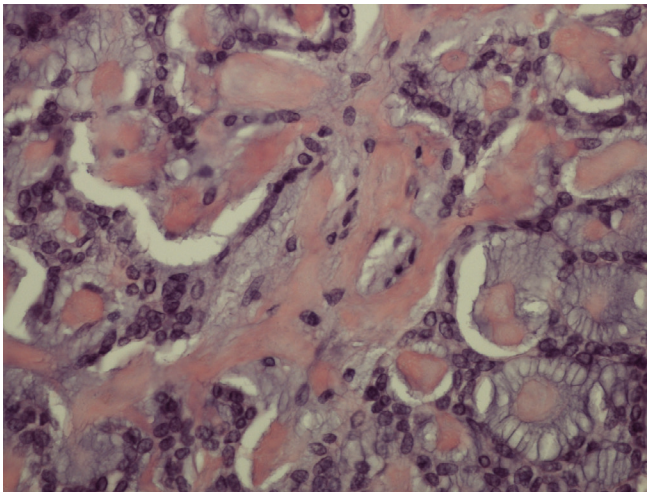


**KAROLIINA HIRVONEN**

**Adenoid Cystic Carcinoma of Salivary Glands:  
Diagnostic and Prognostic Factors and  
Treatment Outcome**



DEPARTMENT OF OTORHINOLARYNGOLOGY — HEAD AND NECK SURGERY  
HEAD AND NECK CENTER, HELSINKI UNIVERSITY HOSPITAL  
AND  
FACULTY OF MEDICINE  
DOCTORAL PROGRAMME IN CLINICAL RESEARCH  
UNIVERSITY OF HELSINKI

Department of Otorhinolaryngology – Head and Neck Surgery  
Head and Neck Center  
Helsinki University Hospital  
and  
Faculty of Medicine, Doctoral Programme in Clinical Research  
University of Helsinki  
Helsinki, Finland

**ADENOID CYSTIC CARCINOMA  
OF SALIVARY GLANDS**

**DIAGNOSTIC AND PROGNOSTIC FACTORS  
AND TREATMENT OUTCOME**

**Karoliina Hirvonen**

ACADEMIC DISSERTATION

To be presented, with the permission of  
the Faculty of Medicine, University of Helsinki, for public examination in the  
Richard Faltin lecture hall at Surgical Hospital, Kasarmikatu 11-13,  
on December 1st, 2017 at 12 noon.

## **Supervised by**

Professor Antti Mäkitie

Department of Otorhinolaryngology – Head and Neck Surgery  
Faculty of Medicine, University of Helsinki, and  
Head and Neck Center, Helsinki University Hospital,  
Helsinki, Finland

Docent Leif Bäck

Department of Otorhinolaryngology – Head and Neck Surgery  
Head and Neck Center, Helsinki University Hospital, and  
Faculty of Medicine, University of Helsinki,  
Helsinki, Finland

## **Reviewed by**

Docent Heikki Teppo

Department of Otorhinolaryngology  
Kanta-Häme Central Hospital  
Hämeenlinna, Finland

Docent Jaana Rautava

Department of Oral Pathology  
University of Turku  
Turku, Finland

## **Opponent**

Professor Vincent Vander Poorten

Department of Otorhinolaryngology – Head and Neck Surgery  
Leuven University Hospital,  
Leuven, Belgium

Cover image: Dr Jaana Hagström

*Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam  
Universitatis Helsinkiensis*

ISBN 978-951-51-3727-2 (paperback)

ISBN 978-951-51-3728-9 (PDF)

ISSN 2342-3161 (paperback)

ISSN 2342-317X (PDF)

<http://ethesis.helsinki.fi>

Layout: Petteri Hirvonen

Hansaprint, Vantaa 2017

***To Petteri, Olavi, Viola, and Laila***

*Success isn't about the end result,  
it's about what you learn along the way.*  
- Vera Wang

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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

- I Hirvonen K, Bäck L, Saarilahti K, Leivo I, Hagström J, Mäkitie AA. Pattern of recurrent disease in major salivary gland adenocystic carcinoma. *Virchows Arch* 2015;467:19-25.
- II Hirvonen K, Bäck L, Haglund C, Leivo I, Jouhi L, Mäkitie AA\*, Hagström J\*. Toll-like receptor 5 and 7 expression in adenoid cystic carcinoma of major salivary glands. *Tumour Biol* 2016;37:10959-10964.
- III Hämetoja H\*, Hirvonen K\*, Hagström J, Leivo I, Saarilahti K, Apajalahti S, Haglund C, Mäkitie AA\*, Bäck L\*. Early stage minor salivary gland adenoid cystic carcinoma has favourable prognosis. *Virchows Arch* 2017 Jun 10. doi: 10.1007/s00428-017-2163-6
- IV Hirvonen K, Rantanen M, Haapaniemi A, Pitkäniemi J, Malila N\*, Mäkitie AA\*. Second primary cancer after major salivary gland carcinoma. *Head Neck* 2017 Sep 29. doi: 10.1002/hed.24937

\*Equal contribution

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## **ABBREVIATIONS**

SGC	Salivary gland carcinoma
ACC	Adenoid cystic carcinoma
MaACC	Major salivary gland adenoid cystic carcinoma
MiACC	Minor salivary gland adenoid cystic carcinoma
MaSGC	Major salivary gland carcinoma
WHO	World Health Organization
RT	Radiotherapy
IMRT	Intensity-modulated radiotherapy
CRT	Chemoradiotherapy
CT	Computed tomography
PET	Positron emission tomography
MRI	Magnetic resonance imaging
DWI	Diffusion-weighted MRI
FS	Frozen section
FNAB	Fine needle aspiration biopsy
OS	Overall survival
DSS	Disease-specific survival
DFS	Disease-free survival
EBV	Epstein-Barr virus
HPV	Human papilloma virus
HNSCC	Head and neck squamous cell carcinoma
OTSCC	Oral tongue squamous cell carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
TLR	Toll-like receptor
LPS	Lipopolysaccharide
N/A	Not available
FCR	Finnish Cancer Registry
SIR	Standardized incidence ratio
SPC	Second primary cancer
TNM	Tumor, node, metastasis classification

## **ABSTRACT**

Salivary gland carcinoma (SGC) is a rare cancer that comprises less than 5% of all head and neck malignancies. In Finland, approximately 60 new SGC cases appear annually. The diagnostics of SGCs is challenging because of their histologic heterogeneity. SGCs are divided into 22 entities. In some studies, mucoepidermoid carcinoma is the most frequent type, followed by adenoid cystic carcinoma (ACC). In a Finnish nationwide study, ACC was the most common histologic type. ACC has three different growth patterns: cribriform, tubular, and solid.

The clinical course of ACC is usually slow but at the same time aggressive, and the long-term prognosis is poor. Perineural invasion is common, and locoregional and distant metastases are relatively usual. The treatment of ACC includes surgery, with its extent depending on the stage and location of the tumor, and post-operative radiotherapy (RT) in some cases. Chemoradiotherapy (CRT) may be used in patients with advanced, recurrent, or metastatic disease, but the impact on survival is modest. The prognosis has great diversity. The location of the tumor, histology, margin status, N class and presence of distant metastases are known factors affecting survival. Despite local aggressive therapy, over half of the patients will have disease recurrence. Distant metastases, the lungs being the most frequent site, are more common than locoregional recurrences.

