most notably the major salivary and lacrimal glands [1]. Because tracer uptake is based on expression of the PSMA epitope in present glandular cells, it can be hypothesized that uptake of PSMA-ligand is associated with gland volume and thus functional capacity of the gland. Two arguments support this hypothesis. First, xerostomia is a well-known side effect of lutetium-177 (^{177}Lu)-PSMA treatment and could be explained by cell loss as a consequence of toxicity. Second, a difference can be noted in ^{68}Ga-PSMA uptake between normal and irradiated submandibular glands, with the latter having decreased glandular function after radiotherapy (Figure 1) [2,3]. Current clinical tomographic imaging modalities such as CT, magnetic resonance imaging (MRI) and ^{18}F-fluorodeoxyglucose (FDG) PET/CT can adequately visualize the parotid and submandibular salivary glands, and clinicians are generally well aware of their normal location and appearance [4].

The sublingual glands are more difficult to detect on CT or FDG PET/CT and they require specific magnetic resonance sequences for good visualization [5]. Imaging of the minor (mucosal) salivary glands with current techniques has been a challenge because until now adequate visualization of these small (1-5mm) glands was impossible. Imaging experts may not even be aware of their existence. In addition, the lack of tools to visualize minor salivary and seromucous glands, and the inability to estimate gland viability limits the options for personalized treatment aiming to preserve the function of these glands, which play a vital role in lubrication of the oral cavity [6,7].

With the introduction of PSMA PET/CT for prostate cancer staging and follow-up, clinicians are confronted with unfamiliar uptake patterns throughout the

head and neck that may lead to misinterpretation of tumor and normal tissues (Figure 1). Adequate knowledge of the normal anatomy and function of the salivary glands is relevant to further improve interpretation of the images. Assessment of salivary gland presence and function by PSMA PET/CT after treatment requires knowledge of quantitative physiological uptake patterns, which currently are not well understood.

This study aimed to describe the physiologic PSMA-ligand uptake distribution characteristics by evaluating tracer maximum standardized uptake values (SUV_{max}) in normal salivary glands, seromucous glands of the upper aerodigestive tract and lacrimal glands on PSMA PET/CT. In addition, this study aimed to identify potential clinical applications of PSMA PET/CT.

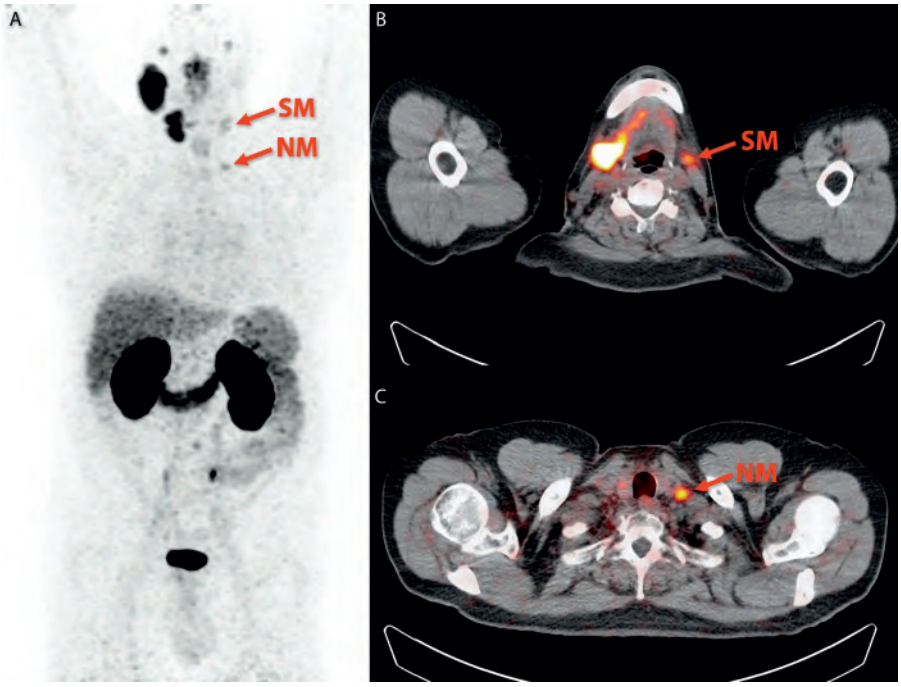

Figure 1: PSMA PET/CT of a patient with a history of cancer of the left parotid gland treated with surgery and radiotherapy, and prostate cancer treated with radiotherapy with current biochemical recurrence. An anterior projection of the body is shown (A), and transverse slices at the level of the neck (B) and upper thoracic outlet (C). The cytologically proven nodal metastasis from prostate cancer in the lower neck (NM) has equivalent uptake as the irradiated submandibular gland (SM) and several normal tissues in the neck (e.g., the glands in the supraglottic larynx). This may lead to confusion and misinterpretation when normal patterns are not adequately recognized.

Materials and methods

The distribution of healthy salivary and seromucous gland tissues in the head and neck area was retrospectively analysed in 30 consecutive patients who underwent total body PSMA PET/CT for staging of prostate cancer in March and April 2016. Exclusion criteria were previous salivary gland disease, aberrant tracer uptake suspected of neoplasia, and previous surgery and/or radiotherapy in the head and neck region.

PSMA PET/CT: Specific patient preparation was not required before PET/CT imaging. Images were acquired from the skull vertex to the thighs using a TruePoint Biograph mCT40 scanner (Siemens, Erlangen, Germany), approximately 60 minutes after intravenous injection of 2 MBq/kg ^{68}Ga-HBED-CC-Glu-NH-CO-NH-Lys (Ahx). A low dose CT scan was performed using Care Dose 4D and Care kV (Siemens, Erlangen, Germany) with the following reference parameters: 40 mAs, 120 kV. Subsequently, PET was acquired according to the European Association of Nuclear Medicine recommendations with the following parameters: PET with time-of-flight and point spread function (TrueX) reconstruction, 4 iterations, 21 subsets, with a filter of 7.5mm full width at half maximum [8].

PSMA-ligand uptake: Tracer uptake in the head and neck was determined visually and quantitatively by a dedicated board-certified head and neck nuclear medicine physician (B.d.K.) experienced in PSMA PET/CT, in consensus in a joint session with an oral and maxillofacial surgeon (T.K.N.) Visibility was defined as visually recognizable by increased tracer uptake relative to surrounding mucosa and other normal tissues. Quantitative evaluation was performed by calculating the maximum standardized uptake value (SUV_{max}) using a freehand isocontour volume of interest and the lean body mass formula, as defined in the European Association of Nuclear Medicine guidelines [8]. In the event of disagreement on gland location margins, a forced consensus was reached. Uptake in the parotid, submandibular, sublingual, pharyngeal, and lacrimal glands was measured bilaterally.

Data analysis: Normal distribution of the SUV_{max} was evaluated per gland location using the Shapiro-Wilk test. The mean SUV_{max} ± standard deviation (SD) and range were calculated according to each gland location. Overall variations in SUV_{max} of the paired glands were reported, and intra-individual differences were

visualized in a boxplot. Statistics were performed using IBM SPSS Statistics for Windows, Version 21.0 (Armonk, NY, USA: IBM Corp.). The boxplot was drawn using GraphPad Prism for Windows, Version 6.02 (GraphPad Software, La Jolla, CA, USA). For this study, individual consent was not required. This was approved by the institutional Medical Research Ethics Committee, protocol number 16-790 and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Results

PSMA PET/CT: The thirty male patients had an average age of 68 (range 54-82 years), the mean administered activity was 173 ± 29 MBq (range 131-273 MBq). The mean time interval between tracer administration and imaging was 66 minutes (interquartile range 58-74 minutes).

PSMA-ligand uptake: All scans clearly depicted anatomical areas of salivary, seromucous, and lacrimal gland concentrations by visualizing high and homogenous uptake of PSMA-ligand (Figure 2). These areas included both major salivary glands and submucosal minor salivary and seromucous glands in the soft palate, pharynx, nasal mucosa, supraglottic larynx, and lacrimal glands. Pharyngeal tracer uptake was concentrated around a bilateral area in the dorsal wall of the nasophanrynx, measurements were presented as 'pharyngeal wall'. See Figures 2 and 3 for an example with anatomic atlas. An annotated full version of the scan is available for review online (Supplementary Data).

The uptake of PSMA-ligand in these glands is summarized in Table I. The highest uptake was seen in the parotid and submandibular glands, with mean SUV_{max} 12.3 ± 3.9 (range 5.2 – 22.9) and 11.7 ± 3.5 (6.0 – 22.2), respectively. For reference, the mean maximal uptake values in the liver and kidneys were 3.7 ± 0.8 (2.4 – 5.0) and 28.8 ± 7.6 (16.9 – 44.5), respectively. The intra-individual differences in SUV_{max} of the paired glands varied per location. Details are listed in Table II and Figure 4.

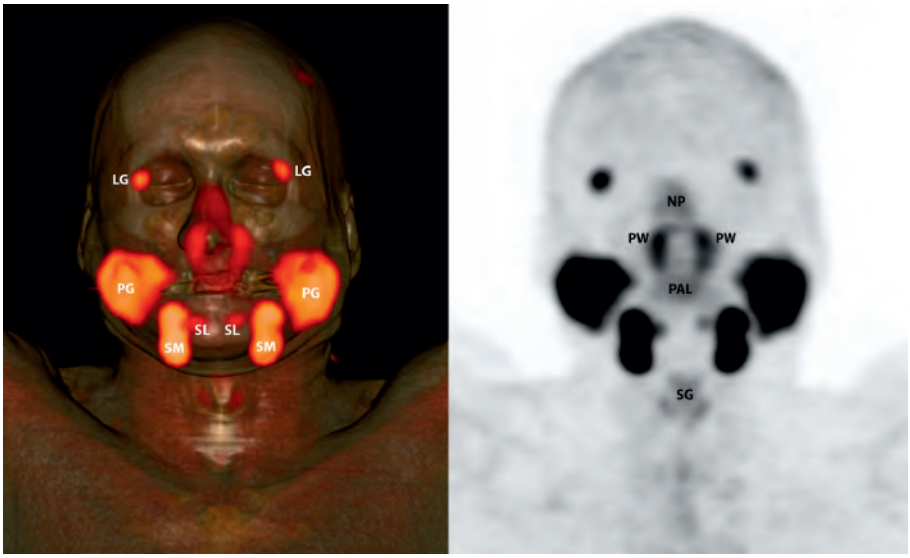

Figure 2: Anterior projection overview of combined PSMA PET/CT (left) and PSMA PET (right), showing tracer uptake in healthy salivary, seromucous, and lacrimal glands in a patient with prostate cancer and no apparent metastatic disease. LG, lacrimal gland; NP, nasal and nasopharyngeal mucosa; PW, pharyngeal wall; PAL, soft palate; PG, parotid gland; SM, submandibular gland; SL, sublingual gland; SG, supraglottic larynx.

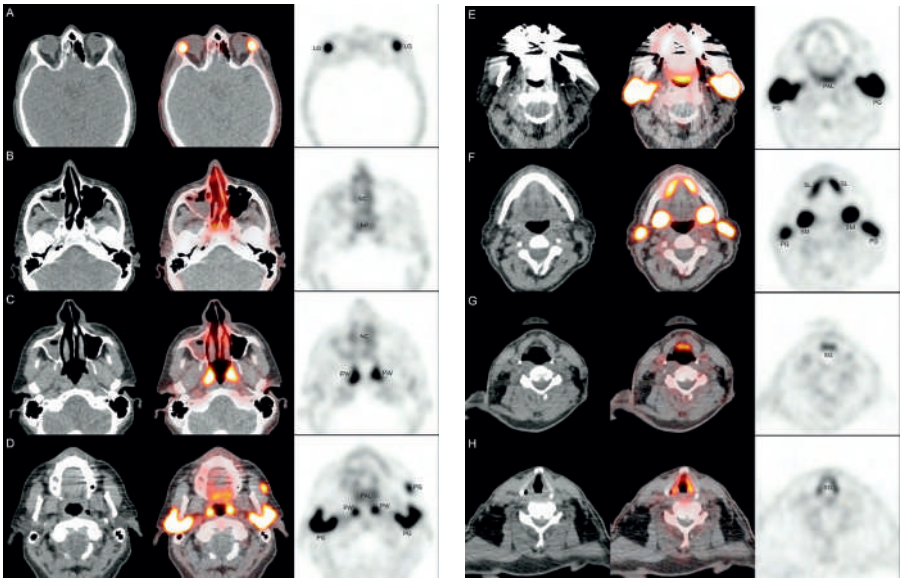

Figure 3: Low dose CT (left), PET/CT (middle) and PET (right) images of the head and neck, showing ^{68}Ga-HBED-CC-Glu-NH-CO-NH-Lys(Ahx) uptake in healthy salivary, seromucous, and lacrimal glands in a patient with prostate cancer and no apparent metastatic disease. LG, lacrimal gland; NC, nasal cavity mucosa; NP, nasopharyngeal mucosa; PW, pharyngeal wall; PAL, soft palate; PG, parotid gland; SM, submandibular gland; SL, sublingual gland; SG, supraglottic larynx.

Table 1: Overall SUV_{max} of healthy salivary, seromucous and lacrimal glands on PSMA PET/CT in 30 patients.

	N	Mean	SD	Range
Parotid	60	12.3	3.9	5.2 – 22.9
Submandibular	60	11.7	3.5	6.0 – 22.2
Sublingual	60	4.5	1.9	1.2 – 8.5
Soft palate	30	2.4	0.5	1.4 – 3.6
Pharyngeal wall	60	4.3	1.3	2.5 – 7.7
Lacrimal glands	60	6.2	2.2	2.5 – 13.6
Nasal mucosa	30	3.4	0.9	2.2 – 5.5
Supraglottic larynx	30	2.7	0.7	1.7 – 3.8

Table 2: Intra-individual differences in SUVmax of paired salivary, seromucous and lacrimal glands on PSMA PET/CT in 30 patients.

	Mean difference (% difference)	SD	Maximum difference (% of the mean)
Parotid	0.59 (4.9%)	1.3	3.2 (26%)
Submandibular	0.17 (1.4%)	1.5	3.8 (32%)
Sublingual	0.14 (3.2%)	1.1	2.6 (58%)
Pharyngeal wall	0.04 (1.0%)	0.9	1.1 (26%)
Lacrimal glands	0.28 (4.6%)	0.5	2.1 (34%)

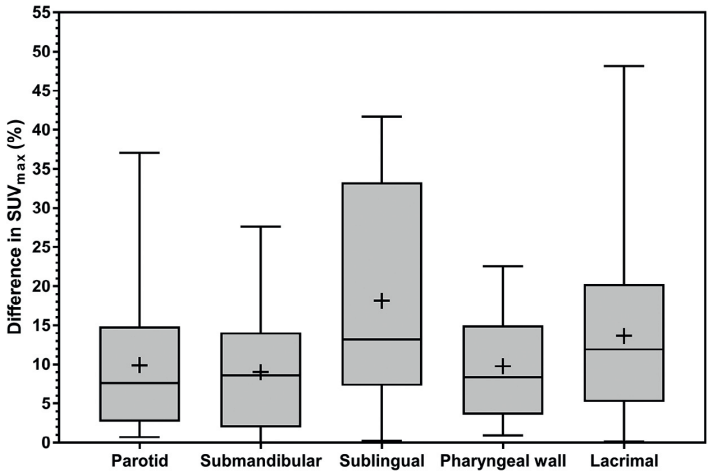

Figure 4: Intra-individual differences in SUV_{max} of paired salivary, seromucous, and lacrimal glands on PSMA PET/CT in 30 patients, showing a mean left/right difference of 9.0-18.1%. Mean (+), median, interquartile range, and range.

Discussion

The results of this study provide a comprehensive qualitative and quantitative overview of tracer accumulation on PSMA PET/CT in salivary, seromucous, and lacrimal gland tissues in the head and neck. The presented distribution patterns of the major salivary glands are comparable to those (to a lesser extent) reported in studies on different topics using the same tracer [9,10]. In addition, our study illustrates the ability of PSMA PET/CT to visualize minor gland locations, for example in the soft palate, pharyngeal wall, nasal mucosa, and supraglottic larynx. This has not been possible with other imaging modalities previously.
Although Demirci et al. described comparable nasopharyngeal tracer concentration around the fossa of Rosenmüller, in our series this area seemed less well defined and spread over a larger area [10]. Awareness of the physiological tracer distribution in the upper aerodigestive tract mucosal and salivary gland tissues can contribute to a correct diagnostic interpretation of PSMA PET/CT.

Imaging of salivary glands: The saliva-producing acinar cells are distributed over 3 paired major and 600-1,000 minor salivary glands, which are organized in small clusters of mainly mucous cells that are located in the mucosa of the palate, lips, buccal mucosa, tongue, and floor of the mouth. In addition, there are many seromucous glands in the oro-, hypo- and nasopharynx, nasal cavities, larynx, trachea, and oesophagus that contain mucous and/or serous cells [7,11]. The nasopharynx, for example, contains 1100-1200 seromucous glands [12]. Anatomic imaging such as CT and MRI generally adequately depicts the parotid and submandibular glands. Therefore, their location, size, and shape are considered common knowledge [4]. The sublingual glands are more difficult to visualize with standard imaging techniques. Until recently, imaging of minor salivary and seromucous glands, located in oral mucosa, lips, tonsils, nasal cavity, nasal sinuses, larynx, trachea, oesophagus, was impossible because of their limited size and poor signal contrast with surrounding tissues; their detection was only possible in case of tumor growth [13]. Today, PSMA PET/CT brings a new technique to clearly depict normal sublingual and minor submucosal gland areas, and to quantify the uptake of the PSMA-ligand as a marker of the presence of glandular cells.

The biomarker PSMA: PSMA was firstly described in 1987 [14]. In 1993, it was successfully cloned and characterized [15]. PSMA is now known as a type 2

transmembrane glycoprotein of the prostate secretory acinar epithelium. Its expression has been reported in normal as well as benign and malignant prostate tumor tissue, with upregulation in advanced prostate carcinoma and metastasis [14–16].

Organ specificity of PSMA: The original assumption of specific binding to prostate epithelium [14,17] was challenged beginning in 1995 after the identification of PSMA epitopes on cells in other organs. Other sites with proven expression include salivary glands, nervous system glia (astrocytes and Schwann cells), kidneys (proximal tubules), the small bowel (jejunal brush border), ductal epithelium of normal breasts, and skeletal muscle [18–21]. The exact function of PSMA in the prostate and kidneys is unclear, although there is suggestive evidence that it may be involved in the metabolism of folate [22]. PSMA was identified not only in healthy tissue, but also in neoplasms, including subtypes of schwannoma and bladder carcinoma [23]. Subsequently, it has been found in the neovascular capillary endothelium of a wide spectrum of solid tumors, i.e., epithelial tumors (carcinomas); neuroendocrine tumors; mesenchymal tumors (soft tissue sarcomas), and melanoma and glioma, which might suggest PSMA involvement in angiogenesis of developing neoplasms [21,24]. Recently, PSMA-ligand uptake and immunohistochemical expression were described in recurrent and distant metastatic adenoid cystic carcinoma and squamous cell carcinoma of the base of the tongue [25,26].