In this thesis study, the clinical data and treatment outcome of major and minor salivary gland ACC were retrieved for the time periods 1974–2009 and 1974–2012, respectively, in the Helsinki University Hospital district area. This cohort included 54 major salivary gland adenoid cystic carcinoma (MaACC) patients and 68 minor salivary gland carcinoma ACC (MiACC) patients. Most (96% of MaACC and 94% of MiACC) patients were treated with curative intent. Local or distant disease recurrence appeared in 62% of MaACC and 52% of MiACC patients during follow up. Recurrence was local in 35% (MaACC) and 30% (MiACC) of the patients, and regional in 8% (MaACC) and 13% (MiACC) of the patients. Distant metastases appeared in 50% (MaACC) and 34% (MiACC) of the patients. Except for three patients, distant metastases appeared within 10 years after diagnosis. For patients treated with curative intent, the 5-year overall survival (OS) and disease-specific survival (DSS) rates were, respectively, 69% and 71% (MaACC) and 70% and 79% (MiACC). The 10-year OS and DSS rates were, respectively, 54% and 62% (MaACC) and 42% and 52% (MiACC), showing a poorer long-term prognosis for MiACC patients. In our study of MiACC, patients with stage I disease had

significantly better survival compared with patients with other disease stages. Our results indicate that stage II MiACC should be considered as a truly advanced disease needing aggressive treatment. In addition, prolonged follow up with imaging should be considered for ACC, as most distant metastases appear within 10 years.

Toll-like receptors (TLRs) are pattern-recognition receptors participating in immunological first-line host defense, particularly in inflammatory responses against exogenous pathogens. TLRs also have a role in innate immunity and are expressed in different cancers. They are thought to have both tumor-progressing and tumor-inhibiting effects. In SGCs, their role remains undetermined. In this study, the expression of TLR5 and 7 in MaACC was investigated. Thirty-four primary tumor samples and six metastases were available for immunohistochemistry. Both TLRs were expressed in both primary tumors and in metastases. Expression was very heterogeneous, and positivity and expression patterns varied within the same tumor. It was found that low TLR5 and 7 expression, respectively, correlated with male gender and a solid growth pattern. A solid growth pattern is known to have a worse prognosis based on many other studies. No correlation was found between survival and either of these TLRs.

Second primary cancers (SPCs) are a cause of significant mortality after many cancer types. They may be treatment-related as well as caused by the influence of lifestyle factors, environmental exposures, syndromes, genetic factors, or a combination of aforementioned. In this study, the risk of MaACC patients developing SPC during their lifetime was investigated. Finnish Cancer Registry data from 1953–2014 were used. Altogether, 1727 MaACCs occurred during this time period and 222 SPCs were detected. MaACC patients had a 43% higher risk for SPC compared with the cancer risk of the general population. The risk was most elevated for thyroid cancer, but the risk for melanoma of the skin, other skin cancers, breast cancer, cancer of the respiratory organs, and cancer of the male genital organs was also significantly higher. The SPC risk was higher during the first years after SGC diagnosis, remained the same as that of the general population between 5 and 10 years after diagnosis, and then started to elevate again, being highest after 20 years of follow up. Based on our findings, a prolonged follow-up time is worth considering in this patient population.

## SUMMARY IN FINNISH

Sylkirauhassyöpä on harvinainen syöpä, joka kattaa alle 5 % kaikista pään ja kaulan alueen pahanlaatuisista kasvaimista. Suomessa todetaan vuosittain noin 60 uutta sylkirauhassyöpää. Näiden kasvainten diagnostiikka on haastavaa niiden histologisen monimuotoisuuden vuoksi. Sylkirauhassyöpät jaetaan 22 histologiseen alatyyppiin. Joissain tutkimuksissa mukoepidermoidi karsinooma on tavallisin alatyyppi ja adenokystinen karsinooma (engl. adenoid cystic carcinoma, ACC) seuraavaksi yleisin. Suomalaisessa maanlaajuisessa tutkimuksessa ACC oli tavallisin histologinen alatyyppi. ACC:llä on kolme erilaista kasvutapaa: kribriforminen, tubulaarinen ja solidi.

ACC:n kliininen eteneminen on yleensä hidas, mutta samaan aikaan aggressiivinen ja pitkän ajan ennuste on huono. Perineuraalinen invaasio on yleistä ja paikallinen uusiutuminen, kaulaetäpesäkkeet sekä kaukoetäpesäkkeet ovat kohtalaisen tavallisia. ACC:n ensisijainen hoitomuoto on kirurgia, jonka laajuuden määrittää kasvaimen luokitus ja sijainti, sekä leikkauksen jälkeinen sädehoito tietyissä tapauksissa. Kemosädehoitoa voidaan käyttää potilaille, joiden syöpä on pitkälle edennyt tai levinnyt, mutta sen vaikutus eloonjäämiseen on vaatimaton. Ennuste vaihtelee paljon. Kasvaimen sijainti, histologia, leikkausmarginaali, paikalliset- ja kaukoetäpesäkkeet ovat tiedettyjä ennusteeseen vaikuttavia tekijöitä. Paikallisesta aggressiivisesta hoidosta huolimatta tauti uusiutuu puolella potilaista. Kaukoetäpesäkkeet ovat tavallisimpia ja keuhkot ovat niiden yleisin sijainti.

Väitöskirjassa selvitettiin Helsingin yliopistollisessa sairaalassa vuosina 1974-2009 hoidettujen pienten sylkirauhasten ja vuosina 1974-2012 hoidettujen suurten sylkirauhasten ACC-potilaiden kliiniset piirteet, hoito ja ennuste. Aineisto koostui 54 potilaasta jolla oli suurten sylkirauhasten syöpä ja 68 potilaasta jolla oli pienten sylkirauhasten syöpä. Suurimman osan (96 % pienten ja 94 % suurten sylkirauhasten syövistä) hoitotavoite oli kuratiivinen. Noin kahdelle kolmasosalle (62 %) suurten sylkirauhasten ja 48 % pienten sylkirauhasten syöpäpotilaista tuli seurannassa paikallinen uusiutuma, kaulaetäpesäke tai kaukoetäpesäke. Uusiutuma oli paikallinen 35 % potilaista suurten sylkirauhasten syövässä ja 30 % potilaista pienten sylkirauhasten syövässä. Kaulaetäpesäkkeitä ilmaantui 8 % suurten ja 13 % pienten sylkirauhasten syöpäpotilaalle. Suurten sylkirauhasten syövässä kaukoetäpesäkkeitä ilmaantui 50 % potilaista ja pienten sylkirauhasten syövässä 34 % potilaista. Kolmea potilasta lukuun ottamatta kaukoetäpesäkkeet ilmaantuivat 10 vuoden sisällä diagnoosista. Kuratiivisesti hoidettujen potilaiden

5 vuoden kokonaiseloönjääminen oli 69 % suurten sylkirauhasten syövissä ja 70 % pienten sylkirauhasten syövissä ja 5 vuoden tautikohtainen eloonjääminen oli 71 % suurten ja 79 % pienten sylkirauhasten syövissä. Vastaavasti 10 vuoden kokonaiseloönjääminen oli 54 % suurten ja 42 % pienten sylkirauhasten syövissä ja tautikohtainen eloonjääminen 62 % suurten ja 52 % pienten sylkirauhasten syövissä. Pienten sylkirauhassyöpien pitkäaikaisennuste näyttää tutkimuksessamme olevan huonompi verrattuna suurten sylkirauhasten syöpiin.