PSMA in imaging: In 1990 the PSMA specific immunoconjugate CYT-356, derived from the prostate-reactive monoclonal antibody 7E11-C5, was labelled with Indium-111 to visualize primary prostate tumors and their metastases using radioimmunoscintigraphy [17]. In recent years, urea-based small-molecule PSMA-inhibiting ligands were developed that could be coupled to ^{68}Ga, with the currently most commonly applied ligand being Glu-NH-CO-NH-Lys(Ahx) coupled to the chelator HBED-CC. This allowed functional imaging with PET. Combined with CT, the imaging modality became known as PSMA PET/CT [27]. This new diagnostic tool is increasingly being used for detection and (re)staging of prostate cancer, with high sensitivity and specificity and with high impact on clinical management of these patients [28].

Expression of PSMA in salivary glands: Histopathological expression of PSMA has not been extensively described. Wolf et al., using immunohistochemical staining, reported that PSMA in human salivary gland specimens is expressed on the epithelium of acinar glandular cells and not the duct cells [29]. Our

preliminary results show staining of serous and mucous acinar cells as well as small (intercalated and striated) duct cells. In the present study, minor salivary glands had lower SUV_{max} values than major glands (Table I). Most likely, this is associated with gland volume. Additionally, it is currently unclear whether this difference in SUV values also reflects a biological difference between the gland types, e.g., a lower number of specific present cells per volume. Direct evidence of a relation between functional capacity and PSMA-tracer uptake is expected to be provided by future research. The limited spatial resolution of current PET/CT scanners may introduce partial volume errors. For objects smaller than the voxel size, the measured tracer concentration is less than the true tracer concentration value in tissue. This could have led to a significant underestimation of the actual tracer uptake in the minor salivary and seromucous glands [30].

The lacrimal glands: Many considerations about the salivary and seromucous glands apply similarly to the serous lacrimal glands that show a comparably high tracer accumulation in PSMA PET/CT. The main lacrimal glands are located in the superolateral orbit and each consists of two lobes that are separated by an aponeurosis [31]. There is currently insufficient knowledge regarding PSMA expression patterns in lacrimal gland cells.

Clinical potential of PSMA-PET/CT: The ability to specifically bind PSMA in salivary and seromucous glandular tissue may open the door to several potential applications of PSMA-ligand binding and imaging of the head and neck area using PET/CT. In oncology, PSMA PET/CT may be used for the detection of recurrent and metastatic salivary gland cancers, squamous cell carcinomas, or even benign salivary gland tumors [25,26]. To differentiate salivary gland tumors from normal glandular tissue, knowledge of the level of physiological uptake is important.
Furthermore, in external beam radiotherapy of the head and neck, it might be possible to selectively spare previously invisible salivary gland locations, especially areas with a high concentration of minor glands. When the location and functional relevance of these glands have been revealed, the amount of radiation dose that passes through these tissues can be reduced by optimization of the treatment plan. This minimize the risk of a persistent xerostomia and associated reduced quality of life [32]. In addition, PSMA PET/CT may identify gland locations devoid of PSMA-ligand uptake, which may not need sparing and thus provide greater flexibility in treatment planning.

Several aspects of PSMA PET imaging require further investigation, such as tissue inhomogeneity and subsequent prediction of response to treatments. Recently, different studies have reported tumor heterogeneity levels, determined on FDG PET/CT alone or combined with MRI, to be significantly associated with survival and responsiveness to radiotherapy. When a comparable determination of metabolic prognosticators in normal tissues would be possible with PMSA PET/CT, treatment plans could be optimized to further reduce toxicity to the salivary glands [33,34].
When a relation of PSMA-ligand uptake with the remaining number of vital glandular cells or actual glandular function after treatment can be determined, PSMA PET/CT could also be used to locally assess salivary gland toxicity after external beam readiotherapy or radionuclide treatment. Moreover, equivalent to prostate cancer, PSMA-ligand uptake suggests the possibility of adjuvant treatment with ^{177}Lu labeled PSMA in selected salivary gland cancer cases [35]. Finally, PSMA PET/CT may also play a future role in the diagnosis and treatment evaluation of benign salivary gland disease such as the sicca syndromes and recurrent plunging ranula.

Conclusions

PSMA PET/CT consistently depicts high tracer uptake in healthy major and minor salivary glands as well as lacrimal and seromucous glands. Minor salivary and seromucous glands can now be selectively visualized on imaging. Potential clinical applications of PSMA-ligand visualized by PET/CT include quantification of present salivary gland tissues and individualization of head and neck cancer treatment by radiotherapy field adaptation and ^{177}Lu-PSMA radionuclide treatment of salivary gland cancers.

Supplementary data related to this article can be found at https://www.oooojournal.net/article/S2212-4403(18)30047-6/fulltext

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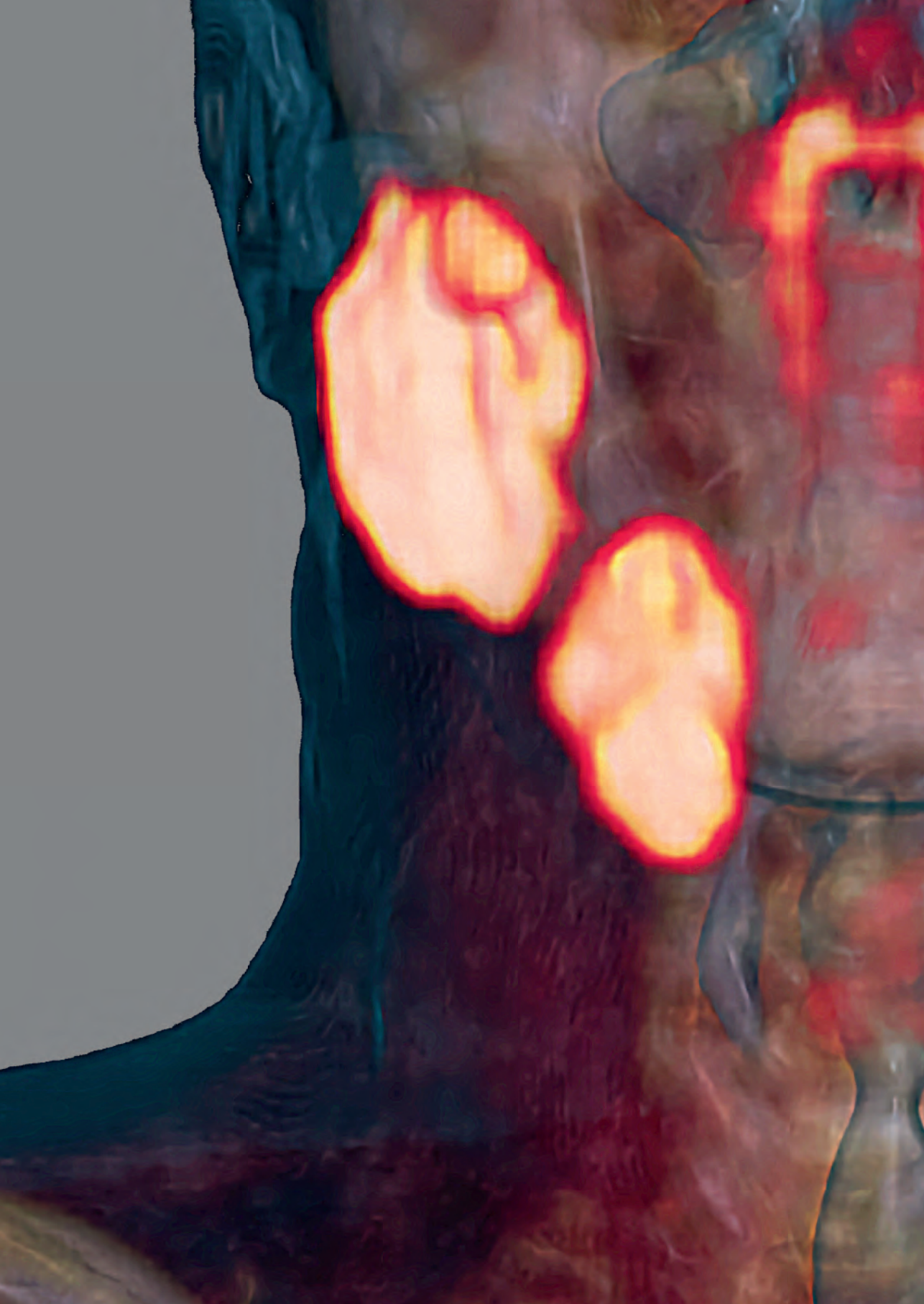

Chapter 7

Prostate specific membrane antigen positron emission tomography/computed tomography imaging as a potential tool to assess and guide salivary gland irradiation

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Abstract

Evaluation of salivary gland damage after head and neck radiotherapy (RT) is difficult with current tools, such as subjective patient-reported outcome measures. We demonstrate the use of prostate-specific membrane antigen positron emission tomography/computed tomography (PSMA PET/CT) as an objective non-invasive tool to visualize damage to salivary glands resulting from RT. In three clinical cases, the PSMA-ligand distribution correlates to the RT dose distribution including intra-gland dose gradients and matches patient-reported toxicity, suggesting a dose-response relation. These findings support further exploration of PSMA PET/CT to guide and evaluate RT, with the ultimate aim to reduce salivary gland toxicity.

Introduction

External beam radiotherapy (RT) can cause damage to salivary glands, resulting in xerostomia and reduction of quality of life [1,2]. Evaluation of glandular function is currently limited to patient-reported outcomes measures, or to laborious and poorly quantifiable procedures such as gland cannulation or planar scintigraphy during stimulated salivation[3]. This limits objective evaluation of toxicity, for example for voxel-based evaluations or anticipated comparison of regular intensity modulated radiotherapy (IMRT) with new modalities like proton treatment.

Based on clinical observations, our hypothesis was that a molecular imaging of the prostate-specific membrane antigen (PSMA) using radiolabelled ligands and positron emission tomography combined with computed tomography (PET/CT) can be used to visualise and quantify viable gland cells in salivary glands. Functional imaging with PSMA PET/CT is generally applied for sensitive and specific (re)staging of prostate carcinoma [4,5]. Normal salivary and seromucous glands have recently been shown to consistently demonstrate high uptake of PSMA-ligand (figure 1) [6,7]. The function of the PSMA epitope in salivary gland tissue has not yet been elucidated, but the expression is reported to be located in secretory glandular (acinar) cells [8]. This theoretically enables sensitive and selective imaging of the presence of acinar gland cells in both major and previously undetectable minor salivary glands, and to quantify loss of these cells in a gland-specific and voxel-based approach [9].

We aimed to identify and introduce PSMA PET/CT as a possible new application to guide radiotherapy, in a first observational report.

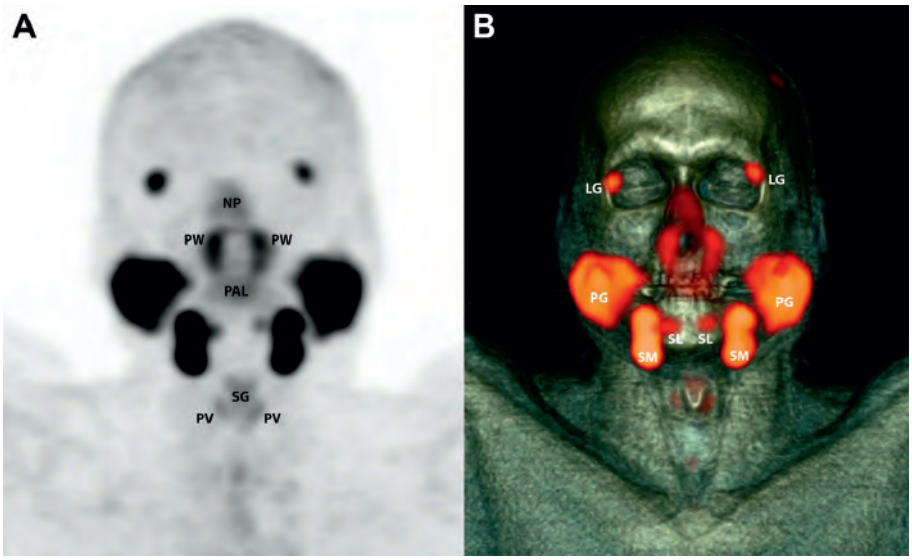

Figure 1: PSMA PET/CT of the head and neck with healthy salivary glands. Coronal maximum intensity projection of normal PSMA distribution in a 60-year-old male with no history of disease in the head and neck, who was scanned for staging of prostate cancer using PET (A) in combination with CT (B). NP = nasal and nasopharyngeal mucosa; PW = posterior pharyngeal wall; PAL = soft palate; SG = supraglottic larynx; PV = plica vocalis; LG = lacrimal gland; PG = parotid gland; SM = submandibular gland; SL = sublingual gland.

Materials and Methods

Three patients who previously received external beam radiotherapy to the head and neck region are presented. These patients were selected and underwent PSMA PET/CT, for a pilot study approved by the local ethical committee. Case A, a 60-year old female with a T3N0 tonsillar carcinoma, was treated with radiotherapy (70Gy) and cisplatin (CCRT). Case B was a 50-year old male treated with CCRT (tumor 70Gy; bilateral neck 54Gy) for a T4N2 nasopharyngeal carcinoma. Case C was a 68-year old male treated for a T3N1 oral cavity carcinoma with CCRT (70Gy, large field involving all macroscopic salivary gland locations except the lips) and a left sided salvage neck dissection.

All patients underwent a PET/CT after intravenous administration of the radiolabelled PSMA-ligand [^{68}Ga]-HBED-CC-Glu-NH-CO-NH-Lys(Ahx), either 100 MBq for whole-body imaging (2 min/bed position) or 50 MBq for the head-neck alone (4 min/bed position). Images were acquired at 45-60 minutes p.i. using a Gemini TOF PET/CT (Philips, Cleveland). The tracer distribution was visually evaluated and compared to the previously irradiated anatomical area

and radiotherapy dose administration. Xerostomia was scored according to the Common Terminology Criteria for Adverse Events version 4 (CTCAE v4).

Results

In case A, PSMA PET/CT showed significantly decreased ligand uptake in the irradiated area, suggesting extensive damage to the left submandibular gland and caudal part of the left parotid gland which received 70 Gy (figure 2). The sublingual glands which received 46 Gy showed partial loss of uptake. All other glands showed normal uptake, with standard uptake values (SUV) comparable to the earlier described values for healthy salivary glands [9]. Clinically there had been grade II xerostomia after treatment, but this had resolved completely at the time of imaging (12 months after RT).

In case B, PET/CT showed loss of PSMA-ligand uptake along the posterior pharyngeal wall glands and in the deep lobe of the left parotid gland, all of which received 70Gy (figure 3). All other gland locations showed normal uptake. Patient reported persistent grade II xerostomia at the time of imaging (11 months after RT).

In case C, PSMA PET/CT showed complete loss of signal in all major and minor salivary gland locations (figure 4), except for notably high uptake in the area of the mucosal lip glands that had not been irradiated. At the time of imaging (240 months after RT) patient reported grade II xerostomia, but with sufficient salivation in the ventral part of the mouth for normal speech and intake and without the need for artificial saliva or other measures.

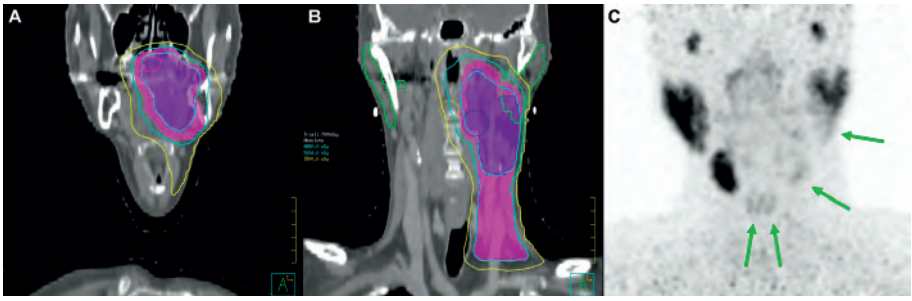

Figure 2: Case A. Coronal slices of radiotherapy dose distribution at the level of the oral cavity (A) and parotid glands (B). Coronal maximum intensity projection of PSMA PET (C), showing the correlation between dose distribution and reduced PSMA uptake in salivary glands: left parotid gland, left submandibular gland, and both sublingual glands (green arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

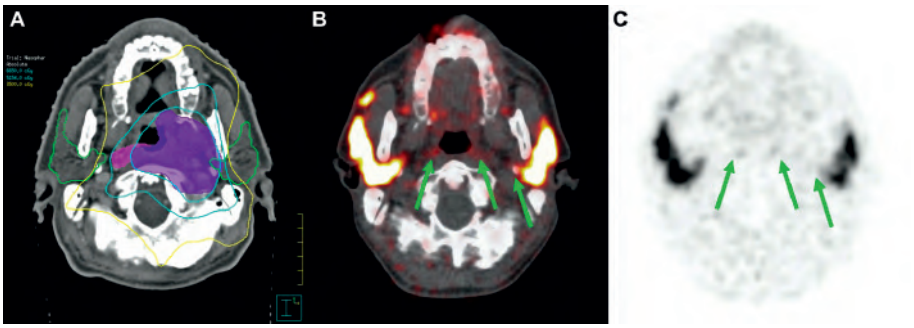

Figure 3: Case B. Transverse slices of radiotherapy dose distribution (A), fused PET/CT (B) and PSMA PET (C) at the level of the parotid and retropharyngeal glands, illustrating complete loss of PSMA uptake in pharyngeal wall glands and in the deep lobe of the left parotid gland (green arrows), which received a high dose.

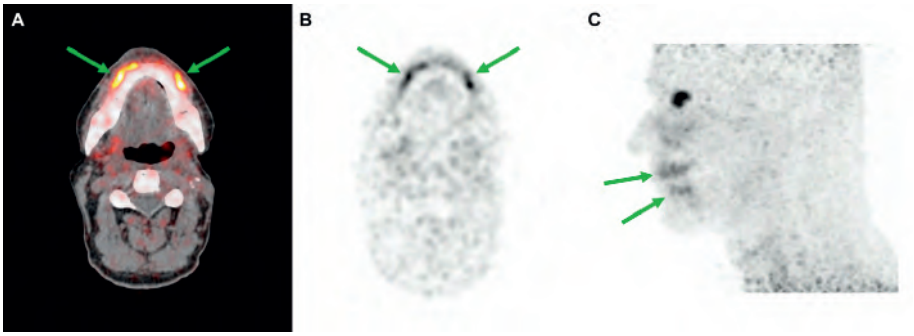

Figure 4: Case C. Transverse slice of fused PET/CT (A), PSMA PET (B), and lateral maximum intensity projection of PSMA PET with indication of prior radiotherapy field in red (C). Complete loss of PSMA uptake is seen in most salivary glands after radiotherapy. Remarkably, PSMA PET indicates hypertrophy of the spared minor salivary glands in the lips (green arrows).

Discussion

In addition to the familiar role of PSMA PET/CT in the assessment of prostate cancer, we propose a new and very different use for this molecular imaging modality. Our clinical findings show that PSMA PET/CT can visualise areas with healthy salivary and seromucous glands after RT, and identify damage by showing specific glands and gland areas with loss of PSMA expression coherent with the dose distribution from prior RT. PSMA PET/CT seemingly detects subclinical damage in case A. The signal gradient over the left parotid gland is in concordance with the dose distribution, and has an inverse relationship between the treatment dose and tracer accumulation. This also indicates a spatial resolution

that is sufficient for interpretation of intra-gland variations. This finding could be elucidated further with prospective research that includes voxel-based evaluations of the dose-effect relation.

In case B, a relationship between clinical symptoms and the distribution of damage in specific gland locations on PSMA PET/CT is suggested. This helps to clarify previously unexplained toxicity in a patient where the major salivary glands were spared and indeed appear to be otherwise unaffected.

The remarkable findings in case C suggest functional compensation by hypertrophy of minor glands of minor glands in the mucosa of the lips on both sides, to explain the residual salivation capacity after complete loss of PSMA-ligand binding in major salivary glands after RT. This finding suggests the relevance of gland locations that are currently not considered in RT planning, especially in case of damage to the major salivary glands, and provides more insight in the functional compensation capacity of minor mucosal gland locations.

The spatial resolution of PET images (generally in the order of 2-5mm FWHM in the head-neck area) allows evaluation of inhomogeneous loss of ligand binding within larger salivary glands. In combination with the high contrast resolution of PET, more and smaller gland locations can be evaluated than with common methods such as planar scintigraphy or functional magnetic resonance imaging (fMRI) [9]. As a result, PET can for the first time allow quantitative assessment of glandular damage using gland-based or voxel-based approaches. As stated before, the expression of PSMA is reported to be located in the secretory glandular (acinar) cells [8]. Although the function of the epitope in the salivary glands is unknown, the level of PSMA-expression per acinar gland is thought to be stable. Therefore, the observed reduction in local PSMA-ligand binding is thought to indicate loss of secretory cells in the affected glands, but this remains to be confirmed in upcoming studies [10]. This method for quantification of cell loss would potentially be more objective and specific than patient-reported outcomes, e.g. independent from factors such as patient experience, medication use, examiner experience etc., and more convenient and potentially better reproducible than salivary gland cannulation or scintigraphy during stimulated salivation. If the suggested relationship can be confirmed, PSMA PET/CT may contribute to the development of new and more accurate dose-effect relations and RT planning

objectives, for the first time with opportunities to derive these per gland subtype and including previously unevaluable gland locations. However, this will require evaluation of potentially interfering factors such as the role of renewed PSMA expression, related to (long term) regeneration of irradiated salivary glands from stem cells.

There are other PET-tracers that accumulate in salivary gland tissues to allow visualisation, with examples including F-18-FDG (metabolic activity), F-18-choline (cell membrane synthesis) or Gallium-68-citrate (non-specific cell activity). However, PSMA-ligands have some unique advantages for this specific application. The uptake in healthy glands is very high, and together with a virtually negative background in all surrounding normal organs this simplifies signal contouring and quantification [9]. But most importantly, histopathological evaluation has proven that PSMA-ligand binds the most relevant cell population, the acinar secretory cells. The distribution of less specific tracers in normal salivary glands is currently not as well understood and is likely to involve other cell types, which brings a risk on signal contamination due to e.g. infiltration with inflammatory cells, and this makes them less suited for measuring salivary gland damage. Tracers for scintigraphy and single photon emission computed tomography (SPECT) imaging like Tc-99m-pertechnetate have a well-understood uptake mechanism, but suffer from low spatial resolution and poor quantification, and they rely on poorly reproducible stimulated salivation to yield a measurable signal. These factors have led to the identification of PSMA PET/CT as a new and promising instrument to quantify specific cell damage in salivary glands, with a better understood biological relation, better spatial resolution, and lower patient burden than currently available alternatives. In conclusion, PSMA PET/CT has the potential to further elucidate the effect of radiation to salivary and seromucous glands. These findings support further exploration of PSMA PET/CT to guide and evaluate RT, with the ultimate aim to reduce salivary gland toxicity.

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Chapter 8

The tubarial salivary glands: A potential new organ at risk for radiotherapy

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Abstract

Introduction: The presence of previously unnoticed bilateral macroscopic salivary gland locations in the human nasopharynx was suspected after visualization by positron emission tomography/computed tomography with prostate-specific membrane antigen ligands (PSMA PET/CT). We aimed to elucidate the characteristics of this unknown entity and their potential clinical implications for radiotherapy.

Materials and methods: The presence and configuration of the PSMA-positive area was evaluated in a retrospective cohort of consecutively scanned patients with prostate or urethral gland cancer (n=100). Morphological and histological characteristics were assessed in a human cadaver study (n=2). The effect of radiotherapy (RT) on salivation and swallowing was retrospectively investigated using prospectively collected clinical data from a cohort of head-neck cancer patients (n=723). With multivariable logistic regression analysis, the association between radiotherapy (RT) dose and xerostomia or dysphagia was evaluated.

Results: All 100 patients demonstrated a demarcated bilateral PSMA-positive area (average length 4 cm). Histology and 3D reconstruction confirmed the presence of PSMA-expressing, predominantly mucous glands with multiple draining ducts, predominantly near the torus tubarius. In the head-neck cancer patients, the mean RT dose to the gland area was significantly associated with physician-rated post-treatment xerostomia and dysphagia ≥ grade 2 at 12 months (0.019/gy, 95%CI 0.005-0.033, p=.007; 0.016/gy, 95%CI 0.001-0.031, p=.036). Follow-up at 24 months had similar results.

Conclusion: The human body contains a pair of previously overlooked and clinically relevant macroscopic salivary gland locations, for which we propose the name tubarial glands. Sparing these glands in patients receiving RT may provide an opportunity to improve their quality of life.

Introduction

The salivary gland system, with its three paired major glands and roughly 1000 minor glands spread throughout the aerodigestive tract submucosa, has been described in detail[1–4]. Its serous, mucous or mixed exocrine acini produce the saliva required for mastication, swallowing, digestion, tasting and dental hygiene. The nearby auditory tube submucosa also contains microscopic seromucous (tubal or Eustachian tube) glands[5]. The recently introduced molecular imaging modality of positron emission tomography/computed tomography with radiolabelled ligands to the prostate-specific membrane antigen (PSMA PET/CT) can visualize these salivary glands with high sensitivity and specificity[6].
Surprisingly, we observed that PSMA PET/CT also depicted an unknown bilateral structure posterior in the nasopharynx, with ligand uptake similar to the known major salivary glands (Fig.1A; Video 1 in the Supplementary material).

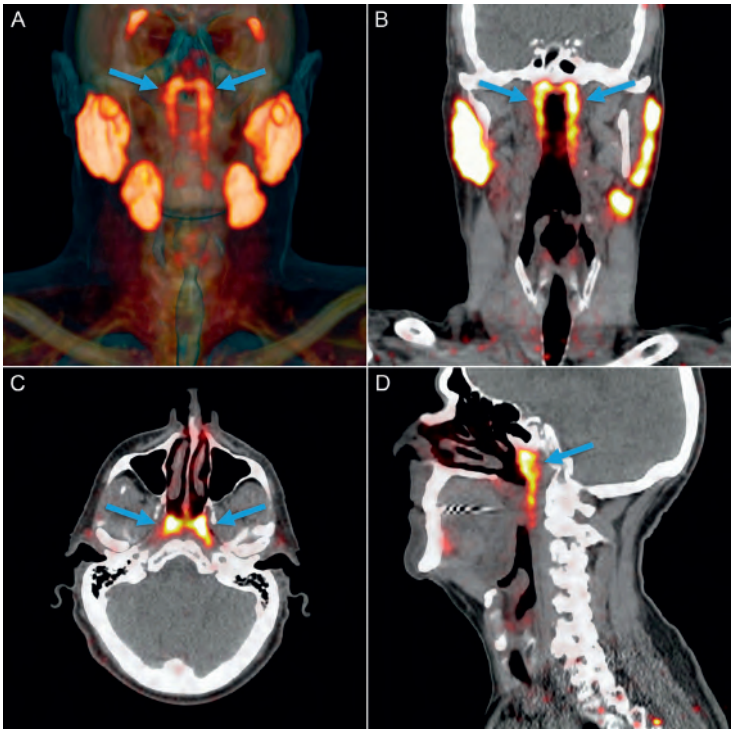

Figure 1: Projections of PSMA PET/CT Overview of the salivary gland tissues as seen on PSMA PET/CT. PSMA PET projected as orange signal on reference CT. The known major salivary glands, which include the parotid, submandibular and sublingual glands, all abundantly express PSMA. An unknown structure in the nasopharynx showed similar imaging characteristics (arrows). A 3-dimensional representation of the PSMA

PET/CT scan is shown in Video 1 in the Supplementary material. Location and extent of the tubarial glands in a random patient. Shown are coronal (B), axial (C) and sagittal (D) slices at the level of the torus tubarius, of PSMA PET projected as yellow signal on grayscale CT. The glandular structure is visible as PSMA-positive tissue (arrows). The coronal slices also show some parts of the parotid and submandibular glands, which demonstrate similar imaging characteristics.

To our knowledge, this structure did not fit prior anatomical descriptions[5,7]. It was hypothesized that it could contain a large number of seromucous acini, with a physiological role for nasopharynx/oropharynx lubrication and swallowing. This could have clinical relevance in oncology, because high-dose external beam radiotherapy (RT) to salivary glands during treatment for head and neck cancer (HNC) or brain metastasis is known to cause damage (toxicity, e.g. interstitial fibrosis, acinar atrophy). This can result in function loss with xerostomia and dysphagia[8,9]. Affected patients experience impaired food intake, digestion, speech problems and increased risk of caries and oral infections, with significant impact on their quality of life[10–12]. The major salivary glands are therefore regarded as organs-at-risk (OAR) and need to be spared when possible. However, since there are no known localized macroscopic glandular structures posterior in the nasopharynx, this area is not included in currently used prediction models for toxicity, nor spared in RT[13,14].

The identification of previously unnoticed salivary gland structures in the posterior nasopharynx could help to explain and avoid radiation-induced side-effects. We performed a comprehensive multi-perspective study with the objective to confirm the presence of this yet unknown glandular entity, and to assess its anatomical and histological characteristics and clinical relevance in RT.

Materials and Methods

Occurrence on molecular imaging

The presence of PSMA-positive tissues in the nasopharynx was evaluated on PSMA PET/CT scans from a retrospective cohort study of 100 consecutively scanned prostate/para-urethral gland cancer patients (from 2017 onwards in NCI and UMCU). Scans were acquired according to routine clinical protocols (Methods 1 in the Supplementary material). The largest cranio-caudal length of the area with uptake was measured on a coronal thick slice and the total tracer uptake in the evaluated region was qualitatively compared with the sublingual glands.

Gland characterization

In a human cadaver study (n=2), the designated area was dissected as 3x3x3cm blocks from bodies retrieved from a body donation program (AUMC, one male, one female). Tissue characteristics were assessed by histochemistry (H&E) and immunohistochemistry (PSMA, alpha-amylase). Immunohistochemistry was performed on a BenchMark Ultra autostainer, Ventana Medical Systems (VMS) (Methods 2 in the Supplementary material). Prostate (for PSMA) and parotid/pancreas (for amylase) samples served as controls. Morphology and anatomical relations were evaluated by 3D-PDF digital reconstruction of anatomy using histological sections (10μm, 1/30). The visibility and anatomical features of the gland area on magnetic resonance imaging (Philips Achieve 3T MRI) were assessed in a healthy volunteer (Methods 3 in the Supplementary material).

Clinical relevance in oncology

The relation of RT dose to the designated area with reported toxicity was evaluated using prospectively collected data from patients treated with curative RT, chemoradiation or bioradiation (accelerated RT+cetuximab) for HNC (UMCG March 2007-June 2016; collected with patient consent in a study approved by the ethical committee; https://clinicaltrials.gov: NCT02435576)[14]. All patients received a CT-scan in a personalized immobilization mask in order to define target volumes (including the primary tumor and lymph node metastasis) and OARs (including the major salivary glands, and swallowing muscles, e.g. pharyngeal constrictor muscle-PCM). This treatment planning aimed to deliver the prescribed dose to the target volumes, while sparing the currently considered OARs. For this study, the location and configuration of the newly detected bilateral gland areas were retrospectively defined as additional OARs by deriving anatomical landmarks from the evaluated PSMA PET/CT and MRI scans, while also considering the cadaver-study findings. The cranial border was defined as the skull base caudally of the sphenoid sinus, and the caudal border as the level of the base of the uvula. The lateral border was defined as the skull base at the cranial side and fatty tissue at the caudal side. The anterior border was the skull base cranially and the dorsolateral pharyngeal wall caudally and the posterior border was the prevertebral long musculature. Delineation of this area was performed on the planning CT-scans of all patients, and its' received radiation dose was determined.

Standardized patient-rated and physician-rated toxicities related to treatment of HNC were prospectively collected at 6, 12, 18 and 24 months after treatment, and were scored using the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire - Head and Neck module (EORTC QLQH&N35) and the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 scores. Multiple imputation was applied to correct for missing data in the follow-up using the MICE package in R[15]. To analyze the association between RT dose to the tubarial gland areas and patient and physician-rated xerostomia and physician-rated dysphagia, multivariable logistic regression analysis was performed to create association models with and without correction for confounding. A two-tailed *P*-value <.05 was considered statistically significant. Baseline toxicity and mean RT dose to the parotid and submandibular glands were considered as confounders for xerostomia, while for dysphagia, additionally mean RT dose to the pharyngeal constrictor muscle was considered.

Results

All 100 consecutive patients (99 male, one female; median age 69.5; range 53-84) demonstrated a clearly demarcated bilateral PSMA-positive area on PSMA PET/CT. It extended from the skull base downward along the posterolateral pharyngeal wall, on the pharyngeal side of the superior pharyngeal constrictor muscle (PCM-superior), with a PSMA-positive mass predominantly overlaying the torus tubarius (Fig.1). The median cranio-caudal length of the detected area was 3.9 cm (range 1.0-5.7 cm). The total tracer uptake in the area of interest determined by visual comparison was on average similar to the uptake in the sublingual glands. This was in line with our earlier quantitative evaluation of tracer uptake in the glands of the head and neck[6]. This was consistently more than the uptake in the palate, which is known to contain a high concentration of minor salivary glands.

The dissected area from human cadavers showed a large aggregate of predominantly mucous gland tissue, with multiple macroscopically visible draining duct openings in the dorsolateral pharyngeal wall (Fig.2). The gland was draped primarily over the torus tubarius, the anatomical structure formed by the cartilage that supports the entrance of the auditory tube. The gland extended caudally to the pharyngeal wall and cranially to Rosenmüllers' fossa. The gland cells showed almost 100%

cytoplasmic expression of PSMA with a luminal preference, comparable to the mucous aspect and PSMA-ligand uptake of minor salivary glands in the palate (Fig.3; Fig.1 in the Supplementary material). There was no amylase expression in the gland cells consistent with very low numbers of serous acini, similar to the sublingual glands. The 3D histology reconstruction illustrates the anatomical distribution of glandular tissue and draining ducts (Fig.4; interactive 3D-PDF Fig.2 in the Supplementary material). MRI images of a healthy volunteer showed a subtle tissue structure with lower signal intensity on the T2 sequence, compatible with glandular tissue, was identified at the expected location of the tubarial gland on the medial side of the torus tubarius (Fig.3 in the Supplementary material). Small T2-intense dots were present within this tissue structure, which may represent the macroscopic duct openings seen in the cadavers and 3D histology reconstruction.

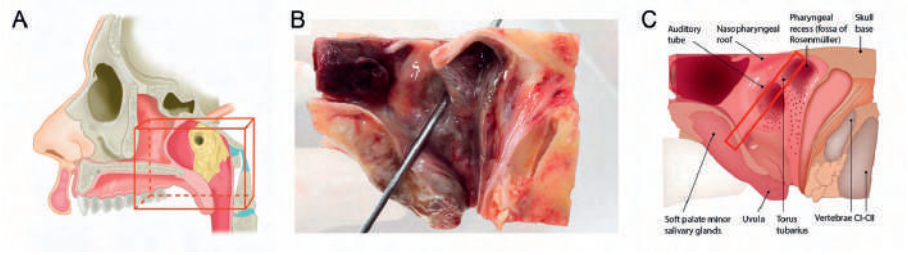

Figure 2: Anatomy of the torus tubarius area Macroscopic views of the torus tubarius area. Global anatomical overview with the area of interest in yellow and dissection planes in red (A) with aligned dissection specimen of the right nasopharynx (B) and annotated graphical overview (C).

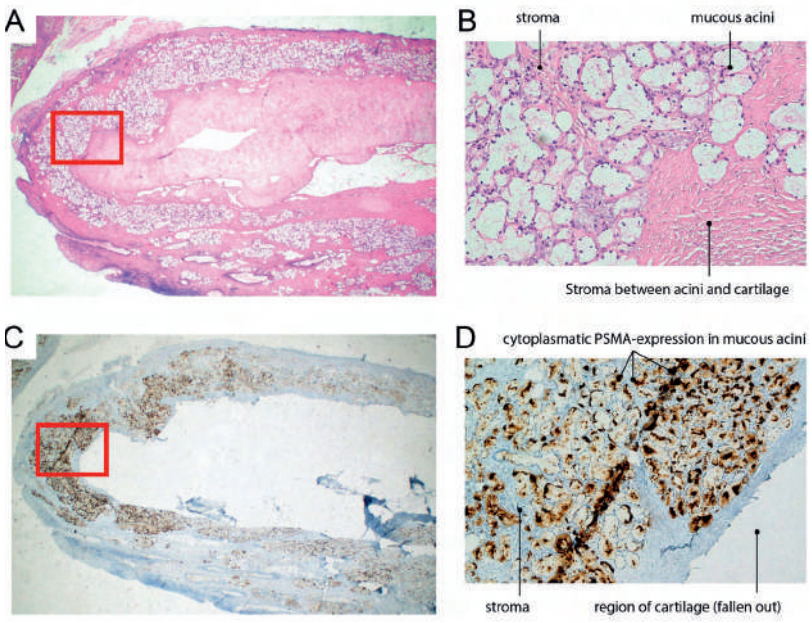

Figure 3: Torus tubarius with overlying gland histology and immunohistochemistry Torus tubarius on HE-staining and PSMA-Immunohistochemistry. Histological slides showing the dorsolateral nasopharynx at the level of the torus tubarius (A, 25x) with detail showing an annotated HE-staining (B, 100x). Correlating PSMA staining (C, 25x) with annotated detail showing gland cells covering the cartilage and with the densest collection of mucous PSMA-positive acini overlying the torus tubarius (D, 100x). For an overview with lower magnification see Fig.1 in the Supplementary material.