Pienten sylkirauhasten syövissä tutkimuksemme mukaan potilaiden, joilla oli TNM-luokituksen mukainen 1. levinneisyysasteen syöpä, ennuste oli merkittävästi parempi kuin niillä potilailla, joilla oli jonkin muun levinneisyysasteen syöpä. Tulostemme mukaan pienten sylkirauhasten 2. levinneisyysasteen syöpää tulisi pitää merkittävästi edenneenä syöpänä ja hoito tulisi näin ollen olla aggressiivinen. Lisäksi on syytä harkita pidennettyä kuvantamisseurantaa, koska suurin osa ACC:n kaukoetäpesäkkeistä ilmaantui 10 vuoden sisällä.

Tollin kaltaiset reseptorit (engl. toll-like receptors, TLRs) ovat proteiineja, jotka osallistuvat elimistön ensivaiheen puolustusreaktioon erityisesti tunnistamalla elimistölle vieraita rakenteita tulehduksellisissa reaktiossa. TLR:t säätelevät myös luontaista immunitettä ja niiden on todettu ilmenevän myös eräissä syövissä. TLR:lla ajatellaan olevan sekä syöpää edistäviä että syöpää hidastavia vaikutuksia, mutta niiden rooli sylkirauhassyövissä on vielä epäselvä. Tässä väitöskirjassa tutkittiin TLR:ien 5 ja 7 esiintymistä suurten sylkirauhasten ACC:ssä. Immunohistokemiallisiin värjäyksiin oli käytettävissä 34 näytettä emokasvaimista ja kuusi etäpesäkkeistä. Molemmat TLR:t esiintyivät sekä alkuvaiheen kasvainnäytteissä että etäpesäkenäytteissä. Esiintyminen oli hyvin monimuotoista ja esiintymisvoimakkuus sekä jakautuminen kudoksessa vaihtelivat samassa kasvainnäytteessä. Vähäinen TLR5:n esiintyminen liittyi miessukupuoleen ja matala TLR7:n esiintyminen solidiin kasvutapaan. Solidin kasvutavan on osoitettu useissa tutkimuksissa liittyvän huonompaan ennusteeseen. Kummallakaan TLR:lla ei havaittu olevan yhteyttä ennusteeseen.

Ensimmäisen syövän jälkeen ilmaantuva uusi syöpä on merkittävä kuolleisuuden aiheuttaja useiden syöpien kohdalla. Uudet syövät voivat ilmentua hoidon seurauksena tai niiden syntyyn voivat vaikuttaa elintavat, elinympäristö, oireyhtymät, geneettiset tekijät sekä näiden tekijöiden yhteisvaikutukset. Väitöskirjassa selvitettiin suurten sylkirauhasten syöpään sairastuneiden potilaiden riskiä sairastua uuteen syöpään pitkän seurannan aikana. Käytössä oli Suomen syöpärekisterin tilastot vuosilta 1953-2014, joissa suurten sylkirauhasten syöpiä oli 1727 potilaalla ja näistä 222 oli todettu uusi syöpä. Suurten

sylikirauhasten syöpäpotilailla oli 43 % korkeampi riski sairastua uuteen syöpään verrattuna normaaliväestön riskiin sairastua ensimmäiseen syöpään. Suurin riski oli sairastua kilpirauhassyöpään, mutta riski sairastua melanoomaan, muihin ihosyöpiin, rintasyöpään, hengityselinten syöpään tai miesten sukupuolielinten syöpään oli myös merkittävästi suurentunut. Riski sairastua uuteen syöpään oli korkeampi ensimmäisinä vuosina sylikirauhassyöpädiagnoosin jälkeen, yhtä suuri kun normaaliväestön riski 5 ja 10 vuoden välillä, jonka jälkeen se jälleen nousi ollen korkein 20 vuoden seurannan jälkeen. Löydöstemme mukaan pidennettyä seuranta-aikaa kannattaa harkita tässä potilasryhmässä.

# 1. INTRODUCTION

Salivary gland cancer is an uncommon disease comprising approximately 3–5% of all head and neck malignancies and approximately 0.5% of all cancers <sup>1</sup>. In this thesis, the term salivary gland carcinoma (SGC) is systemically used because practically all salivary gland cancers are carcinomas, i.e. epithelial in origin. In the Finnish population with 5.5 million inhabitants, the annual incidence of SGCs is 0.6–0.7 per 100,000 persons <sup>2</sup>.

Most SGCs are located in the parotid gland (59–81%), followed by the submandibular gland (6–21%), and the minor salivary glands (7–22%). For unknown reasons, tumors of the sublingual glands are rare (<1%). <sup>3,4</sup> The ratio of benign to malignant tumors varies between sites. In the parotid gland, 15–31% of tumors are malignant, while submandibular gland and minor salivary gland tumors are malignant in 40–60% and 40–90% of cases, respectively. Most (approximately 90%) sublingual tumors are malignant. <sup>4,5</sup> The etiology of SGC is still largely unknown. Prior radiotherapy (RT) and ionizing radiation have associated with an increased incidence of SGC in several studies <sup>6-8</sup>.

SGCs are extremely heterogeneous histologically and have a great diversity of morphological features in both cells and tissues <sup>9</sup>. SGCs are divided into 22 histologic categories according to the latest WHO classification <sup>10</sup>. Mucoepidermoid carcinoma is usually noted as the most common type <sup>4</sup>. In a Finnish series between 1991 and 1996, adenoid cystic carcinoma (ACC) was the most common histologic subtype, followed by mucoepidermoid carcinoma and acinic cell carcinoma <sup>11</sup>. ACC has three different growth patterns. Cribriform is the most common, followed by tubular and solid. Solid is the rarest and the most aggressive type. <sup>9,12</sup>

ACC accounts for approximately 22–28% of all SGCs <sup>11,13,14</sup>. There is a wide age distribution, with a peak incidence during the fifth and sixth decades <sup>15,16</sup>. ACC most often occurs in minor salivary glands, with the palate being the most common location <sup>15,17,18</sup>. The most common symptom of ACC is a slowly growing mass followed by pain caused by perineural invasion (typical of ACC). However, symptoms vary depending on the location. In parotid tumors, facial nerve palsy may occur, while in minor salivary glands, the primary symptoms typically include a mass or ulceration in the palate, dyspnea, nasal obstruction, epistaxis, or eye symptoms. <sup>15</sup>

The treatment of ACC consists of surgery followed by RT in selected cases. Chemoradiotherapy (CRT) is mainly used in advanced, recurrent, or metastatic disease, and there is some evidence that it improves local control <sup>19, 20</sup>. Treatment options for patients with metastatic disease are observation and supportive care, palliative systemic therapy, or inclusion in clinical trials <sup>15</sup>. In selected cases, surgical resection of pulmonary metastases may be beneficial <sup>21</sup>. ACC has a high metastatic rate and poor long-term prognosis. The main factors impacting on survival are tumor location, histology, the presence of neck and distant metastases, and the surgical margin status <sup>22, 23</sup>.

New knowledge on the molecular pathogenesis and on molecular markers is important to identify prognostic markers and to develop alternative treatment modalities. Toll-like receptors (TLRs) have been studied in different cancers, as they are associated with tumor progression and also have tumor growth-inhibiting effects <sup>24</sup>. TLRs in ACC have not previously been studied.

Overall cancer survival has increased. In Finland, the 5-year relative cancer survival rate was 16% in men and 25% in women in 1961–63, while the respective figures were 34% and 46% in 1982–1984, and 66% and 69% in 2012–2014 ([www.cancer.fi](http://www.cancer.fi)). Higher cancer survival and the fact that the population is aging have led to the increased detection of second primary cancers (SPCs) <sup>25</sup>.

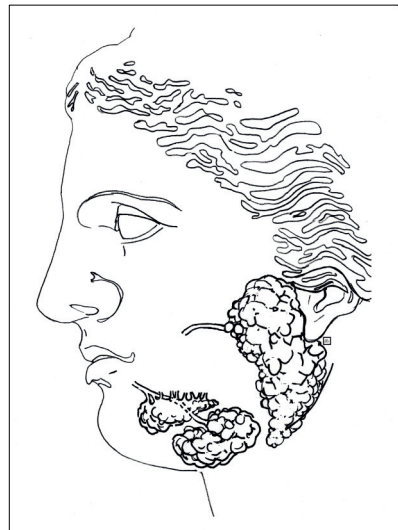
The present study aimed to determine the incidence and long-term outcome of both major and minor salivary gland ACC (MaACC and MiACC, respectively) in a population-based study in the Helsinki University Hospital district area. In addition, the objective was to assess TLR5 and 7 as potential prognostic markers in MaACC. The study also aimed to define the SPC risk of major SGC (MaSGS) patients in a nationwide Finnish Cancer Registry (FCR) series.

## 2. REVIEW OF THE LITERATURE

### 2.1 SALIVARY GLAND CARCINOMA (SGC)

#### 2.1.1 ANATOMY AND MORPHOLOGY OF THE SALIVARY GLANDS

The salivary glands are divided into major and minor glands. The major salivary glands include three paired glands, the parotid, submandibular, and sublingual glands. The minor salivary glands consist of 600 to 1000 glands distributed throughout the mouth, oropharynx, upper respiratory tract, sinonasal tract, and sinuses. <sup>26, 27</sup> The salivary glands have secretory acini and secrete saliva, which can be serous, mucous, or mixed. The parotid duct, or Stensen's duct, secretes almost completely serous saliva into the oral cavity. Within the parotid gland, there may be one or up to more than 20 lymph nodes, mainly in the superficial lobe of the gland. The weight of the parotid gland is 15–30 g, while the submandibular gland weighs approximately 7–16 g. Saliva produced by the submandibular gland is mostly serous, but also a mixture of mucous and serous. The main secretorial submandibular duct is called Wharton's duct, which opens in the floor of the mouth. The smallest major salivary gland, the sublingual gland, weighs approximately 2–4 g and secretes via several ducts into the floor of the mouth or into Bartholini's duct, which then leads to Wharton's duct. Saliva produced by the sublingual gland is mostly of the mucous type. <sup>26, 27</sup> Figure 1 illustrates the location and secretorial ducts of the major salivary glands. The minor salivary glands are either mucous or seromucous. Their size varies from 1–5 mm. <sup>26, 27</sup> The minor salivary glands located in the bronchi, nasal cavity, and larynx secrete mucus instead of saliva, but are still often classified as minor salivary glands, as they mimic the morphology and function of the glands located in the oral cavity <sup>9</sup>.



**Figure 1.**  
*Location and secretorial ducts of the major salivary glands. Courtesy of Dr Seppo Piirainen.*

### **2.1.2 EPIDEMIOLOGY OF AND RISK FACTORS FOR SGC**

SGCs represent approximately 0.5% of all malignancies and 3–5% of all head and neck malignancies <sup>1</sup>. There are approximately 60 new SGC cases in Finland annually. According to the FCR, the corresponding age-adjusted incidence rates between the years 2010–2014 were 0.6 for females and 0.7 for males per 100,000 person-years ([www.cancer.fi](http://www.cancer.fi)). This is similar to other Nordic countries <sup>2</sup>. Inuits in Greenland, Northwestern Canada, and Alaska have a high incidence of SGC. The age-standardized incidence rates between the years 1969–1988 were 6.0 for females and 3.7 for males per 100,000 person-years. <sup>28</sup> The risk remained high after immigration, which supports the idea of important genetic or early-acting environmental factors in the etiology <sup>29</sup>.

There are few known risk factors for SGCs. Exposure to ionizing radiation and prior RT treatment are related to an increased incidence of SGC, especially for mucoepidermoid carcinoma <sup>6-8</sup>. In a case-control study with 64 SGC patients, Spitz et al. <sup>8</sup> also demonstrated that heavy alcohol consumption, hair dye use, and higher education among women are associated with SGCs. In another study with 498 MaSGC patients, these authors showed that an agricultural occupation and previous primary cancer, especially nonmelanoma skin cancer in men, is related to an excess SGC risk <sup>30</sup>.

Lymphoepithelial SGC represents around 1% of SGCs but covers most of the Inuit SGCs. Lymphoepithelial SGC has histological similarities with nasopharyngeal cancer. <sup>26, 28, 31, 32</sup> Epstein-Barr virus (EBV) infection is associated with both nasopharyngeal and lymphoepithelial cancer <sup>33</sup>. EBV, however, seems not to have a role in other SGC types <sup>34</sup>. Patients with Acquired Immunodeficiency Syndrome have an increased risk of lymphoepithelial SGC, which indicates that immunosuppression and oncogenic viral infection might have etiological importance <sup>32</sup>.

Most SGCs are located in the parotid gland (59–81%), followed by the submandibular gland (6–21%) and minor salivary glands (7–22%) <sup>3</sup>. Of all parotid gland lesions, approximately 15–31% are malignant, whereas in submandibular glands and minor salivary glands, the corresponding figures vary between 40–60% and 40–90%, respectively <sup>5</sup>. In a recent Finnish series of 83 submandibular tumors, 30% were malignant <sup>35</sup>.

SGCs are divided into 22 histological subtypes according to the 2017 WHO classification (presented in Table 1). In contrast to the 24 categories of the 2005 WHO classification, the new WHO classification has 22 SGC categories, including

three subentities in the category for poorly differentiated carcinoma. One new SGC type, secretory carcinoma (previously called mammary analogue secretory carcinoma), was added to the latest classification. In addition, one cancer type with uncertain malignant potential, sialoblastoma, is now included in the new classification.<sup>10</sup> Malignant tumors of the salivary glands are staged according to the tumor, node, metastasis (TNM) classification updated by the UICC (presented in Table 2)<sup>36</sup>.