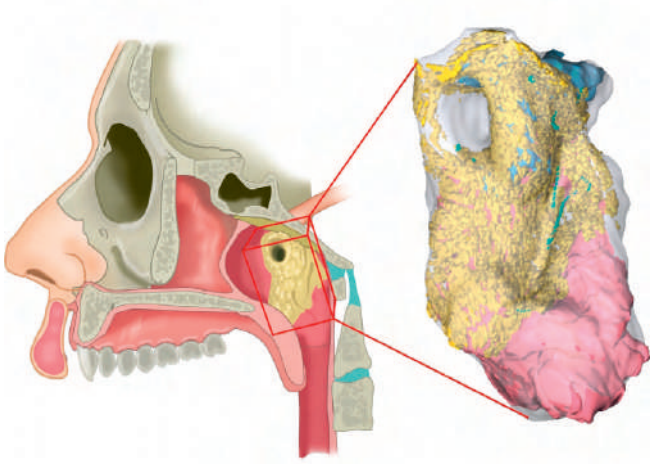

Figure 4: Interactive 3D-reconstruction of histological slides. Schematic representation of region of the torus tubarius with overlying gland, in its anatomical setting (left) and as 3D-histology reconstruction (right). The glandular tissue is shown in yellow (acini) and light blue (ducts). This dorsomedial view, demonstrates the relation of the tubarial gland to the underlying torus tubarius cartilage (dark blue) and muscle (pink). An interactive 3D-PDF with the full reconstruction data can be found in Fig.2 in the Supplementary material.

The characteristics of the 723 evaluable respondents (initial cohort 750), included in the clinical study, are listed in Table 1 in the Supplementary material. The derived gland OAR delineations are illustrated in an anatomical atlas (Fig.4 in the Supplementary material). The RT dose distribution and delineation of the new gland are shown in Fig.5 for an example patient.

For crude physician-rated toxicity, the mean RT dose to the new glands was associated at all time points with ≥ grade 2 xerostomia (oral intake alterations, e.g. copious water, other lubricants, diet limited to purees and/or soft, moist foods), and with ≥ grade 2 dysphagia (symptomatic and altered eating/swallowing) (Table 1 and Table 2 in the Supplementary material). After correction for confounding factors, this association was reduced but still significant at 12- and 24-months. In other words, the effect of RT on dysphagia and xerostomia was explained by RT dose in multiple organs at risk, among which the new glands. However, independently from the dose to the parotid glands as the most relevant OAR, an increase in RT dose to the new glands lead to an increase in toxicity risk (normal tissue complication probability, NTCP) (Fig.5 in the Supplementary material). For patient-rated xerostomia, the crude association between mean RT dose to the new glands and moderate to severe toxicity was present at all time points, but was reduced and no longer significant after correction for confounding.

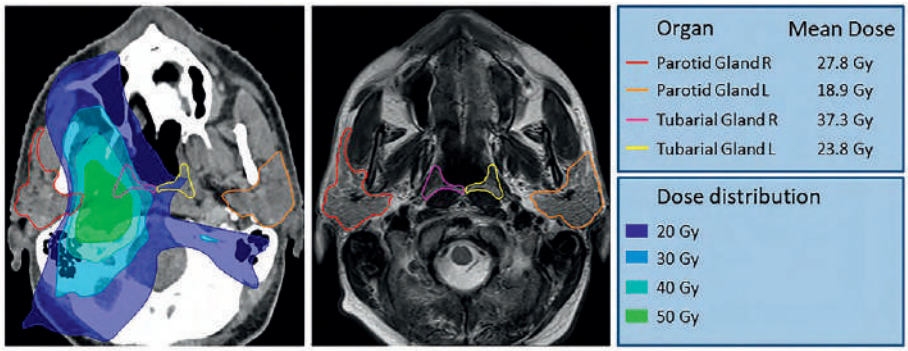

Figure 5: Radiotherapy evaluation of region of the torus tubarius with overlying gland Evaluation of radiotherapy dose to the newly described glands overlying the torus tubarius: Axial CT-slice with projected radiation dose distribution as color-wash (left) and MR-slice (right, 3-Tesla, T2-dixon with gadolinium) of a 53-year old patient who received radiotherapy for cT3N1M0 oropharyngeal cancer.

Table 1: Associations of toxicity and mean dose to the new glands at 12 and 24 months Associations of xerostomia and dysphagia with mean dose to newly described glands overlying the torus tubarius. The mean RT dose was associated with grade 2 or higher physician-rated xerostomia and dysphagia at 12 and 24 months post-treatment (For association data including 6 and 18 months, see Table 2 in the Supplementary material). The effect was independent of baseline toxicity and still significant after correction for RT dose in the surrounding OARs that were considered as confounders, i.e., the crude effect was explained by RT dose in multiple OARs among which the new glands had a significant association. The crude association between mean RT dose to the new glands and moderate to severe *patient-rated* xerostomia was present at all time points, but these associations were reduced and not significant after correction for confounders. PAR=parotid; SM=submandibular gland; BL=baseline xerostomia/dysphagia; PCM=pharyngeal constrictor muscle.

	12 Month			24 Month		
	Coef. (gy^{-1})	95% CI	p	Coef. (gy^{-1})	95% CI	P
Physician rated Xerostomia (≥ grade 2)						
Crude	0.047	0.037-0.057	<.001	0.043	0.033-0.053	<.001
Corrected (PAR, SM, BL)	0.019	0.005-0.033	.007	0.018	0.003-0.033	.02
Physician rated Dysphagia (≥ grade 2)						
Crude	0.041	0.033-0.049	<.001	0.038	0.030-0.046	<.001
Corrected (PAR, SM, PCM, BL)	0.016	0.001-0.031	.04	0.02	0.006-0.034	.006
Patient rated Xerostomia (moderate to severe)						
Crude	0.025	0.018-0.032	<.001	0.022	0.013-0.031	<.001
Corrected (PAR, SM, BL)	0.001	-0.011-0.013	.88	0.001	-0.014-0.016	.9

Discussion

To our knowledge, this is the first description of paired macroscopic (sero) mucous gland locations in the human posterolateral nasopharyngeal wall, and an indication of their clinical relevance in RT for HNC. Based on its predominant location over the torus tubarius, we propose the name "tubarial glands". These gland locations were present as macroscopic structures in the PSMA PET/CT scans of all 100 studied individuals, and in two investigated cadavers (one of each gender). Microscopically, they indeed showed salivary gland tissue, highly concentrated bilaterally near the torus tubarius, with macroscopically visible draining duct openings towards the nasopharyngeal wall. High-dose RT to this area lead to significant clinical toxicity. These findings support the identification of the tubarial glands as a new anatomical and functional entity, representing a part of the salivary gland system[3,4,7].

Interpretation of findings

Several factors can explain why these glands have not been noticed previously as macroscopic gland locations. The occurrence of acinar cell groups in the nasopharynx has been reported, but in a spread out pattern in a large region instead of localized tissue in an organized clustered glandular structure[3]. The newly detected tubarial glands involve flat submucosal glandular structures at a poorly accessible anatomical location under the skull base, an area that can only be visualized using nasal endoscopy. The macroscopically visible excretory duct openings may have been noticed, but have not been interpreted as part of a larger gland. Conventional imaging modalities (ultrasound, CT, MRI) have never allowed visualization of this submucosal structure and interpretation as a salivary gland, although an indication of its presence may have been visible in various prior functional imaging modalities upon retrospective evaluation [16]. Modern multiparametric MRI imaging could only be used to identify the tissue compartment containing the gland, after analyzing the information provided by PSMA-PET/CT and histology. As a result of these coinciding factors, the discovery depended on the introduction of molecular imaging with radiolabeled PSMA-ligands. This provided the required high sensitivity and specificity for detection of salivary gland cells, with a very high contrast-ratio relative to the surrounding PSMA-negative tissues. In combination with 3D anatomical reconstruction of histological information, this allowed us to realize that these cells in fact form distinctive macroscopic gland locations.

Based on the described anatomical and histological characteristics, and on the demonstrated relation between high-dose RT to the tubarial gland regions and toxicity (xerostomia and dysphagia), we assume the physiological function of the tubarial glands is the moistening and lubrication of the nasopharynx and oropharynx. Although this interpretation of physiology requires confirmation with additional research, it does suggest an opportunity for sparing in RT for patients treated for HNC to avoid toxicity.

One could question whether the tubarial glands should be considered as separate organs, and as major or minor salivary gland locations. However, we think these qualification systems may not be suited and relevant to interpret and appreciate this finding. The accepted definition of an organ is that it consists of more than one kind of tissue, with a definite shape and structure, and performs specific tasks[17].

Indeed, the cadaver study confirmed the presence of a defined structure containing acini and draining ducts, and the association of xerostomia and dysphagia with RT dose in the clinical cohort indicated a specific function that can be disrupted. When compared to the known major salivary glands, the tubarial glands had the most similarities with the sublingual glands based on the predominant mucous acini (hence the negative amylase staining), similar PSMA-ligand uptake, and the presence of multiple draining ducts[6,18]. It can be argued that the tubarial glands do not have a capsule as opposed to the major salivary glands, however the sublingual glands also have an unencapsulated part that consists of 8-30 minor mixed glands[19]. Additionally, the type and frequency of salivary gland tumors that occur in the nasopharynx and sublingual glands seem to be similar, with rare occurrences of benign tumors and adenoid cystic carcinoma as the most frequent malignant tumor[20–26]. As a consequence of these considerations, our interpretation of the tubarial glands could be guided by the classification that is applied to the sublingual glands, and they would qualify as a fourth pair of major salivary glands. On the other hand, the tubarial glands have many similarities with the palatal conglomerate of microscopic glands, which are classified as minor salivary glands. Based on the overlapping spectrum of their characteristics it could be argued that all salivary glands together could be interpreted as a continuum, formed by smaller and larger collections of acini that together form a salivary gland system. In this approach, the tubarial glands should not be classified as separate organs or as minor or major salivary glands, and can better be interpreted as macroscopic parts of the composite salivary gland organ system.

Regardless of the classification of the tubarial glands as either a conglomerate of minor glands, a major gland, a separate organ, or as a new part of an organ system, the tubarial glands are macroscopic glandular tissue locations with clinical relevance. Therefore, they require a name that allows unique identification in daily clinical practice. The proposed name tubarial glands is based on their anatomical location, in coherence with the naming strategy for the other macroscopic salivary glands (parotid, submandibular, sublingual). This also prevents confusion with the microscopic tubal glands lining the auditory tube. Other names that were considered included Eustachian or Rosenmüllers' glands, but these did not optimally match the anatomical location, and eponymous medical nomenclature is no longer considered desirable.

Strengths and Limitations

The clinical relevance of the tubarial glands was derived from a retrospective evaluation of multiple confounding OARs. Since all salivary glands are situated closely together, they often receive a comparable RT dose in treatment for HNC. As an example, in our data the dose to the parotid glands was highly correlated with the tubarial gland dose, with a correlation coefficient of 0.84. This means that reported xerostomia and dysphagia caused by RT dose to the tubarial glands, also includes a toxicity effect caused by dose to the parotid glands. This phenomenon in statistics, referred to as multicollinearity, complicates measuring a difference in toxicity effects caused by RT to different glands. The same applies to several muscles involved in swallowing in relation to dysphagia. Therefore, a large cohort was required to be able to measure such effects of RT dose to the tubarial glands, after correction for known toxicity effects to adjacent structures. External validity is likely to be warranted by this cohort size and inclusion of unselected consecutive patients. Regarding the internal validity, depending on the time point, 9-37% of the toxicity data are missing (complete) at random (MCAR or MAR) due to death, recurrence, or limited follow-up. A high compliance rate of (88-91%) ensures a low probability of data missing not at random (MNAR).

The association between RT dose to the tubarial glands and crude physician-rated toxicities was present at all time points ($p<.05$). After correction for confounders this association was still present for xerostomia at 12 and 24 months and for dysphagia at 6, 12 and 24 months. The fact that these association after correction were not significant at all four time points can probably be explained by multicollinearity. Similarly, radiation to the oral cavity (which also includes palatal minor glands) might play a role, although RT to this area was not a significantly contributing factor in currently used prediction models[13,14]. More extensive and comprehensive modelling is expected to clarify the influence of multicollinearity. The absence of a significant correlation with patient-rated xerostomia after correction for confounders, may be explained by the inherently higher variability in subjective evaluations of toxicity by patients.

The logical next step seems to be optimization of radiotherapy fields to the tubarial glands as new OARs. Since the PCM superior is close to the tubarial glands, sparing both structures simultaneously seems attractive. Still, we prefer to acquire external validation in an independent dataset, and advise to change clinical protocols

only in the setting of continued monitoring of the anticipated clinical benefits. Also, a prospective confirmation of concordance between the tubarial glands as visualised on PSMA PET/CT, with the delineation based on anatomical landmarks is desirable, prior to clinical implementation as a relevant AOR.

We conclude that the human nasopharynx contains previously overlooked bilateral macroscopic salivary glands. Our findings indicate that sparing these tubarial glands could provide an opportunity to prevent side effects from radiotherapy and better maintain patients' quality of life.

Acknowledgements

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Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2020.09.034

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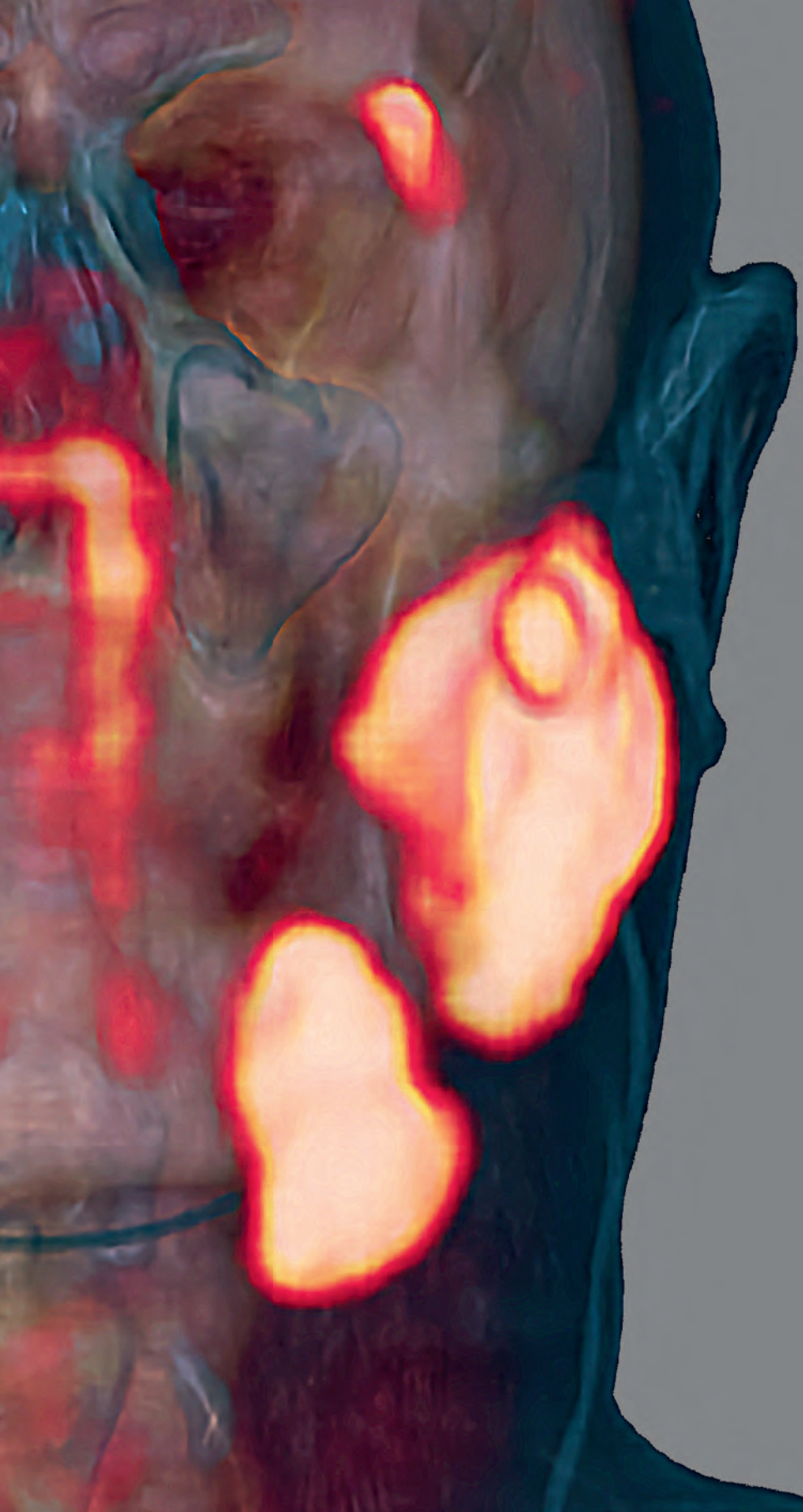

Chapter 9

The tubarial glands paper: A starting point. A reply to comments

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Radiotherapy and Oncology 2021; 154: 308-311.

Introduction

The findings in Chapter 9 led to widespread international media attention which helped in generating reactions in the form of letters-to-the-editor with relevant comments and interpretations of our findings [1,2]. Our response below was published as a letter too.

Dear Editor,

We thank the authors of the letters for their constructive comments and thereby their contribution to the interpretation of the glandular structure as described in the tubarial gland paper and the clinical consequences of its presence [3]. Their comments will be discussed by topic in this summarized reply. Our reply to the comments in a point-by-point response to each letter is provided in the Supplemental material.

The glands clustered in the mucosa of the auditory tube and regions adjacent to the tubarius torus have been earlier described by anatomists

The words "new" and "organ" in the title seem to have been a trigger for massive media attention[4]. This might have caused some distraction from the core message of the manuscript. We therefore thank Mudry and Jackler for their view on the focus of our paper: "reclassification of a well-known anatomical feature rather than a striking discovery of an unrecognized structure somehow overlooked by generations of anatomists", and its possibly important role in preserving salivary gland function in radiotherapy for head and neck cancer[5,6]. It is indeed important to emphasize that clusters or a high density of seromucous glands in this area have been described before. In the writing of the original paper, we realized as authors that we were standing on the shoulders of giants in anatomy. For that reason, we reviewed commonly used anatomical atlases and available papers in English and non-English from the past two centuries. Some (but not all) of these giants were mentioned in the references list of our paper and they referred to the even older papers of e.g. Moos, in German, dating back to 1874[5–7]. We thank the authors of the letter for adding more and even older references and anatomical interpretations.