**Table 1.** WHO histological classification of malignant epithelial salivary gland tumors according to WHO/IARC, 4<sup>th</sup> edition<sup>10</sup>

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Mucoepidermoid carcinoma
Adenoid cystic carcinoma
Acinic cell carcinoma
Polymorphous adenocarcinoma
Clear cell carcinoma
Basal cell adenocarcinoma
Intraductal carcinoma
Adenocarcinoma, not otherwise specified
Salivary duct carcinoma
Myoepithelial carcinoma
Epithelial-myoepithelial carcinoma
Carcinoma ex pleomorphic adenoma
Secretory carcinoma
Sebaceous adenocarcinoma
Carcinosarcoma
Poorly differentiated carcinoma
Undifferentiated carcinoma
Large cell neuroendocrine carcinoma
Small cell neuroendocrine carcinoma
Lymphoepithelial carcinoma
Squamous cell carcinoma
Oncocytic carcinoma
<i>Uncertain malignant potential</i>
Sialoblastoma

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**Table 2.** *TNM classification of malignant tumors of the major salivary glands according to UICC, 8<sup>th</sup> Edition* <sup>36</sup>

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<b>T-Primary tumor</b>	
Tx	Primary tumor cannot be assessed
To	No evidence of primary tumor
T1	≤2 cm, no extraparenchymal extension
T2	>2 cm, but ≤4 cm, no extraparenchymal extension
T3	>4 cm, and/or extraparenchymal extension
T4a	Invasion of skin, mandible, ear canal, or facial nerve
T4b	Invasion of base of skull, pterygoid plates, or encloses carotid artery
<b>N-Regional lymph nodes</b>	
Nx	Cervical nodes cannot be assessed
No	No cervical nodes
N1	One ipsilateral metastasis ≤3 cm
N2a	One ipsilateral metastasis >3 cm, but ≤6 cm, no extranodal extension
N2b	Multiple ipsilateral metastases, all ≤6 cm, no extranodal extension
N2c	Bilateral or contralateral metastases, all ≤6 cm, no extranodal extension
N3a	Metastasis >6 cm, no extranodal extension
N3b	Metastasis in single or multiple lymph nodes with clinical extranodal extension
<b>M-Distant metastasis</b>	
Mo	No distant metastasis
M1	Distant metastasis
<b>Stage grouping</b>	
Stage I	T1NoMo
Stage II	T2NoMo
Stage III	T3NoMo
	T1-3N1Mo
Stage IVA	T1-3N2Mo
	T4aN0-2Mo
Stage IVB	T4b, any N, Mo
	Any T, N3, Mo
Stage IVC	Any T, any N, M1

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### **2.1.5 DIAGNOSIS OF SGC**

Painless swelling or palpable resistance of the salivary gland site is the most common symptom in SGC patients<sup>13,37</sup>. Pain is also fairly often experienced; pain was reported by half of the patients in a Finnish series<sup>37</sup>. Patients with minor SGC have different symptoms depending on the site and size of the tumor. These symptoms include painless swelling (due to mouth tumors) and nasal obstruction (due to tumors originating from the sinus area).<sup>38</sup> Strong indicators of malignancy include a rapid increase in the size of the mass, local soft tissue invasion, facial nerve or other neuropathy, and the presence of abnormal regional lymph nodes<sup>13,39</sup>. Electromyography can be used preoperatively to examine the function of the facial nerve more precisely than in clinical examination<sup>40</sup>. Symptom duration varies from less than a month to up to 10 years<sup>13,37</sup>.

Patients with SGCs must undergo a distinct clinical head and neck examination. Cranial nerve function must be assessed. Imaging modality options include ultrasound with or without fine needle aspiration biopsy (FNAB), computed tomography (CT), and magnetic resonance imaging (MRI). FNAB has a specificity of 98% (95% CI: 97–98%) and a sensitivity of 78% (95% CI: 74–82%) in differentiating malignant and benign parotid gland lesions<sup>41</sup>. In a Finnish series of 47 malignant salivary gland tumors, the sensitivity and specificity of FNA was 55% and 92%, respectively, in differentiating malignant and benign lesions<sup>42</sup>. With submandibular tumors, the sensitivity and specificity were 90% and 100%, respectively<sup>35</sup>. Analyses of histological fragments found in FNAB and the use of immunohistochemistry in challenging cases are valuable tools in SGC diagnosis. This method was crucial for the diagnosis in 44% of examined SGCs in a Finnish study<sup>43</sup>. Open biopsy is not recommended due to the risk of nerve damage and seeding of tumor cells<sup>4</sup>.

Frozen sections (FS) can be used during surgery if the diagnosis is preoperatively unclear. FS is used to differentiate between benign and malignant tumors or low- or high-grade malignancies, to determine the extent of the tumor as well as the excision margins, and to assess loco-regional lymph node participation<sup>4</sup>. FS has better sensitivity and specificity than FNAB. In a series of 138 parotid tumors, Fakhry et al.<sup>44</sup> determined that the sensitivity and specificity of FS was 98% and 80%, respectively. However, parotid tumors are often not homogeneous and the FS diagnosis can therefore be misleading. The final clinical decision must be made after careful examination of the whole tumor specimen.<sup>44</sup> The experience of the pathologist is important when considering the diagnosis of SGCs. Determining the correct diagnosis is challenging due to the complexity of the classification and the rarity of the disease<sup>45</sup>.

Ultrasound may be used to detect superficial lesions and to show regional nodes. It is very cost-effective, but it may not always assess deeper structures. CT and MRI reveal the exact location and extent of the tumor. They can also show the perineural extension of the tumor. MRI more accurately shows invasion in soft tissues, diffuse growth patterns, perineural invasion, and lymphadenopathy. MRI is usually the modality of choice.<sup>27, 46</sup> Diffusion-weighted MRI (DWI) is used both in primary diagnosis and in follow up. DWI is especially useful in differentiating pleomorphic adenoma and lymphomas from SGC.<sup>47</sup>

In a study by Razfar et al.<sup>48</sup>, PET (positron emission tomography) -CT displayed a sensitivity of 74%, a specificity of 100%, positive predictive value of 100%, and a negative predictive value of 62% in SGCs. PET-CT is accurate in initial staging and detecting metastatic disease, and may assist in deciding between curative and palliative treatment<sup>48</sup>. The most important application of PET-CT is in follow-up imaging after treatment.

#### **2.1.6 TREATMENT OF SGC**

The standard treatment for SGCs is surgery with or without RT. However, treatment must be individualized for each patient<sup>49</sup>. When the tumor is located in the superficial lobe of the parotis, superficial parotidectomy (removal of all parenchyma superficial to the facial nerve) is the treatment of choice. In partial parotidectomy, only the tumor and surrounding glandular cuff is removed. When the deep parotid lobe is involved, total parotidectomy (removal of all parotid parenchyma) is considered. If the facial nerve must be sacrificed, the procedure is called radical parotidectomy. If the surgery extends to the skin, mastoid, mandible, masticatory muscles, or infratemporal fossa, it is called extended radical parotidectomy.

Stage III and IV parotid tumors often require at least total parotidectomy. However, it is debatable whether total parotidectomy should be performed, even in low-stage tumors, as both the superficial and deep parotid lobes contain lymph nodes and thus have direct contact with the cervical lymphatic field.<sup>50</sup>

The principal goal is that if facial nerve function is normal before operation, it should not be sacrificed. However, if the nerve is preoperatively paralyzed, adherent, or surrounded by tumor, it must be sacrificed. If possible, immediate surgical reconstruction should be performed to achieve the best possible functional outcome. The greater auricular nerve is commonly used for cable

grafting, as it is easily accessed and has a similar, monofascicular structure to the facial nerve.<sup>50-52</sup> The sural nerve is also often used<sup>53, 54</sup>.

For submandibular tumors, in addition to removing the whole gland, clearance of the submandibular triangle (including level I nodes) is recommended. Depending on the tumor type, resection of the close structures (lingual and hypoglossal nerves and digastric, mylohyoid, hyoglossus, and stylohyoid muscles) may be required.<sup>50, 51</sup> In sublingual cancer surgery, the whole gland must be removed. Due to gland location and missing of the encapsulating cervical fascia, removal of the floor of the mouth mucosa, lingual nerve, or part of mandible or submandibular gland is sometimes necessary.<sup>50, 51</sup>

In minor SGCs, wide local resection with clear tumor margins is the treatment of choice. Resectability is planned preoperatively based on location, stage, and histology.<sup>55</sup> Surgery can often influence speech, swallowing, and velopharyngeal competence, as common locations include the palate and base of the tongue<sup>51</sup>.

Neck dissection should be performed when there are clinically apparent metastases or when the primary tumor has a significant risk of developing occult metastases. However, it is debatable how to treat a clinically negative neck, as detecting the true frequency of positive lymph nodes is challenging.<sup>56</sup>

Postoperative RT is recommended in patients with high risk factors such as T3 or T4 tumors, incomplete or close resection margins, high grade, vascular or perineural invasion, positive lymph nodes, as well as in the case of recurrent disease or if the tumor has invaded skin, bone, nerve, or extra-capsular tissue<sup>57, 58</sup>. Surgery combined with postoperative RT compared with surgery alone is superior in terms of 5- and 10-year local control in these cases. However, the role of adjuvant RT in T1 and T2 patients with clear surgical margins has not been confirmed. Primary RT is mainly considered for patients with inoperable disease, unresectable tumor, or for those who refuse surgery.<sup>57</sup> Chemotherapy is mostly reserved for palliative treatment in cases of symptomatic recurrent disease where further surgery or RT does not play a role<sup>57</sup>.

### **2.1.7 PROGNOSIS OF SGC**

The prognosis depends on the localization of the gland, stage, histology, grade, disease extension, facial nerve paralysis, and lymph node status<sup>49, 51</sup>. The stage appears to be superior compared with grading in prognostication<sup>49</sup>. Patient age and positive surgical margins are important factors for locoregional control in the

management of parotid malignancies <sup>49</sup>. Distant metastases are most commonly found in the lungs (80%), followed by bone (15%), liver, and other sites (5%). Distant metastases are the primary cause of death. The metastasis rate is highest (50%) in ACC, adenocarcinoma NOS, carcinoma ex-mixed tumor, small-cell carcinoma, and ductal carcinoma. <sup>49</sup> In a Finnish nationwide series from 1991 to 1996, the 10-year disease-specific survival (DSS) for SGC patients was 64% <sup>11</sup>, and the 5-year relative survival for SGC patients diagnosed in Finland between 2010–2014 was 61% (95% CI: 53–70) <sup>2</sup>. In a German study on 63 SGC patients, the 5-year OS and DFS were 63 and 58%, respectively <sup>59</sup>.

## **2.2 SALIVARY GLAND ADENOID CYSTIC CARCINOMA (ACC)**

### **2.2.1 EPIDEMIOLOGY AND CLINICAL FEATURES OF ACC**

ACC comprises approximately 22% of all SGCs <sup>13</sup>. In Finnish material covering the years 1991–1996, there were 65 ACCs, which formed 27% of all SGC malignancies <sup>11</sup>. In Danish material for the years 1990–2005, altogether 240 ACCs were identified. This series represented 28% of all SGCs during this time period and corresponded to an incidence of 3/1,000,000/year. <sup>14</sup>

ACCs are most commonly located in the minor salivary glands. The most frequent location is the palate, followed by the paranasal sinuses and other oral cavity sites <sup>17, 18, 60</sup>. Spiro <sup>13</sup> reported the location distribution as follows: 19% parotid, 16% submandibular, and 65% minor salivary glands. In the Danish population, 32% of the tumors were parotid, 23% were submandibular, 4% were sublingual, and 40% were of minor salivary gland origin <sup>14</sup>. In a Finnish single-institution series of submandibular tumors, ACC was the most common histology and accounted for 68% of cases <sup>35</sup>.

ACC can also appear in any site where mucous glands exist. Sites other than the head and neck include the breasts, vulva, esophagus, cervix, bronchi, and skin <sup>61-64</sup>. ACC is extremely rare in these locations. Unlike in salivary glands, ACC has a very good prognosis and a low metastasis rate in the breasts <sup>65</sup>.

ACC most often occurs in the fifth and sixth decades, but all age groups are represented <sup>16, 60</sup>. It is extremely rare in children. Female predominance has been reported by many studies, but some have also shown male predominance or no gender predilection. <sup>16, 66, 67</sup>

### 2.2.2 HISTOPATHOLOGY OF ACC

ACC consists of two principal cell types: ductal and modified myoepithelial cells that usually have hyperchromatic, angular nuclei and often a clear cytoplasm <sup>26</sup>. ACC is divided histologically into cribriform, tubular, and solid growth patterns (Figure 2). The cribriform pattern is the most common. The solid pattern is the rarest and has the poorest prognosis. The solid pattern is associated with a high frequency of distant metastases and thus with a high stage. <sup>17, 26, 67</sup> The tubular growth pattern has the best prognosis. Tumors often have different growth patterns, and the dominant pattern determines their classification. <sup>46</sup> Perineural invasion is common, even among early-stage tumors <sup>17, 26</sup>.

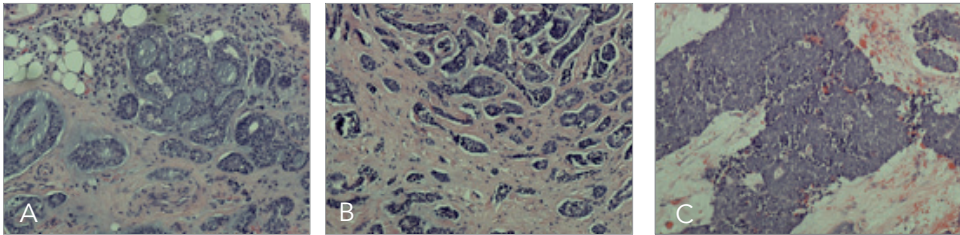
ACC is often graded histopathologically into three grades according to its growth pattern. Grade I includes no solid pattern, grade II includes less than 30% solid pattern, and grade III more than 30% solid pattern <sup>68</sup>. High-grade transformation (HGT) is a rare phenomenon, involving ACC mostly in sinonasal and palatal minor salivary glands and in the submandibular gland. It is histologically a component of conventional ACC and another distinct anaplastic cell population that lacks ductal and myoepithelial dedifferentiation. While these components can be separate, transitional zones exist. HGT ACC appears to have the poorest prognosis and has a high component of lymph node metastases. <sup>69</sup>

### Molecular biology

The molecular pathogenesis of ACC is poorly understood. Studies on chromosomal translocations have been performed to find molecular diagnostic tools as well as target-specific therapies. The most important finding so far was reported by Persson et al. <sup>70</sup>, who observed a frequent t(6;9) (q22-23;p23-24) translocation in ACC that regularly results in a fusion of the myeloblastosis (*MYB*) oncogene with the transcription factor *I/B* (*NFIB*). This fusion oncogene is highly overexpressed and is specific to ACC <sup>71</sup>. A novel finding by Mitani et al. <sup>72</sup> was an *MYBL1-NFIB* gene fusion resulting in a t(8;9) translocation and several rearrangements in the *MYBL1* gene in 35% of t(6;9)-negative ACCs.