The core of our message is the fact that the gland tissue in this area has not been considered as an organ at risk (OAR) in radiotherapy. One should realize that an OAR (as mentioned in the original title) is a radiotherapy term and does not only concern organs, but also comprises all tissues near the clinical target volume that can get damaged by radiation. In the original paper, we thus did not claim to have discovered a new organ, as suggested by Cohen Goldemberg et al., Narayan et al. and Schumann, but introduced a potential new organ at risk[8–

10]. The new techniques of PSMA PET/CT and 3D reconstruction of histological slides that were presented in the paper, opened a new perspective that did not fit previous anatomical descriptions. To our knowledge, there has not been a prior interpretation of the gland tissue in this area as a localized and organized macroscopic glandular structure, similar to one of the major salivary glands (the sublingual gland). Earlier contributions by colleagues to the knowledge of the embryology and anatomy of the nasopharynx fit into this new perspective, but in our opinion, it is not the other way around. Additionally, anatomical knowledge of the area had until now not been translated into clinical consequences, regarding the evaluation of the potential role as an OAR.

Therefore, this bilateral gland structure that we named "the tubarial glands" can be regarded as a potential OAR. Earlier, this realization led to our evaluation of toxicity in a cohort of 723 head and neck cancer patients, in which that theory was confirmed. As far as the anatomical classification is concerned, we took a rather neutral position on the interpretation of findings and wrote in the discussion section: "Regardless of the classification of the tubarial glands as either a conglomerate of minor glands, a major gland, a separate organ, or as a new part of an organ system, the tubarial glands are macroscopic gland tissue locations with clinical relevance". We thank Nascimento et al., Iwanaga et al., Mudry et al., Cohen Goldemberg et al., Narayan et al. and Schumann for their comments regarding the aspects mentioned above [4,8–12].

It is too early to call this gland a salivary gland

We thank Bikker and Vissink for their constructive input concerning the interpretation of the glandular structure that was described in the tubarial gland paper [3,13]. They argue that it is too early to call this gland a salivary gland for several reasons.

First, the question is raised whether the acinar cells actually produce saliva, as the authors of the letter seem to have set this as a criterium for the glands to be called salivary glands. The problem in answering that question, is that saliva is not strictly defined, as all fluids that contribute to the fluid in the oral cavity together are called saliva. These fluids are produced in the major (parotid, submandibular and sublingual) and minor (spread out microscopic) salivary glands and gingival crevice and have widely ranging compositions [14]. As an example, the amylase concentration varies by

tenfold between the parotid and sublingual gland and the uncontaminated crevicular fluid (formed by serum and locally generated materials) contains no amylase [14–16] . Therefore, in our opinion, the uninvestigated fluid composition argument to call the tubarial glands salivary glands does not seem to hold.

Second, Bikker and Vissink argue that the tubarial glands do not contribute to the oral fluid, but they do not provide evidence for this statement. In addition, one could question this criterium for calling the tubarial glands actual salivary glands. Functional and histological characteristics rather than location of the gland or its fluid, should determine whether it can be considered a salivary gland. However, we have been able to perform a simple in vivo pilot study (n = 1) in a healthy volunteer, in which 1 ml of blue dye in thickened slimy saline was put directly next to the tubarial gland on the cranial side of the soft palate. This subsequently led to staining of the pharyngeal wall, but also of the palatopharyngeal arch, which is the dorsal border of the oral cavity (Fig. 2). Based on this finding, one could say it is not unthinkable that the excreted fluid from the tubarial glands reaches the oral cavity. If this contribution to the oral cavity fluid is used as a criterium for saliva, the fluid from the tubarial glands might thus be considered as saliva.

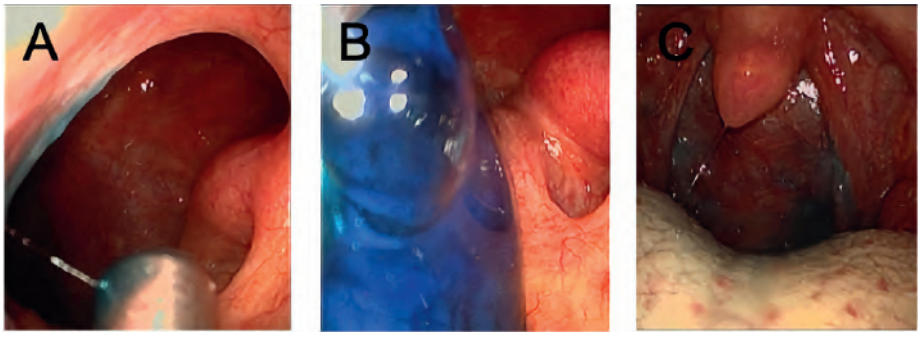

Figure 2: Torus tubarius (A). Thickened saline with blue dye applied right next to the tubarial gland area (B). Blue dye on the palatopharyngeal arch, which is part of the oral cavity (C).

Third, the authors of the letter mention the absence of a main excretory draining duct as an argument against the categorization as a major gland, but they at the same time admit that this is not really an argument, because part of the sublingual gland has similar ducts as the tubarial glands. The relativity of this argument was already discussed in the original paper. Additionally, the excretory duct openings of the tubarial glands are macroscopically visible as small holes filled with a secrete

(see Fig. 2B and 2C in the original paper), in contrast to and larger than labial duct openings.

Finally, Bikker and Vissink mention that salivary glands are known to secrete amylase. This was shown by Veerman et al. who investigated sectreted saliva that was collected from the parotid, submandibular and sublingual glands [14] . We investigated tubarial gland cells by immunohistochemistry (IHC), instead of collecting excreted fluid because of the risk of contamination by saliva of other glands. Our findings by IHC showed that amylase was expressed in neither the tubarial, nor the palatal and sublingual glands, in contrast to expression in the parotid gland (Fig. 3). Since these three gland locations thus did not differ using this sensitive method, there was no urgent reason to measure other proteins or enzymes as is suggested by the authors of the letter. However, an Alcian blue staining was performed for the 3D reconstruction slides, showing presence of mucin in the gland cells of the pharyngeal wall next to the auditory tube (Fig. 4). Further characterization of the tubarial glands by its protein excretion profile and stimuli for excretion as suggested, is however thankfully welcomed to provide more insight. The original authors invite the authors of the letter to do so in a constructive cooperation.

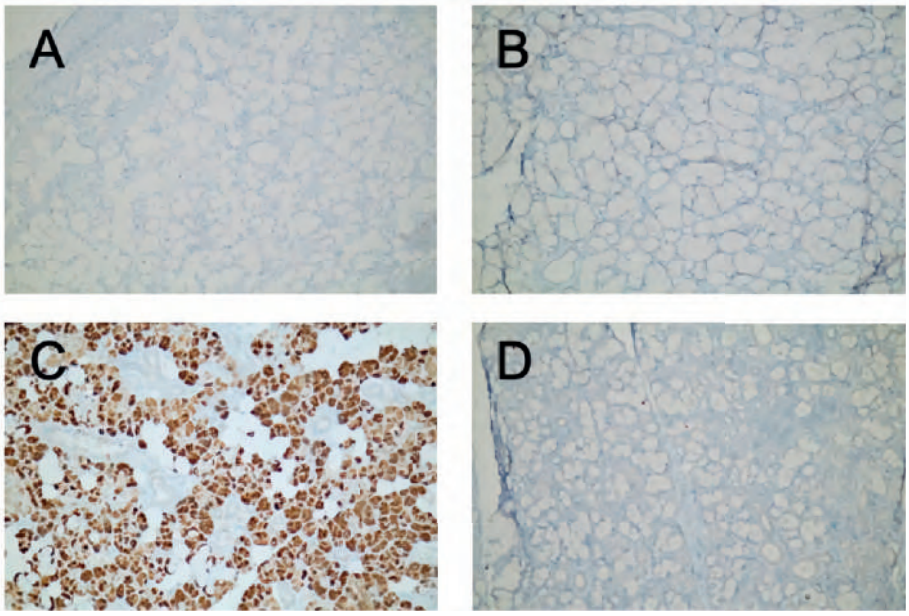

Figure 3: Amylase immunohistochemistry: tubarial gland (A), palatal glands (B), parotid gland (C), sublingual gland (D).

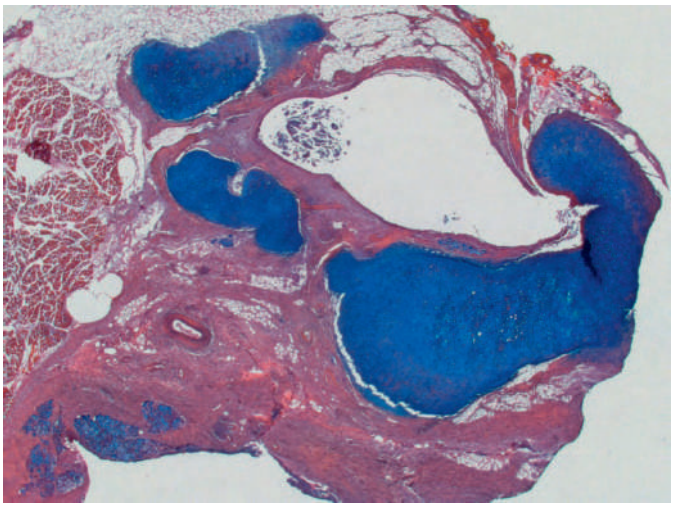

Figure 4: Alcian blue staining of the auditory tube deep to the tubarial gland, showing mucine in the glands of the pharyngeal wall, near/possibly as part of the tubarial gland.

If the newly described gland structures have a similar PSMA-expression profile, similar histology as the sublingual glands, with a similar amylase absence on IHC, similar multiple draining ducts, and a similar profile of benign and malignant tumors, according to the WHO International Classification of Diseases for Oncology (ICD-O), one could say there are sufficient arguments to call these glands salivary glands. But we do agree that further characterization of the tubarial gland fluid and its course downwards through the pharynx and over the dorsal tongue would provide more insight in its function and contribution to the saliva in the oral cavity.

In conclusion, we think that the arguments in favor of a classification of the tubarial glands as salivary glands outperform the arguments against it. We again emphasize the fact that the major/minor gland classification system has its limitations. However, we thankfully endorse the suggestions that were made for further characterization of this newly interpreted part of the salivary glands system, not only for academical reasons, but also to better understand the potential benefit for head and neck cancer patients.

No convincing evidence for the presence of tubarial salivary glands

We thank Iwanaga et al. for their interpretation and comments regarding the presented evidence [12]. They state that the existence of the tubarial salivary glands is not proven for three reasons. First, PSMA PET/CT does not prove existence of a salivary gland. Second, histology does not prove existence of a salivary gland. Finally, it is anatomically incorrect to say this represents a newly discovered salivary gland.

This interpretation is not in agreement with statements in the original paper, as we show in the point-by-point response in the Supplementary material We thank Narayan et al. for forth bringing their interpretation of our paper [14]. Similar to the points mentioned earlier in this response to the letters, it interestingly shows that important nuances in the discussion section regarding interpretation of the findings by the original authors, seem not to have been noted by Narayan et al. Also, the radiotherapy term organ at risk (OAR) seems to have been incorrectly interpreted as the anatomical term "organ". These misunderstandings might have resulted from the monodisciplinary anatomical background of the authors of the letter as in contrast to the multidisciplinary (7 specialty) team of authors of the original paper. The suggestions for further characterization are, however, thankfully taken. As is said in the title of our response, the original paper is a starting point.

The study design created considerable gender imbalance, with possible clinical consequences

We thank the authors Ellsworth et al. for their remarks on gender imbalance. We should have realized and mentioned these concerns in the limitations section [17] . To provide more insight in the female tubarial glands, we have now been able to examine six additional female patients that were scanned with PSMA PET/CT for another study. We can report that all scanned women had the expected visual indications of tubarial glands, with a median cranio-caudal length of 3.3 cm (range 2.2–4.6 cm). This number is too low for an actual statistical comparison between the sexes, but it is comparable to the length of the gland in the 99 men in the paper: median 3.9 cm (range 1.0–5.7 cm).

There are more clinical concerns than only in radiotherapy

We thank Thakar et al. for their contribution regarding the clinical implications of the findings reported in our paper [18]. The realization of the presence of these relatively large glandular structures might indeed have clinical consequences in more (aspects of) diseases than we described in our paper. They report diagnostic concerns in juvenile nasal angiofibroma (JNA) evaluated by PSMA PET/CT. Also, pathological concerns are mentioned in diagnosing tumors in the nasopharynx that should take the tubarial glands into account. But perhaps most importantly regarding our paper, they emphasize the relevance for head and neck radiotherapy and suggest routinely implementation as an organ at risk. The authors of the original paper made recommendations regarding implementation: "We prefer to acquire external validation in an independent dataset and advise to change clinical protocols only in the setting of continued monitoring of anticipated clinical benefits". For this reason, we referred to our original paper as "a starting point" in the title of this reply to the comments in 8 letters-to-the-editor.

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Appendix A Supplementary data

Supplementary data to this article are available online at https://doi.org/10.1016/j.radonc.2020.12.001

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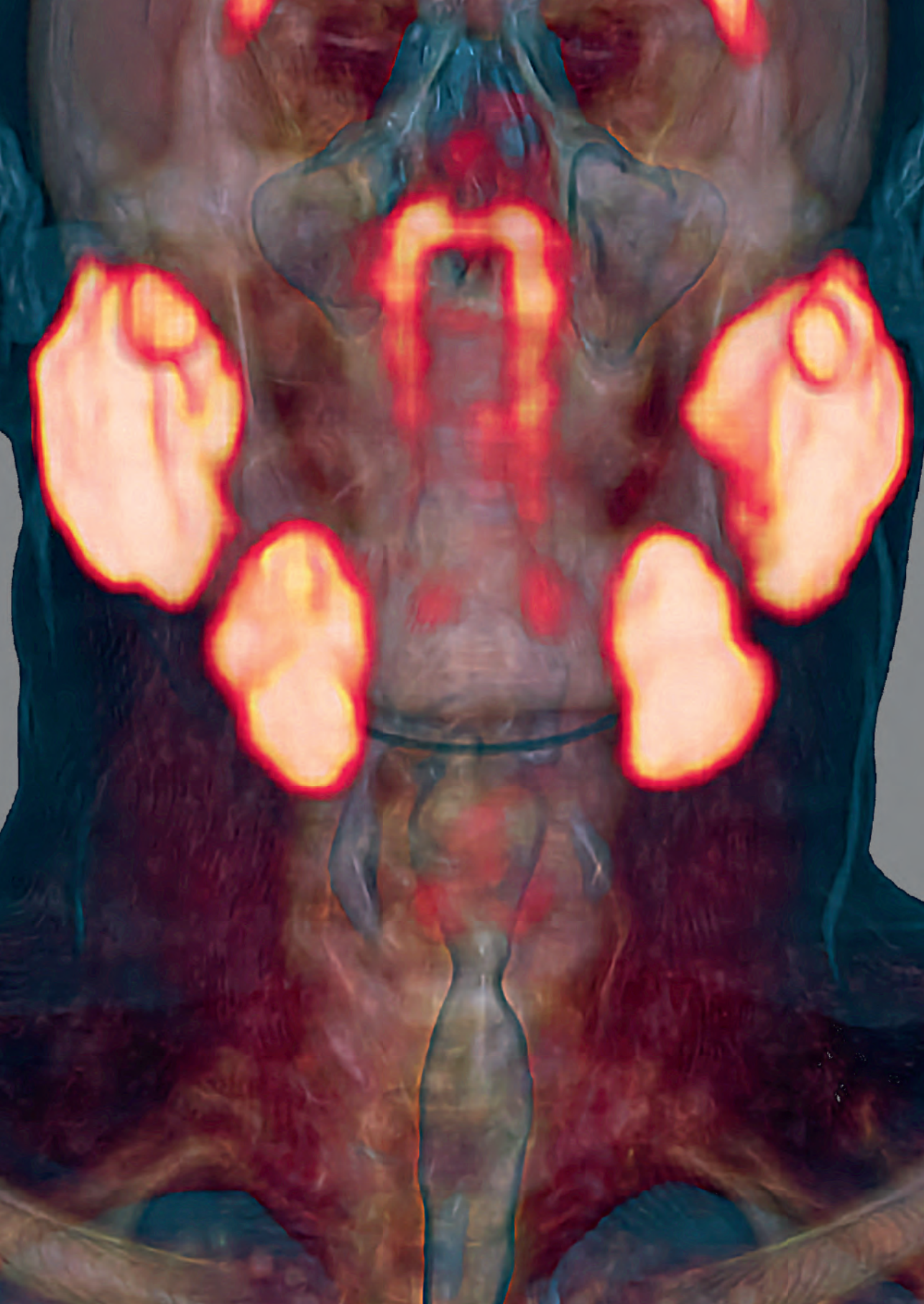

Chapter 10

English summary

New epidemiological and PSMA-expression based insights in salivary gland tumors

Chapter 1:

Humans have three pairs of major salivary glands and about a thousand minor glands spread out in the airways and digestive tract. These glands produce saliva which is important for several biological and mechanical functions. Saliva helps in digestion, moistening and lubrication of food, prevention of dental decay and wear, killing of microorganisms such as bacteria and viruses, protection against chemical agents and dehydration. It also plays a role in hemostasis, and woundhealing.

The salivary glands contain many other anatomical structures such as lymph nodes and lymph vessels, blood vessels and nerves. The facial nerve, which controls the muscles of facial expression and runs through the parotid can make surgery of particularly this gland challenging.

Salivary gland tumors are rare tumors. In around 85% these tumors are benign, but malignant salivary tumors also occur. The most frequent salivary gland tumor is the salivary gland pleomorphic adenoma (SGPA). This tumor has a diversity of morphological presentations with a varying combination of different cell types (epithelial, myoepithelial and mesenchymal). The SGPA sometimes turns into a malignant tumor, called the carcinoma ex pleomorphic adenoma (CEPA) despite is benign background. The malignant salivary gland tumors have a surprising wide range of histological presentations. The 2017 classification of the world health organization (WHO) mentions 20-25 different primary salivary gland cancers. These tumors have malignant characteristics such as invasive growth, mitotic figures and necrosis and genetic mutations.

Conventional imaging modalities (ultrasound, CT, MRI) show the major salivary glands in various detail. In tumor diagnostics, MRI depicts tumors best. However, these modalities have never allowed visualization of minor salivary glands. The salivary glands are now visible on nuclear scans with prostate specific membrane antigen for patients with prostate cancer (PSMA PET/CT), although possibilities for head and neck cancer have until recently not had any attention.

Because of the interesting nature of salivary glands and salivary gland tumors, this thesis will explore a main topic in both fields: epidemiology of salivary gland tumors and imaging of the salivary glands by PSMA PET/CT.

-Part 1-

Epidemiology of salivary gland tumors

Chapter 2:

Whereas salivary gland pleomorphic adenoma (SGPA) is the most common type of salivary gland tumor, relatively little was known about its epidemiology because national cancer registries do not register this disease. Therefore, our objective was to establish pleomorphic adenoma incidence trends, rates of secondary malignant transformation and recurrence and associated factors in the Netherlands. Data on incidence, epidemiology, secondary malignant transformation and recurrence were retrieved from the Dutch pathology registry (PALGA) for the years 1992, 1997, 2002, 2007, and 2012. Multivariate analysis was performed to discover the risk factors for recurrence. This resulted in 3,506 recorded pleomorphic adenoma cases, implying an overall European standardized rate of 4.2-4.9 per 100,000 person-years. Our figures showed a female preponderance (1:1.43) with an annual 1% rise in female incidence (95% confidence interval [CI]: 0.2-1.8) and a bimodal age distribution in women ($p<0.0001$). The overall 20-year recurrence rate was 6.7%, and median time to first recurrence was 7 years. Positive and uncertain resection margins and younger age at diagnosis were risk factors for recurrence, with odds ratios (ORs) of 4.62 (95%CI 2.84-7.51), 4.08 (95%CI 2.24-7.43), and 0.42 (95%CI 0.29-0.63) respectively. Tumor locations in the minor salivary glands had lower odds of recurrence than tumors in the parotid (OR 0.24; 95% CI: 0.07-0.77; $p<0.016$). Malignant transformation occurred in 0.15% of SGPAs (3.2% of recurrences).

In conclusion, this first nationwide study clearly showed sex differences in pleomorphic epidemiology, suggesting an underlying hormonal mechanism. Long-term recurrence risks were low, and secondary malignant transformation risks were very low.

Chapter 3:

Risk of recurrence and risk of malignant transformation are notorious during treatment of pleomorphic adenoma. Therefore, treatment of primary and recurrent pleomorphic adenoma is aimed at preventing these problems while avoiding damage to anatomical structures such as the facial nerve. Our objective was to systematically investigate the natural history of recurrent pleomorphic adenoma and address the current rational for its treatment.

The follow-up data of two nationwide series of pleomorphic adenoma was pooled with a focus on risk of malignant transformation and analyzed against the literature. The combined nationwide data (n=9,003 patients) showed 3.1% with 1st recurrence of which 6.2% were malignant. In the literature 1st recurrence rate was >7% at 20 years follow-up. Malignant transformation occurred in 0-7%, and facial nerve damage increased from with each surgery 3-16% at 1st recurrence to 18-30% at 2nd recurrence.
Recurrent pleomorphic adenoma showed a characteristic course with surgery being unreliable and damage to the facial nerve. The risk of malignant transformation was low. This might give flexibility towards a more conservative approach of management.

Chapter 4:
Supposed risk of malignant transformation of salivary gland pleomorphic adenoma is an important reason for aggressive retreatment in recurrent pleomorphic adenoma. However, although the diagnostic category "carcinoma ex-pleomorphic adenoma" suggests that malignant transformation of a pleomorphic adenoma is a regular event, this has until now not been shown to occur in sequential lesions of one patient. Here we show the molecular events in transformation to a malignancy of a pleomorphic adenoma of the parotid gland. Detailed molecular analysis revealed a *LIFR*/*PLAG1* translocation characteristic for pleomorphic adenoma and next to this a *PIK3R1* frameshift mutation and several allelic imbalances. In subsequent malignant recurrences the same *LIFR*/*PLAG1* translocation, *PIK3R1* frameshift mutation and allelic imbalances were present in addition to *TP53* mutations.
This case thus not only shows the malignant transformation of pleomorphic adenoma but also demonstrates that molecular analysis can be of help to recognize malignancy in the rare case of recurrent pleomorphic adenoma.

Chapter 5:
Calculation of the risk of a second primary tumor or malignant transformation after a benign primary tumor (e.g. a pleomorphic adenoma) is impossible in cohorts without central registration of follow-up, because the exit date (by death or international emigration) is unknown. Our aim was to develop and validate a method to address this problem.
We used a cohort with a tumor (similar to a benign primary tumor) with a high survival probability, that in contrast to benign tumors, did have follow-up data: basal cell carcinoma (BCC). In women with BCC, registered in the former Dutch Comprehensive Cancer Centre South Registry (1992-2012), the breast cancer risk

was assessed comparing our new method (mortality projection) with the gold standard (registered person-time). For our method, national age- and gender-specific mortality data was projected on the cohort to estimate person-time and the Standardized Incidence Ratio (SIR). For validation, the error of mortality projection and its effect on the number of expected breast cancer cases were calculated. This resulted in a cohort of 6,949 women. The person-times, calculated with both methods were 42,418 and 42.443 person-years (error 0.06%). The expected number of breast cancers based on the projection method was 148 and 147 if based on the gold standard. The observed number of breast cancers was 162, resulting in almost identical SIRs of 1.09 (95%CI 0.93-1.26) and 1.10 (95%CI0.94-1.27) respectively.
In conclusion, the mortality projection method is robust and can be applied to calculate risks and rates of a second primary tumor or malignant transformation in a cohort lacking follow-up data.

Chapter 6:
Salivary and mammary gland tumors show morphological similarities and share various characteristics, including frequent overexpression of hormone receptors and female preponderance. Although this may suggest a common etiology, it remains unclear whether patients with a salivary gland tumor carry an increased risk of breast cancer (BC). Our aim was to determine the risk of BC in women diagnosed with salivary gland carcinoma (SGC) or pleomorphic adenoma (SGPA). BC incidence (invasive and in situ) was assessed in 2 nationwide cohorts: one comprising 1,567 women diagnosed with SGC and one with 2,083 women with SGPA. BC incidence was compared with general population rates using standardized incidence ratio (SIR). BC risk was assessed according to age at SGC/SGPA diagnosis, follow-up time and (for SGC patients) histological subtype. Our results showed that the mean follow-up was 7.0 years after SGC and 9.9 after SGPA diagnosis. During follow-up, 52 patients with SGC and 74 patients with SGPA developed BC. The median time to BC was 6 years after SGC and 7 after SGPA. The cumulative risk at 10 years of follow-up was 3.1% after SGC and 3.5% after SGPA (95% Confidence Interval (95%CI) 2.1-4.7% and 2.6%-4.6%, respectively). BC incidence was 1.59 times (95%CI 1.19-2.09) higher in the SGC-cohort than expected based on incidence rates in the general population. SGPA-patients showed a 1.48 times (95%CI 1.16-1.86) higher incidence.
As an overall interpretation, women with SGC or SGPA have a slightly increased risk of BC. The magnitude of risk justifies raising awareness, but is no reason for BC screening.

-Part 2-

Molecular imaging of PSMA in salivary glands

Chapter 7:

Prostate-specific membrane antigen (PSMA) positron emission tomography/computed tomography (PET/CT) is used for detection and (re)staging of prostate cancer. However, healthy salivary, seromucous, and lacrimal glands also have high PSMA-ligand uptake. This study aimed to describe physiologic PSMA-ligand uptake distribution characteristics in the head and neck to aid in PSMA PET/CT interpretation and to identify possible new clinical applications for PSMA-ligand imaging.

Thirty consecutive patients who underwent PSMA PET/CT for prostate cancer were evaluated. Tracer maximum standardized uptake values (SUV_{max}) in the salivary, seromucous, and lacrimal glands were determined visually and quantitatively. Overall and intraindividual variations were reported. The scans showed that all gland locations had increased tracer uptake. The mean SUV_{max} ± standard deviation varied: parotid 12.3 ± 3.9; submandibular 11.7 ± 3.5; sublingual 4.5 ± 1.9; soft palate 2.4 ± 0.5; pharyngeal wall 4.3 ± 1.3; nasal mucosa 3.4 ± 0.9; supraglottic larynx 2.7 ± 0.7; and lacrimal 6.2 ± 2.2. The parotid had the largest overall variation in SUV_{max} (5.2-22.9), and the sublingual glands had the largest mean intraindividual difference (18.1%).

In conclusion, major and minor salivary and seromucous glands consistently have high PSMA-ligand uptake. Minor gland locations can be selectively visualized by this technique for the first time. This provides potential new applications such as quantification of present salivary gland tissues and individualization of radiotherapy for head and neck cancer or lutetium-177-PSMA radionuclide treatment.

Chapter 8:

Evaluation of salivary gland damage after head and neck radiotherapy (RT) is difficult with current tools, such as subjective patient-reported outcome measures. We demonstrate the use of prostate-specific membrane antigen positron emission tomography/computed tomography (PSMA PET/CT) as an objective non-invasive tool to visualize damage to salivary glands resulting from RT. In three clinical cases, the PSMA-ligand distribution correlates to the RT dose distribution including intra-gland dose gradients and matches patient-reported toxicity,

suggesting a dose-response relation. These findings support further exploration of PSMA PET/CT to guide and evaluate RT, with the ultimate aim to reduce salivary gland toxicity.

Chapter 9:

The presence of previously unnoticed bilateral macroscopic salivary gland locations in the human nasopharynx was suspected after visualization by positron emission tomography/computed tomography with prostate-specific membrane antigen ligands (PSMA PET/CT). We aimed to elucidate the characteristics of this unknown entity and their potential clinical implications for radiotherapy.

The presence and configuration of the PSMA-positive area was evaluated in a retrospective cohort of consecutively scanned patients with prostate or urethral gland cancer (n=100). Morphological and histological characteristics were assessed in a human cadaver study (n=2). The effect of radiotherapy (RT) on salivation and swallowing was retrospectively investigated using prospectively collected clinical data from a cohort of head-neck cancer patients (n=723). With multivariable logistic regression analysis, the association between radiotherapy (RT) dose and xerostomia or dysphagia was evaluated.

All 100 patients demonstrated a demarcated bilateral PSMA-positive area (average length 4 cm). Histology and 3D reconstruction confirmed the presence of PSMA-expressing, predominantly mucous glands with multiple draining ducts, predominantly near the torus tubarius. In the head-neck cancer patients, the mean RT dose to the gland area was significantly associated with physician-rated post-treatment xerostomia and dysphagia ≥ grade 2 at 12 months (0.019/gy, 95%CI 0.005-0.033, p=.007; 0.016/gy, 95%CI 0.001-0.031, p=.036). Follow-up at 24 months had similar results.

In conclusion, the human body contains a pair of previously overlooked and clinically relevant macroscopic salivary gland locations, for which we propose the name tubarial glands. Sparing these glands in patients receiving RT may provide an opportunity to improve their quality of life.

Chapter 10:

In a letter-to-the-editor, we thank the authors of 9 letters for their constructive comments and thereby their contribution to the interpretation of the glandular structure as described in the tubarial gland paper and the clinical consequences of its presence. A first comment concerned the novelty of our report on clustered

glands, since these have been earlier described by anatomists. Our manuscript however mentioned earlier descriptions in papers and indirectly their references (dating back to 1874) of a higher density of glands in this area. The novelty concerned the visualization and interpretation of the gland conglomerate as comparable to the major salivary glands with new techniques. And because of this new interpretation, possible clinical implications as "a potential new organ at risk" could be evaluated in a large clinical study. The words "new" and "organ" in the title seem to have been a trigger for massive media attention. This might have caused some distraction from the core message of the manuscript.

The core of our message is the fact that the gland tissue in this area has not been considered as an organ at risk (OAR) in radiotherapy. One should realize that an OAR does not only concern organs, but it comprises all tissues near the clinical target volume that can get damaged by radiation. The new techniques of PSMA PET/CT and 3D reconstruction of histological slides that were presented in the paper, opened a new perspective that did not fit previous anatomical descriptions.

Another comment was that it is too early to call this gland a salivary gland, because of uncertainties regarding the fluid composition, and the location of the gland in the nasopharynx. These issues are addressed by discussing the definition of saliva and comparing the characteristics of tubarial glands to those one of the major salivary glands, the sublingual gland. Also, relevant results of additional research are presented, that suggest presence of tubarial gland fluid in the oral cavity. Also, some anatomists have questioned the evidence for presence of tubarial salivary glands, but seem to have missed the nuanced discussion in our paper regarding the positioning of our findings.

An important issue that should have had more emphasis in our paper was the gender imbalance in the patients whose data were used for defining the position of the tubarial glands.

Finally, consequences of the realization that a major glandular structure is present in the nasopharynx, for other diseases are discussed. We conclude that the tubarial gland paper is only a starting point and new questions will need additional interesting research.

Chapter 11

Conclusions and future perspectives

In general, ongoing developments in medical sciences provide new possibilities for in depth evaluation of the origin of disease, for use of diagnostic methods and new treatments and for evaluation of treatment outcomes. This asks for constant awareness of the reality that current knowledge of today is not a fact of life, or a fact of medicine but is just the perspective that we (can) have today based on current techniques.

In patients with rare diseases like salivary gland tumors, it can be even harder to discern new patterns in pathophysiology, diagnostic methods or treatment outcome. This asks for strategies such as the use of big data from nationwide registries, multidisciplinary, multicenter and multi-organization and sometimes international cooperation, as was shown throughout this thesis. Nationwide databases with salivary gland tumor data from the Netherlands and Denmark were used to evaluate incidence of the salivary gland pleomorphic adenoma (SGPA) in Chapter 2 and 3 [1,2]. Incidence of adverse and relatively uncommon outcomes like recurrence and malignant transformation could be investigated in these large cohorts. It must be said that these investigations were only possible thanks to the existence of the high quality nationwide tumor and pathology registries. And having said this, the quality of the input determines the quality of the output. Ongoing research is expected to provide more insight in the difficulties in correct registration of a rare disease like the malignantly transformed SGPA. Molecular diagnostics can be of help as was shown in Chapter 4, in a patient with the first full genetic story behind malignant transformation of the SGPA, the most common salivary gland tumor[3]. Future investigations of more of these rare cases could determine what other pathways can lead to malignant transformation. Also, the optimal way of use of molecular techniques in the diagnostic process could then be further clarified.

Zooming out to the epidemiology-level again, the possibility of connecting nationwide databases with patient data of salivary gland tumors and breast cancer, provided a cohort in which the risk of breast cancer could be investigated. In case of the SGPA, which lacked follow-up data in the nationwide databases, this follow-up problem had to be explored and solved first, as is shown in Chapters 5 and 6 [4]. The increased relative risk supports further exploration of the mechanisms behind this phenomenon which had been noted on a smaller scale by clinical observations and smaller studies. The relation between sex hormones and female

reproductive cancers (breast, ovarian, endometrium cancer) has received a lot of attention because of the role of these hormones in activating specific signaling pathways after binding to e.g. the estrogen receptor in these tissues [5,6]. However, other entities, like the salivary gland tumors that also have these receptors, seem to claim their small share of attention as has been shown by publications over the years [4,7,8]. And a relation of salivary gland tumors with other tumor types with hormone receptors might follow.

The fact that new medical techniques can not only shed a new light on the origin of diseases and diagnostical possibilities and therapy outcomes, but also on anatomical perspectives, was shown in the chapters of the second part of this thesis (Chapter 7, 8, 9 and 10)[9–12].

Shifting the focus of attention of a new diagnostic modality for prostate cancer, to the use for head and neck cancer lead to an array of surprising findings. Prostate specific membrane antigen (PSMA) positron emission tomography / computed tomography (PET/CT) for the first time showed PSMA-ligand binding to minor salivary and seromucous glands in Chapter 7. This offers possibilities for radiotherapy treatment planning aiming for sparing of these glands in order to preserve quality of life for patients. Loss of PSMA-ligand binding capacity after (partial) gland radiation could been seen on PSMA PET/CT scans in Chapter 8. Further research is expected to answer questions on how these scans can be used in radiotherapy for head and neck cancer patients. The finding of a large bilateral collection of gland tissue in the dorsolateral nasopharyngeal wall in Chapter 9 showed that new perspectives regarding the understanding of anatomy and possible clinical consequences are still possible. Although this perspective of a possible new "organ at risk" (which is a term in radiotherapy for organs and tissues at risk for radiation damage) was carefully reported with nuances regarding its interpretation, the popular media interpreted and reported it as a new organ (Figure 1). In Chapter 10 we therefore pointed out that we were standing on the shoulders of giants in anatomy in describing this new perspective regarding presence of nasopharyngeal glandular tissue. The PSMA PET/CT scan made it possible for the first time, to delineate and interpret these gland collections as macroscopic glands, which could be a potential new organ at risk in radiotherapy. In general, standing on shoulders is not enough to translate anatomical knowledge or new findings into clinical relevance. Researchers and clinicians need to connect

topics, techniques, new developments, new insights and people to make small steps and hopefully sometimes even leaps forward. Our description of the use of PSMA PET/CT for the head and neck region and the tubarial glands as a possible new organ at risk in radiotherapy can be regarded as a beginning. It is a first step, exploring a new perspective. Many aspects of the use of the scans and the interpretation of the gland tissue and its role in treatment of cancer and role in other (such as auto-immune) diseases have to be clarified. Preferably, our findings regarding the relevance in head and neck radiotherapy are validated in a separate cohort. Also, further characterization of the gland tissue and its excreted fluid is expected to provide further insight in their interpretation as glands. Looking beyond PSMA, it would not be surprising if new molecular tracers would provide new insights regarding our understanding of tissues and diseases in the near future.