C-kit is a tyrosine kinase receptor that is overexpressed in 90% of ACC. While it correlates with tumor grade in ACC, the exact mechanism is still unclear <sup>73, 74</sup>. An increase in MIB-1 antibody expression is associated with more aggressive tumor behavior and a poor prognosis <sup>75</sup>. Neurotrophin-3 receptor TrkC/NTRK3 is also found to be expressed in ACC, suggesting the existence of an autocrine signaling loop in these tumors <sup>76</sup>. Immune checkpoint inhibitors, for example the role of PD-1 inhibition, have also been investigated in ACC <sup>77</sup>

Ho et al.<sup>78</sup> found a low exonic somatic mutation rate and high mutational diversity in their report on 60 ACC tumor/normal pairs. Potential driver mutations were identified, and include those in *PIK3CA*, *TP53*, *PTEN*, *SsMARCA2*, *KDM6A*, and *CREBBP*. These findings indicate aberrant epigenetic regulations in ACC oncogenesis<sup>78</sup>.



**Figure 2.** Different growth patterns of ACC (magnification x 20): cribriformic (A), tubular (B), and solid (C).

### 2.2.3 DIAGNOSIS OF ACC

The symptoms of ACC are dependent on the location of the tumor and do not differ from the SGCs in general (2.1.5). However, perineural invasion and resultant pain are more common findings in ACC than in other SGCs<sup>79-81</sup>. ACC has also been described to grow slower than other SGCs<sup>60, 82</sup>. The first diagnostic procedure is FNAB, as in other SGCs<sup>60, 82</sup>. The imaging modalities used are in general the same as in other SGCs, with MRI usually being the imaging modality of choice. Figure 3 presents an MRI of ACC of the sinonasal area extending into the orbit.



**Figure 3.** T2-weighted coronal MR image of sinonasal ACC extending into the orbit. The bright T2 signal is typical of ACC (A). T1-weighted contrast-enhanced axial MRI of ACC extending into the orbit and causing exophthalmus. The tumor has spread along the maxillary nerve through the foramen rotundum into the right sinus cavernosus (B). Courtesy of Dr Antti Markkola.

## **2.2.4 TREATMENT OF ACC**

### **Treatment of the primary tumor**

The “gold-standard” treatment for ACC is radical surgical resection (including free margins) followed by RT. However, the treatment strategy depends on the site, stage, histological grade, and biological behavior of ACC. <sup>17, 60</sup> ACC has a distinct tendency to spread along the nerves and subperiosteal and perichondral areas; careful consideration of nerve sacrifice and the liberal use of FSs are necessary <sup>50</sup>. Treatment protocols of parotid, submandibular, and sublingual tumors are the same as in SGCs in general (2.1.6). With MiACC, surgical margins are reported to be inadequate in 27–64% of cases; the situation is poorest in the oral cavity and sinonasal area <sup>50</sup>. MiACC is often diagnosed at an advanced stage, especially in the sinonasal area and larynx <sup>50, 55</sup>. The use of endoscopic transnasal surgery has been investigated and supported by some studies, especially in ACC of the ethmoid area <sup>50</sup>.

### **Treatment of the neck**

In parotid ACCs, neck dissection should be performed on patients with clinical evidence of nodal metastases. It has been shown that neck levels I–V are affected in N+ parotid tumors with all histologies <sup>83</sup>. Therefore, it is recommended to remove lymph nodes from all neck levels <sup>50</sup>. Neck metastases are more common in the submandibular gland than in the parotis. This may be due to direct invasion of the tumor or affected soft tissues into adjacent nodes and perineural spread. <sup>50, 54, 51</sup> It has been discussed that all except T1 submandibular carcinomas should warrant elective neck dissection <sup>51</sup>.

It is debatable when to perform elective neck dissection. The incidence of occult metastases in ACC varies between 15–44%, and such metastases are more common in oral cavity/oropharyngeal carcinomas than in sinonasal and MaACCs <sup>84</sup>. In a multicenter study by Amit et al. <sup>85</sup> with both major and minor ACCs included, the occult nodal metastasis rate was 17%. Occult metastases were mainly located in the ipsilateral levels I–III. No significant difference in survival was observed between patients who had or did not have elective neck dissection <sup>85</sup>. ACC with high-grade transformation has a high lymph-node metastasis rate (43–57% of patients), and elective neck dissection is therefore highly recommended for patients with this rare histologic entity <sup>69</sup>.

## **Oncological treatment**

Post-operative RT is recommended if primary surgery has positive or close resection margins, if there is infiltration of major nerve, bone, or muscle, in the case of high-grade histology, paranasal sinus location, and high T and N classification <sup>55, 86</sup>. There is evidence that adjuvant RT increases the survival of ACC patients <sup>87, 88</sup>. In a study of ACCs by Lee et al. <sup>88</sup>, postoperative RT resulted in a 9.5% and 11.6% improvement in 5-year overall survival (OS) compared with surgery alone for pT1-T2 and pT3-4 cancers, respectively. The survival benefit was 23.4% for tumors with positive lymph nodes and 17.1% for positive margin status with combined therapy. Patients with negative margins did not have better survival after adjuvant RT combined with surgery alone. <sup>88</sup> Many centers consider adjuvant RT as the standard of care <sup>12, 18</sup>. The aim is to initiate RT in four weeks after surgery. Intensity-modulated radiotherapy (IMRT) is the treatment of choice. IMRT provides better local control and has fewer side effects, as the surrounding structures receive less radiation. The radiation dose is usually between 66–70 Gy. Primary RT can be used in unresectable cases, if resection would cause unacceptable functional and cosmetic outcome, and in palliative care. <sup>86</sup>

Gentile et al. <sup>89</sup> investigated proton beam therapy with or without chemotherapy for 14 patients with unresectable ACC of the nasopharynx with skull base involvement. Proton beam therapy showed a promising local control rate with three late local failures <sup>89</sup>. This therapy is not currently available in Finland. Patients who would benefit from this modality can be sent to some of the proton centers in Northern Europe. When compared with IMRT only or fractionated stereotactic RT, raster-scanned carbon ion boost combined with IMRT led to better locoregional control, progression-free survival, and OS <sup>90</sup>. CRT with different agents can be used in the management of high-risk tumors. There is some evidence that this approach improves local control. <sup>19, 20</sup>

## **Treatment of metastatic and recurrent ACC**

The current options for patients with metastatic disease are observation and supportive care, palliative systemic therapy, or inclusion in clinical trials investigating novel chemotherapeutic agents <sup>60</sup>.