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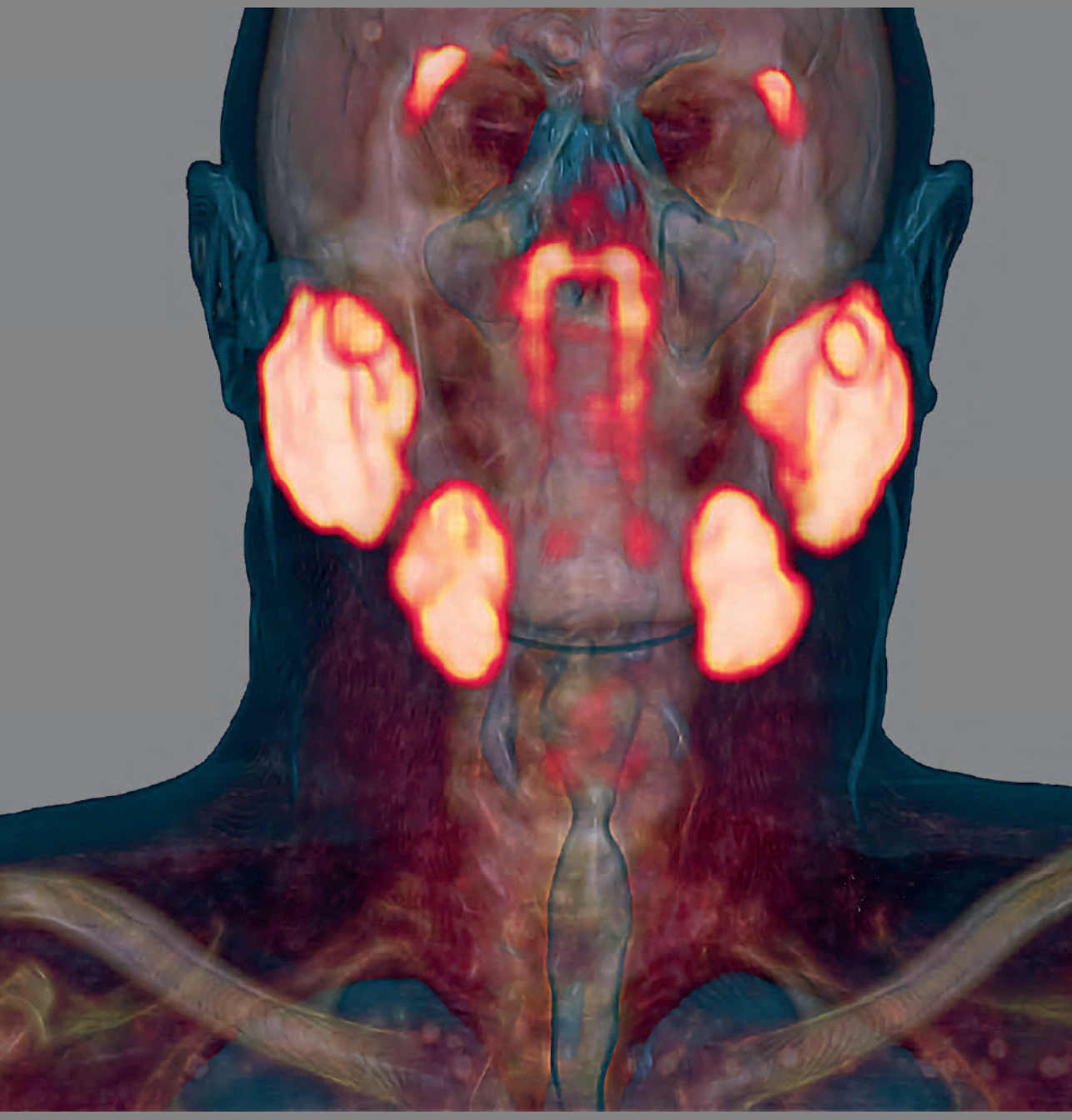
Figure 1: Title of an interview in the New York Times October 19, 2020, as an example of the interpretation of the tubarial glands by the popular media.



Chapter 12

Epilogue

Samenvatting (Dutch summary)

Contributing authors and author contributions

Dankwoord (Acknowledgements)

Curriculum vitae

List of publications

12.1 Samenvatting (Dutch summary)

Nieuwe epidemiologische en op PSMA-expressie gebaseerde inzichten bij speekselkliertumoren

Hoofdstuk 1:

De mens heeft -zo leren de leerboeken ons- drie paar grote speekselklieren en ongeveer duizend kleine speekselklieren verspreid over het bovenste deel van de luchtwegen en het spijsverteringsstelsel. Het speeksel dat deze klieren produceren is belangrijk voor verscheidene biologische en mechanische functies. Zo helpt speeksel bij de vertering, bevochtiging en het smeren van voedsel, preventie van cariës (tandbederf) en slijtage van het gebit. Daarnaast doodt speeksel micro-organismen zoals bacteriën en virussen en beschermt het tegen schadelijke chemische stoffen en uitdroging. Ook heeft het een rol bij bloedstolling en wondgenezing.

De speekselklieren bevatten ook andere anatomische structuren, zoals lymfeklieren, lymfvaten, bloedvaten en zenuwen. De nervus facialis, de aangezichtszenuw die de gezichtsuitdrukkingen verzorgt, loopt door de glandula parotis. Chirurgie van deze klier kan hierdoor uitdagend zijn.

Speekselkliertumoren zijn zeldzame tumoren. Zo'n 85% van deze tumoren is goedaardig, maar maligne speekselkliertumoren komen ook voor. De meest voorkomende speekselkliertumor is het pleiomorf adenoom. Deze tumor kan zich op verschillende manieren morfologisch presenteren, met daarbij een variërende combinatie van verschillende celtypes (epitheliaal, myoepitheliaal en mesenchymaal). Ondanks zijn goedaardige aard ontwikkelt het pleimorf adenoom zich soms tot een maligne tumor en wordt dan een carcinoom ex pleiomorf adenoom genoemd. De maligne speekselkliertumoren hebben een verrassend breed palet van histologische presentaties. De 4e editie van de World Health Organisation (WHO) classificatie van tumoren uit 2017 noemde 20-25 verschillende speekselkliertumoren met kwaadaardige eigenschappen, zoals invasieve groei, mitosen (delingen), necrose (dood weefsel) en genetische mutaties. Conventionele beeldvormende technieken (echografie, CT, MRI) kunnen de speekselklieren met een verschillende mate van detail zichtbaar maken. Voor het diagnostische onderzoek van deze klieren is de MRI het meest geschikt.

Het afbeelden van de kleine speekselklieren is met de bovengenoemde radiologsiche technieken nooit gelukt. Bij patiënten met prostaatkanker bleek bij toeval dat deze speekselklieren sinds kort wel gevisualiseerd kunnen worden op nucleaire scans van het prostaat specifieke membraan antigen (PSMA PET/CT). Echter, aan mogelijkheden die dit biedt voor de hoofd-halsoncologie is tot voor kort weinig aandacht besteed.
In dit proefschrift zullen wetenschappelijk relevante onderwerpen over speekselklieren en speekselkliertumoren worden uitgelicht.

- Deel 1-

Hoofdstuk 2:
Hoewel het pleiomorf adenoom de meest voorkomende speekselkliertumor is, is er betrekkelijk weinig bekend over de epidemiologie, aangezien deze goedaardige tumor internationaal niet wordt geregistreerd in de landelijke kankerregistraties. Deze studie richtte zich op het in kaart brengen van incidentie, trends, secundaire maligne transformatie en factoren geassocieerd met het ontwikkelen van een recidief voor de Nederlandse populatie. Data werd geëxtraheerd uit de Nederlands Pathologie Database (PALGA) van de jaren 1992, 1997, 2002, 2007, en 2012. Er werd een multivariate analyse uitgevoerd om het risicofactoren voor optreden van een recidief vast te stellen. De zoekvraag resulteerde in 3506 patiënten met een pleiomorf adenoom, wat neerkomt op een Europees gestandaardiseerde incidentie van 4.2-4.9 per 100,000 persoon-jaren. De studie liet een hogere incidentie bij vrouwen zien (1:1.43) met een jaarlijkse groei van 1% (95% betrouwbaarheidsinterval [BI]: 0.2-1.8) en een bimodale leeftijdsverdeling ($p<0.0001$). Het deel van patiënten van de totale populatie dat na 20-jaar een recidief kreeg, was 6.7%, en mediane tijd tot eerste recidief was 7 jaar. Positieve en onzekere resectiemarges alsmede een jongere leeftijd bij diagnose waren risicofactoren voor recidief, met respectievelijk een odds ratios (ORs) van 4.62 (95% BI 2.84-7.51), 4.08 (95% BI 2.24-7.43), en 0.42 (95% BI 0.29-0.63). Tumorlokalisaties in de kleinere speekselklieren hadden een lagere odds voor recidief dan tumoren in de parotisklier (OR 0.24; 95% BI: 0.07-0.77; $p<0.016$). Maligne transformatie ontstond in 0.15% van de pleiomorfe adenomen (3.2% van de recidieven).
Concluderend werd in deze epidemiologische studie op basis van nationale data aangetoond dat er geslachtsverschillen zijn in het voorkomen van het pleiomorf

adenoom, wat een onderliggend mechanisme suggereert. Het lange termijnrisico op recidief was laag en de kans op maligne ontaarding was zeer laag.

Hoofdstuk 3:

Het risico op een recidief en het risico op maligne ontaarding van een pleiomorf adenoom zijn berucht. Daarom is de behandeling van een primaire tumor en ook van een recidief gericht op het voorkomen van deze problemen. Tegelijkertijd dient schade aan anatomische structuren zoals de nervus facialis te worden vermeden. Het doel van deze studie was om het natuurlijk beloop van een recidief pleiomorf adenoom te beschrijven en daarmee de huidige ideeën achter de behandeling te beschouwen.

De follow-up data van twee landelijke series van pleiomorfe adenomen werden samengevoegd met speciale aandacht voor het risico op maligne ontaarding en geanalyseerd tegen de achtergrond van de bestaande literatuur. De samengevoegde data (n=9.003 patiënten) lieten bij 3.1% een eerste recidief zien, waarvan 6.2% maligne was. In de literatuur was de kans op een eerste recidief >7% na 20 jaar follow-up. Maligne ontaarding trad op bij 0-7%. Letsel van de nervus facialis nam bij elke operatie toe van 3-16% bij het eerste recidief naar 18-30% bij het tweede recidief.

Recidief pleiomorf adenoom liet een karakteristiek beloop zien met onzekere uitkomsten van chirurgische behandeling met steeds weer kans op een nieuw recidief en op letsel van de nervus facialis. Het risico op maligne ontaarding was laag. Dit maakt enige flexibiliteit met betrekking tot een afwachtend beleid denkbaar.

Hoofdstuk 4:

Het veronderstelde risico op maligne ontaarding van pleiomorf adenoom van de speekselklier is een belangrijke reden voor het instellen van een agressief beleid bij een recidief. Ondanks het bestaan van de diagnostische categorie 'carcinoom ex pleiomorf adenoom' is de maligne verandering tot op heden nog niet aangetoond in één casus. In deze studie laten we voor het eerst op moleculair niveau alle genetische stappen zien in de maligne ontaarding van een pleiomorf adenoom van de glandula parotis. Gedetailleerde moleculaire analyse onthulde de voor pleiomorf adenoom karakteristieke *LIFR*/*PLAG1* translocatie, met daarnaast optreden van een *PIK3R1* frameshift mutatie en verschillende allel instabiliteiten. In opeenvolgende maligne recidieven kwamen dezelfde *LIFR*/*PLAG1* translocatie, *PIK3R1* frameshift mutatie, de allel instabiliteiten naast een *TP53* mutatie voor.

Deze casus laat dus niet alleen de maligne ontaarding van een pleiomorf adenoom zien, maar demonstreert ook dat moleculaire analyse kan helpen om een maligniteit te herkennen in het zeldzame geval van een recidief pleiomorf adenoom.

Hoofdstuk 5:

Speekselklier- en borstkliertumoren laten vergelijkbare morfologiëen zien en delen verscheidene eigenschappen, waaronder frequente overexpressie van hormoonreceptoren en een oververtegenwoordiging van vrouwen. Hoewel dit een gemeenschappelijke etiologie suggereert, is het nog onduidelijk of patiënten met een speekselkliertumor een verhoogd risico hebben op het ontwikkelen van borstkanker. Ons doel was om het risico op het ontwikkelen van borstkanker te bepalen bij vrouwen gediagnosticeerd met een carcinoom of pleiomorf adenoom van de speekselklier.

Borstkankerincidentie (invasief en situ) werd vastgesteld in twee landelijke cohorten: het ene omvatte 1567 vrouwen gediagnosticeerd met speekselkliercarcinoom en het andere 2083 vrouwen met een pleiomorf adenoom. De borstkankerincidentie werd vergeleken met die in de algehele populatie, gebruikmakend van gestandaardiseerde incidentie ratio (SIR). Dit borstkankerrisico werd berekend, rekening houdend met leeftijdsgroep bij speekselkliertumordiagnose, follow-up tijd en (voor de speekselklier- carcinoom patiënten) histologisch subtype. De resultaten laten zien dat de gemiddelde follow-up 7 jaar was na speekselklierkanker en 9.9 jaar na pleiomorf adenoom. Tijdens de follow-up ontwikkelden 52 patiënten met een eerder speekselkliercarcinoom en 74 patiënten met een eerder pleiomorf adenoom borstkanker. De gemiddelde tijd tot de diagnose van borstkanker was 6 jaar na speekselkliercarcinoom en 7 jaar na pleiomorf adenoom. Het cumulatieve risico bij 10 jaar follow-up was 3.1% na speekselkliercarcinoom en 3.5% na pleiomorf adenoom (95% BI 2.1-4.7% en 2.6%-4.6%, respectievelijk). De borstkankerincidentie was hiermee 1.59 keer (95% BI 1.19-2.09) hoger in het speekselkliercarcinoom cohort dan de verwachtte incidentie in de algemene populatie. Patiënten met een pleiomorf adenoom hadden een 1.48 (95% BI 1.16-1.86) keer hogere incidentie dan de algemene populatie.

Dus, vrouwen met een speekselkliercarcinoom of pleiomorf adenoom hebben een licht verhoogd risico op BK. De omvang van het verhoogde risico vraagt om alertheid, maar is geen reden voor borstkankerscreening in deze groep patiënten.

-Deel 2-

Hoofdstuk 6:

Prostaat specifiek membraan antigen (PSMA) positron emissie tomografie/ computed tomografie (PET/CT) wordt gebruikt voor de detectie en (her-) stadiëring van prostaatkanker. Echter, gezonde speekselklieren, seromuceuze klieren en traanklieren hebben ook een hoge PSMA-ligand opname. Deze studie heeft als doel het beschrijven van de fysiologische PSMA-ligand opnamedistributiekenmerken in het hoofd-halsgebied, om zo de PSMA PET/CT te helpen interpreteren en om eventuele nieuwe klinische toepassingen van het PSMA-ligand afbeelding te identificeren.

Dertig opeenvolgende patiënten die een PSMA PET/CT ondergingen voor prostaatkanker werden geëvalueerd. Maximale gestandaardiseerde opname waarden (SUV_{max}) van het traceermiddel (tracer) in de speeksel-, traan- en seromuceuze klieren werden visueel en kwantitatief bepaald. Inter- en intra-individuele variaties werden weergegeven. De scans lieten zien dat alle klierlocaties een verhoogde opname van de tracer hadden. De gemiddelde SUV_{max} ± standaardafwijking varieerde: parotis 12,3 ± 3,9; submandibularis 11,7 ± 3,5; sublingualis 4,5 ± 1,9; palatum molle 2,4 ± 0,5; farynxwand 4,3 ± 1,3; neusmucosa 3,4 ± 0,9; supraglottische larynx 2,7 ± 0,7;en traanklier 6,2 ± 2,2. De parotis had de grootste algehele variatie in SUV_{max} (5,2-22,9) en de glandula sublingualis het grootste gemiddelde intra-individuele verschil (18,1%).

Concluderend hadden de grote en kleine speeksel- en seromuceuze klieren consistent een hoge PSMA-ligand opname. Kleine klierlocaties werden voor het eerst selectief gevisualiseerd met behulp PSMA PET/CT. Dit biedt potentieel nieuwe mogelijkheden zoals het kwantificeren van aanwezig klierweefsel en individualisering van radiotherapie bij hoofd-halskanker of lutetium-177-PSMA radionuclidetherapie.

Hoofdstuk 7:

Evaluatie van speekselklierschade na radiotherapie voor hoofd-halstumoren is moeilijk uit te voeren met de bestaande onderzoekstechnieken, waarbij van subjectieve door de patiënt gerapporteerde uitkomstmaten gebruik gemaakt wordt. Wij tonen aan dat PSMA PET/CT als een objectieve niet-invasieve methode gebruikt kan worden om bestralingsschade aan de speekselklieren zichtbaar te maken. In drie klinische casusbeschrijvingen correleert de PSMA-ligand distributie

met de radiotherapie dosisdistributie inclusief de dosisverdeling over de klier. Hierbij komt dit overeen met de door patiënt gerapporteerde toxiciteitsklachten, hetgeen een dosis-response relatie suggereert. Deze bevindingen ondersteunen verdere exploratie van het gebruik van PSMA PET/CT om radiotherapie te sturen en te evalueren, met als uiteindelijk doel om de speekselklierschade te beperken.

Hoofdstuk 8:

De aanwezigheid van eerder niet opgemerkte bilaterale macroscopische speekselklierlocaties in de menselijke nasofarynx werd vermoed na visualisering door PSMA PET/CT. Het doel van dit onderzoek was het ophelderen van de eigenschappen van deze onbekende entiteit en de potentiële klinische implicaties voor radiotherapie. De aanwezigheid en vorm van het PSMA-positieve gebied werd geëvalueerd in een in retrospectief cohort van opeenvolgend gescande patiënten met kanker van de prostaat of para-urethrale klier (n=100). Morfologische en histologische eigenschappen werden bepaald in een humane kadaverstudie (n=2). Het effect van bestraling op mondbevochtiging en slikken werd retrospectief onderzocht door gebruikmaking van prospectief verzamelde klinische data van een cohort hoofd-hals-kankerpatiënten (n=723). Met multivariabele logistische regressieanalyse werd de associatie tussen radiotherapie dosis en xerostomie en/of dysfagie geëvalueerd.

Alle 100 patiënten vertoonden een afgegrensd dubbelzijdig PSMA-positief gebied (gemiddelde lengte 4 cm). Histologie en 3D-reconstructie bevestigden de aanwezigheid van PSMA die voornamelijk door muceuze klieren met meerdere afvoergangetjes, met name gelegen bij de torus tubarius, tot expressie werd gebracht. Bij de hoofd-halskankerpatiënten, was de gemiddelde radiotherapie dosis significant geassocieerd met de door de behandelaar gerapporteerde xerostomie en dysfagie ≥ graad 2 na 12 maanden (0,019/gy, 95% BI 0,005-0,033, p=,007; 0,016/gy, 95% BI 0,001-0,031, p=,036). Follow-up na 24 maanden toonde vergelijkbare resultaten.

Concluderend bevat het menselijke lichaam een paar eerder niet opgemerkte en klinisch relevante speekselklierlocaties, waarvoor wij de naam glandulae tubariales voorstellen. Het sparen van deze klieren bij patiënten die bestraald worden, zou een kans kunnen bieden om hun kwaliteit van leven te verbeteren.

Hoofdstuk 9:

In een 'letter-to-the-editor', bedanken wij de auteurs van negen ingezonden brieven voor hun constructieve commentaren en daarmee hun bijdrage aan de interpretatie van de klierstructuur zoals beschreven in ons artikel over de glandulae tubariales. Een eerste brief betrof het commentaar op de noviteit van onze melding van een clustering van speekselkliertjes, die naar de mening van de auteurs eerder beschreven zijn door anatomen. Ons manuscript refereerde aan meer compact klierweefsel in dit gebied dat teruggaand tot 1874 in artikelen en daarin genoemde referenties teruggevonden kan worden.

De noviteit betrof vooral de visualisatie en interpretatie met nieuwe PET/CT-technieken, die een vergelijking van het klierconglomeraat met de drie bekende paren grote speekselklieren mogelijk maakten. En vanwege deze nieuwe interpretatie, konden klinische implicaties van dit mogelijk nieuwe 'organ at risk' (OAR) voor patiënten in een grote klinische studie worden geëvalueerd.

De woorden 'nieuw' en 'orgaan' in de titel hebben een enorme hoeveelheid media-aandacht tot gevolg gehad. Dit heeft wellicht de aandacht afgeleid van de kernboodschap van het manuscript, die zich richtte op de status van het klierweefsel als OAR bij bestraling. Men dient zich te realiseren dat een OAR niet alleen organen betreft, maar alle weefsels behelst die beschadigd kunnen raken in de buurt van het doelvolume.

De nieuwe technieken van PSMA PET/CT en 3D reconstructie van histologische coupes die worden gepresenteerd in het artikel, openen een nieuw perspectief dat niet past bij de eerdere anatomische beschrijvingen in de periode van het einde van de 19^{e} eeuw.

Een ander commentaar kritiseerde de naamgeving van deze klier als speekselklier, vanwege onzekerheden over de samenstelling van de vloeistof en de locatie van de klier in de neuskeelholte. De kritiek wordt gepareerd met een uitleg van de definitie van speeksel en de vergelijkbaarheid van eigenschappen van de glandula tubarialis met de glandula sublingualis, een van de grote speekselklieren. Ook worden resultaten gerapporteerd van aanvullend onderzoek die de aanwezigheid van speeksel uit de glandulae tubariales in de mondholte suggereren.

Sommige anatomen die hadden gereageerd, plaatsten vraagtekens bij het bewijs voor de aanwezigheid van de glandulae tubariales. Blijkens hun reacties hadden zij onvoldoende rekening gehouden met onze nuances in de discussie van het artikel.

Een belangrijk onderwerp dat meer nadruk in het artikel had moeten hebben volgens andere briefschrijvers is de disbalans in verdeling over de geslachten van patiënten van wie anatomische gegevens zijn gebruikt.
In een laatste commentaar wordt de nieuwe blik die PSMA PET/CT op het hoofd-halsgebied biedt, in de context geplaatst van een mogelijke rol bij andere aandoeningen zoals het juveniel nasaal angiofibroom.
In onze reacties hebben wij duidelijk gemaakt dat het artikel over de nieuwe bevinding van de glandulae tubariales slechts een start voor nieuw onderzoek behelst. De nieuwe vragen die worden opgeworpen, vragen om additioneel wetenschappelijk onderzoek.

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Part 1: Epidemiology of salivary gland tumors

1. **General Introduction** MV

2. **Salivary gland pleomorphic adenoma in the Netherlands: A nationwide observational study of tumor incidence, malignant transformation, recurrence and risk factors for recurrence.** Valstar MH, de Ridder M, van den Broek EC, Stuiver MM, van Dijk BAC, van Velthuysen MLF, Balm AJM, Smeele LE.
Oral Oncology 2017; 66: 93-99.

Study concepts and design	MV, MdR, LS, AB, MvV
Data acquisition	MV, MdR, EvdB
Data analysis and interpretation	MV, MdR, EvdB, MvV, LS, AB
Statistical analysis	MV, MdR, MS, BvD
Manuscript preparation	MV, MdR
Manuscript editing and review	EvdB, MS, BvD, MvV, AB, LS, MV, MdR

3. **Natural history of Recurrent Pleomorphic Adenoma: Implications on management.** Valstar MH, Andreasen S, Bhairosing P, McGurk M.
Head Neck. 2020 Aug;42(8): 2058- 2066.

Study concepts and design	MV, SA, MM
Data acquisition	MV, SA, MM, PB
Data analysis and interpretation	MV, SA, MM
Manuscript preparation	MV, SA, MM
Manuscript editing and review	MV, SA, MM, PB

4. **Malignant transformation of salivary gland pleomorphic adenoma: Proof of principle.** Valstar MH, Mast H, ten Hove I, Moonen LR, Balm AJM, Smeele LE, Koljenović S, Dinjens WNM, van Velthuysen MF.
The Journal of Pathology: Clinical Research 2021; 7(5):432-437

Study concepts and design	MV, MvV, WD, LM, AB, LS
Data acquisition	MV, MvV, HM, SK, WD, LM
Data analysis and interpretation	MV, MvV, SK, WD, LM
Manuscript preparation	MV, HM, MvV, WD
Manuscript editing and review	MV, MvV, WD, HM, ItH, LM, AB, LS, SK

5. **Risk of breast cancer in women with a previous salivary gland carcinoma or pleomorphic adenoma in the Netherlands.**
Valstar MH, Schaapveld M, van den Broek EC, van Velthuysen MF, de Ridder M, Schmidt MK, van Dijk BAC, Balm AJM, Smeele LE.
Cancer Medicine 2021 Jan; 10(1): 424-434.

Study concepts and design	MV, EvdB, MS, AB, LS, MvV, BvD
Data acquisition	MV, MdR, EvdB, BvD
Data analysis and interpretation	MV, MS, EvdB, MvV, MKS, BvD, AB, LS
Statistical analysis	MV, MS, EvdB
Manuscript preparation	MV, MS, EvdB
Manuscript editing and review	MV, MdR MS, EvdB, MvV, MKS, BvD, AB, LS

Part 2: Molecular imaging of PSMA in salivary glands

6. **Physiological distribution of PSMA-ligand in salivary glands and seromucous glands of the head and neck on PET/CT**
   Klein Nulent TJW, Valstar MH, de Keizer B, Willems SM, Smit LA, Al-Mamgani A, Smeele LE, van Es RJJ, de Bree R, Vogel WV
   Oral Surgery Oral Medicine Oral Pathology Oral Radiology. 2018; 125(5): 478-486.

Study concepts and design	TKN, MV, WV, BdK, RvE, LS
Data acquisition	TKN, WV, BdK
Data analysis and interpretation	TKN, MV, BdK, WV, AA, LS, RvE, RdB, WV
Statistical analysis	TKN, MV, WV
Manuscript preparation	TKN, MV, WV
Manuscript editing and review	TKN, MV, BdK, SW, LAS, AA, LS, RvE, RdB, WV

7. **Prostate specific membrane antigen imaging as a potential tool to assess and guide salivary gland irradiation: a case series.**
   Valstar MH, Owers EC, Al-Mamgani A, Smeele LE, van de Kamer JB, Sonke JJ, Vogel WV.
   Physics and Imaging in Radiation Oncology. 2019; 4 (9): 65-68.

Study concepts and design	MV, EO, AA, JvdK, JJS, WV
Data acquisition	MV, EO, WV
Data analysis and interpretation	MV, EO, AA, JvdK, JJS, WV
Manuscript preparation	MV, EO, WV
Manuscript editing and review	MV, EO, AA, LS, JvdK, JJS, WV

8. **The tubarial salivary glands: A potential new organ at risk for radiotherapy.**
Valstar MH, de Bakker BS, Steenbakkers RJHM, de Jong KH, Smit LA, Klein Nulent TJW, MD, van Es RJJ, Hofland I, de Keizer B, Jasperse B, Balm AJM, van der Schaaf A, Langendijk JA, Smeele LE, Vogel WV
Radiotherapy and Oncology. 2021; 154: 292-298.

Study concepts and design	MV, BdB, RS, KdJ, LAS, TKN, RvE, AB, JL, LS, WV
Data acquisition	MV, BdB, RS, KdJ, LAS, TKN, IH, BdK, BJ, AvdS, JL, WV
Data analysis and interpretation	MV, BdB, RS, LAS, TKN, RvE, IH, BdK, BJ, AB, AvdS, JL, LS, WV
Statistical analysis	MV, AvdS, JL, RS, WV
Manuscript preparation	MV, WV
Manuscript editing and review	MV, BdB, RS, KdJ, LAS, TKN, RvE, IH, BdK, BJ, AB, AvdS, JL, LS, WV

9. **The tubarial glands paper: A starting point. A reply to comments**
Valstar MH, de Bakker BS, Steenbakkers RJHM, de Jong KH, Smit LA, Klein Nulent TJW, MD, van Es RJJ, Hofland I, de Keizer B, Jasperse B, Balm AJM, van der Schaaf A, Langendijk JA, Smeele LE, Vogel WV
Radiotherapy and Oncology 2021; 154: 308-311.

Study concepts and design	MV, WV
Data acquisition	MV, BdB, WV
Data analysis and interpretation	MV, LAS, WV
Manuscript preparation	MV, BdB, WV
Manuscript editing and review	MV, BdB, RS, KdJ, LAS, TKN, RvE, IH, BdK, BJ, AB, AvdS, JL, LS, WV

12.3 Dankwoord (Acknowledgements)

De periode van 8 jaar promotieonderzoek laat zich het best omschrijven als een ontdekkingsreis langs fascinerende onderwerpen, ervaringen en mensen.

Graag wil ik allen danken voor de prettige samenwerking en bijdragen in de vorm van tijd en toewijding die de totstandkoming van dit proefschrift mede mogelijk hebben gemaakt. De voltooiing was echter ook niet mogelijk geweest zonder de steun van het thuisfront. Een persoonlijk woord van dank richt ik graag tot de volgende personen:

Professor Smeele, beste Ludi, dank voor de mogelijkheid om mij bijna onbeperkt te kunnen storten op onderzoek naar speekselkliertumoren en gerelateerde onderwerpen vanuit het Antoni van Leeuwenhoek. Mede door de zeldzaamheid van deze tumoren bleken nog veel vragen onbeantwoord te zijn en hebben we er een paar kunnen beantwoorden. Jouw steun en vertrouwen vormden een goede basis voor alle samenwerkingen binnen en buiten de deur van het NKI-AVL. Daarbij zorgden jouw visie en heldere redenaties er op lastige momenten voor, dat de punten op de horizon zichtbaar bleven. De afspraak bij het startpunt was dat het geen eindeloos promotietraject of "Opus magnum" hoefde te worden. Het scheelde niet veel, maar…bij dezen (zoals je het later noemde).

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Dr. van Velthuysen, beste Loes, jij stond mede aan de wieg van het pleiomorfe adenomen project en daarmee aan de wieg van dit proefschrift. Het is prachtig hoe we mede dankzij jouw vertrouwen en inzet, via de big data van het eerste landelijke cohort patiënten met deze tumor, zijn uitgekomen bij de eerste volledige beschrijving van de genetica van maligne ontaarding van een pleiomorf adenoom in een patiënt. Dank daarvoor. En er zit nog meer in de pen, waarvoor ook dank aan professor Slootweg voor de inzet en bijdrage. Helaas kon nog niet alles mee in dit proefschrift.

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Dr. van Dijk, beste Boukje, dank voor de prettige betrokkenheid vanuit de Nederlandse Kankerregistratie op diverse momenten bij meerdere projecten.

Dr. Schmidt, beste Marjanka, dank voor het enthousiaste meedenken over het al dan niet aanwezig zijn van een genetische verklaring voor de verhoogde kans op borstkanker na een speekselkliertumor.

Dr. Andreasen †, dear Simon up above, it felt like you were my Danish research-counterpart in salivary gland epidemiology. The world lost a wonderful person, a talented doctor and researcher when you passed away. Thank you for your effort and precious time. My heart goes out to your wife Caroline and son.

Professor McGurk, dear Mark, thank you very much for your multiple inspiring salivary gland surgery activities and taking interest in our pleomorphic adenoma epidemiology project. Your remarks regarding the absence of nationwide data-based incidence numbers of this disease, in an early publication, sparked my initiative to go out there and get these data. Teaming up with you and Simon in the international study combining Danish and Dutch nationwide data was a real pleasure.

Beste Professor Winand Dinjens, dr. Senada Koljenović, Hetty Mast, Laura Moonen en Ivo Ten Hove, dank voor de enthousiaste inzet samen met Loes vanuit het Erasmus MC, tijdens de genetische ontdekkingstocht naar de achtergrond van ontsporing van een pleiomorf adenoom tot een maligniteit. Voor het eerst kon dit bij opeenvolgende tumoren in een patiënt worden vastgelegd.

Drs. Klein Nulent, beste Thomas, met in het kielzog dr. Robert van Es, professor Stefan Willems, dr. Bart de Keizer en professor Remco de Bree, dank voor de prettige samenwerking bij het ontginnen van het onderzoeksgebied naar mogelijkheden van PSMA PET/CT voor speekselklier(tumor)onderzoek. Het bleek het startschot voor evaluatie van de kliercollectie in de nasopharynx, de glandula tubarialis.

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Professor Langendijk, dr. Steenbakkers en dr. van der Schaaf, beste Hans, Roel en Arjen, dank voor de enorme bijdrage vanuit Groningen waardoor we de aannemelijkheid van klinische relevantie van het tubarialis kliergebied bij bestraalde patiënten konden niet aantonen, maar vaststellen.

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Marloes, Farzaneh, Richard en Piet, onze halfjaarlijkse weekenden met de gezinnen zijn een dierbare traditie geworden. Piet, jouw bijdrage als classicus en enige niet-medicus aan het gezelschap, aan de naamgeving van de klierstructuur in de nasopharynx, heeft zelfs de landelijke media gehaald. Dank.

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konden wedijveren wie als eerste zijn proefschrift kon afronden. Aangezien deze wetenschappelijke interesse een resultaat is van jullie "Nature and Nurture", kunnen we dit proefschrift als een family effort zien en gaan we samen over de finish!

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12.4 Curriculum vitae

The author of this thesis, Matthijs H. Valstar was born on April 29, 1978 in Zwolle, the Netherlands. He is the son of Gerda Valstar-Nieuwenhuisen and Johan Valstar and grew up with his younger sisters Anneleen and Harriet. After completing secondary school at the Carolus Clusius College in Zwolle, he started Medical School at the University of Groningen in 1996 and graduated as a Medical Doctor in 2003. Clinical experience as a junior doctor was gained as an emergency department physician, partially in combination with a Master of Science in Dentistry from 2005 until 2008 at the University of Amsterdam. He started the specialist training in maxillofacial surgery in 2008 supervised by prof. dr. van den Akker. After a program at the Amsterdam University Medical Center (AUMC, location AMC), the Netherlands Cancer Institute (NCI) Antoni van Leeuwenhoek (supervised by prof. dr. Smeele), the Isala Clinics in Zwolle and again the AUMC (supervised by prof. dr. de Lange), he completed the training program in 2012.

Later that year, the author started a PhD-research project at the NCI under the inspiring supervision of prof. dr. Smeele and professor dr. Balm, parallel with clinical work as a maxillofacial surgeon. The research activities resulted in the "PALGA-prize" at the 2015 Dutch Pathologists conference. In 2016, he completed a two-year postgraduate master degree in Clinical Epidemiology at the University of Amsterdam, supervised by dr. Stuiver. During this master, he contributed as a member of the education committee to the introduction of digital classes. he master-thesis on the nationwide cohort of pleomorphic adenoma patients led to other projects among which that on malignant transformation, supervised by dr. Loes van Velthuysen. In 2019, the author focused his clinical activities as an oral and maxillofacial surgeon in the Flevohospital, Almere and joined the partnership in 2020. In 2021, the author was delegated by the Dutch Association of Nuclear Medicine for the 2-yearly Science and Innovation Award of the Dutch Federation of Medical Specialists for the research project on the tubarial glands. This multi-center seven-specialty project, that was managed together with dr. Wouter Vogel, was extensively reported on both in medical and in public international media [1–4].

Since 2016 the author is married to Anne Brecht Francken. They are the loving parents of their two daughters Olivia and Philippa, who were born in respectively 2008 and 2011. In his spare time, he pursues his hobbies being a hockey-coach, making race cycling trips and playing some piano and the drums.

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12.5 List of publications

Malignant transformation of salivary gland pleomorphic adenoma: Proof of principle. **Valstar MH**, Mast H, ten Hove I, Moonen LR, Balm AJM, Smeele LE, Koljenović S, Dinjens WNM, van Velthuysen MF
The Journal of Pathology: Clinical Research 2021; 7(5):432-437

The tubarial glands paper: A starting point. A reply to comments.
Valstar MH, de Bakker BS, Steenbakkers RJHM, de Jong KH, Smit LA, Klein Nulent TJW, van Es RJJ, Hofland I, de Keizer B, Jasperse B, Balm AJM, van der Schaaf A, Langendijk JA, Smeele LE, Vogel WV.
Radiotherapy and Oncology. 2021; 154: 308-311

Risk of breast cancer in women with a previous salivary gland carcinoma or pleomorphic adenoma in the Netherlands.
Valstar MH, Schaapveld M, van den Broek EC, van Velthuysen MF, de Ridder M, Schmidt MK, van Dijk BAC, Balm AJM, Smeele LE.
Cancer Medicine. 2021; 10(1):424-434

The tubarial salivary glands: A potential new organ at risk for radiotherapy.
Valstar MH, de Bakker BS, Steenbakkers RJHM, de Jong KH, Smit LA, Klein Nulent TJW, van Es RJJ, Hofland I, de Keizer B, Jasperse B, Balm AJM, van der Schaaf A,
Langendijk JA, Smeele LE, Vogel WV.
Radiotherapy and Oncology. 2021; 154: 292-298

Validated prognostic nomograms for patients with parotid carcinoma predicting 2-and 5-year tumor recurrence-free interval probability. Peeperkorn S, Meulemans J, Lierde CV, Laenen A, **Valstar MH**, Balm AJ, Delaere P, vander Poorten V
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Natural history of Recurrent Pleomorphic Adenoma: Implications on management.
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Head Neck. 2020; 42(8):2058-2066

High CXCR4 expression in adenoid cystic carcinoma of the head and neck is associated with increased risk of locoregional recurrence.
Klein Nulent TJW, van Es RJJ, **Valstar MH**, Klein Gunnewiek R, Smit LA, Smeele LE, Zuithoff NPA, de Keizer B, de Bree R, Willems SM.
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Prostate-specific membrane antigen (PSMA) expression in adenoid cystic carcinoma of the head and neck.
Klein Nulent TJW, **Valstar MH**, Smit LA, Smeele LE, Zuithoff NPA, de Keizer B, de Bree R, van Es RJJ, Willems SM.
BMC Cancer. 2020; 20(1):519

Prostate specific membrane antigen positron emission tomography/computed tomography imaging as a potential tool to assess and guide salivary gland irradiation.
Valstar MH, Owers EC, Al-Mamgani A, Smeele LE, van de Kamer JB, Sonke JJ, Vogel WV.
Physics and Imaging in Radiation Oncology 2019;9: 65-68

Clinicopathological characteristics and outcome of 31 patients with ETV6-NTRK3 fusion gene confirmed (mammary analogue) secretory carcinoma of salivary glands.
Boon E, **Valstar MH**, van der Graaf WTA, Bloemena E, Willems SM, Meeuwis CA, Slootweg PJ, Smit LA, Merkx MAW, Takes RP, Kaanders JHAM, Groenen PJTA, Flucke UE, van Herpen CML.
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Salivary gland pleomorphic adenoma in the Netherlands: A nationwide observational study of tumor incidence, malignant transformation, recurrence, and risk factors for recurrence.
Valstar MH, de Ridder M, van den Broek EC, Stuiver MM, van Dijk BAC, van Velthuysen MLF, Balm AJM, Smeele LE.
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