The lungs are the most common location for distant metastases. However, lung metastases do not often appear as solitary lesions. Metastases may be relatively stable for months and routine thoracotomy is not advisable. <sup>17, 91</sup> Girelli et al. <sup>21</sup> stated that lung metastasectomy should be considered as a therapeutic option to achieve local control when complete surgical resection is possible and when

pulmonary metastases appear more than 36 months after primary treatment. It is still not clear whether incomplete lung resection provides symptomatic benefit <sup>21</sup>.

Chemotherapy has not yet proved to be beneficial, as many ACC tumors have slow growth kinetics. A low response rate in metastatic disease has been shown in many studies, but no standard treatment modality exists. <sup>82</sup> Trials have included small populations with great heterogeneity concerning histology, the state of recurrence, and prior therapies. Furthermore, as ACC has a slow growth pattern, response assessment is difficult. Systemic therapy is mainly reserved for patients with symptoms, rapidly progressive disease, or both. <sup>92</sup>

Targeted therapy is under research. Several potential therapeutic targets have been studied in ACC. These include the tyrosine kinase inhibitors imatinib, gefitinib, and lapatinib, the monoclonal antibodies cetuximab and trastuzumab, and the proteasome inhibitor bortezomib. No real antitumor activity has been shown. There is evidence of disease stabilization for a few months <sup>93</sup>. There are a few case reports of tamoxifen hormone therapy in recurrent ACC, where disease progression slowed down <sup>94</sup>. Several clinical trials are ongoing with the aim to find clinically relevant antitumor activity for these and related targeted agents <sup>82</sup>.

### **2.2.5 PROGNOSIS OF ACC**

Despite local aggressive therapy, approximately 60% of patients will have disease recurrence <sup>95</sup>. Factors that increase local recurrence rate are positive surgical margins, major nerve involvement, and primary tumor origin in a minor salivary gland <sup>96</sup>. The local recurrence rate was 40% in a study of MiACCs <sup>23</sup>. Clinically evident neck metastases at diagnosis are reported to occur in 3–16% of patients, depending on the site of origin and series <sup>23, 84</sup>.

Solid growth pattern, tumor size over 3 cm, primary tumor origin in the nasal cavity/paranasal sinuses or orbit, positive lymph nodes, age over 70 years, or local recurrence all increase the risk for distant metastases <sup>12, 22, 96</sup>. The low long-term survival in ACC is due to frequent distant metastases and the failure to control them. Distant metastases are reported to appear in 35–55% of patients and to be more common than regional recurrences. <sup>67, 91</sup> In a study that only included early-stage (T1-2/No) ACCs, 20% of patients developed distant metastases <sup>12</sup>. These metastases are thought to develop via a hematogenous route. The most common location is the lungs <sup>18, 22, 60, 67, 91</sup>. Another common location is bone, and in this case disease progression is usually rapid <sup>96</sup>. Other locations for distant metastases are the liver, skin, breasts, and rarely the brain <sup>82</sup>. Lung metastases seem to appear

earlier than metastases in other sites. However, according to a study by Van der Wal et al. <sup>91</sup>, patients with lung metastases die approximately a year later than patients with metastases in other locations. In a study by Amit et al. <sup>22</sup>, patients with bone and brain metastases had the poorest outcome, with a 31% and 25% median 5-year survival, respectively. Patients with lung and liver metastases had a 66% and 84% median 5-year survival, respectively <sup>22</sup>.

ACC is thought to have a prolonged natural course, with slow growth and sometimes also extended survival after recurrent disease <sup>97, 98</sup>. Survival figures are presented in Table 3. Five-year OS rates vary between 76–92% <sup>14, 22, 23, 81, 87, 88, 99</sup>. In a Danish series of 240 ACC patients, the 15-year OS rate was 46%, and recurrences appeared as late as after 15 years after primary treatment <sup>14</sup>.

**Table 3.** *Survival rates of patients with ACC*

Study, number of patients	Survival rates (%)			
		5-year	10-year	15-year
Bjorndal et al. 2015; n = 201 <sup>14</sup>	OS	80	58	46
	DSS	90	75	68
	DFS	72	60	55
Ellington et al. 2012; n = 3026 <sup>81</sup>	OS	90	80	69
Amit et al. 2014; n = 489 <sup>22</sup>	OS	76		
	DSS	80		
	DFS	68		
Ciccolallo et al. 2009; n = 2611 <sup>99</sup>	OS	78	65	
He et al. 2017; n = 130 (only MiACCs) <sup>23</sup>	OS	81		
	DFS	56		
Lee et al. 2017; n = 61 <sup>88</sup>	OS	81		
	DFS	56		
Shen et al. 2012; n = 101 <sup>87</sup>	OS	92		
	DFS	63		

In several studies, perineural invasion has associated with local recurrence and margin involvement, but not with rate of metastasis and survival <sup>66, 100</sup>. Other studies have found perineural invasion to be a prognostic factor for OS and DFS

<sup>23, 100, 101</sup>. In an international study by Amit et al. <sup>22</sup>, age, site, N classification, and the presence of distant metastases were independent prognostic factors for OS and DSS and margin status was for OS. In the study by He et al. <sup>23</sup> on MiACC, a solid histologic type and positive surgical margins were independent prognostic markers for OS.

Nomograms are statistical tools that can be used to accurately predict the outcome of an individual patient. They apply also multiple other variables in addition to TNM. <sup>102</sup> Ganly et al. <sup>103</sup> created predictive nomograms for ACC. They used an international database and created four different nomograms based on 438 MaACCs. The 10-year OS nomogram had seven variables: age, gender, clinical T class, tumor site, margin status, pathologic N status and M status; the concordance index was 0.71. Other nomograms predicted the 10-year recurrence-free probability, distant recurrence-free probability, and cancer-specific mortality; the concordance indices were 0.66, 0.64, and 0.70, respectively <sup>103</sup>.

## **2.3 TOLL-LIKE RECEPTORS**

### **2.3.1 TLRs IN IMMUNE DEFENCE AND CANCER**

TLRs are transmembrane pattern-recognition receptors, which participate in immunological first-line host defense, especially in inflammatory responses against different exogenous pathogens. TLRs regulate the innate and adaptive immunity of epithelial cells located at mucosal sites such as skin and respiratory, gastrointestinal, and genitourinary tracts. <sup>104-106</sup> TLRs recognize pathogen-associated molecular patterns and activate immune cells and immune cascades to target the pathogen <sup>24, 107</sup>. Furthermore, TLRs can recognize damage-associated molecular patterns released from injured and inflamed tissue <sup>24, 108</sup>. TLR signaling also regulates apoptosis by expressing either antiapoptotic proteins or apoptosis inhibitors <sup>24</sup>.

The name ‘Toll-like receptor’ comes from their similarity to the protein encoded by the *Drosophila* Toll gene, which was identified in 1985 <sup>109</sup>. There are ten known TLRs in humans and 12 functional TLRs in mice. TLRs 3, 7, 8, and 9 mainly function intracellularly on endosome membranes and detect bacterial DNA and viral RNA <sup>105, 107, 108</sup>. TLRs 1, 2, 4, 5, 6, and 10 are mainly expressed on the cell surface, where they recognize microbial membrane components such as lipopolysaccharides, lipoproteins, and lipids <sup>24, 105</sup>.

There is a well-established connection between the development of cancer and chronic inflammation caused by infection, environmental agent exposure, genetic disease or metabolic disorders. Approximately 20% of cancers are presumed to be associated with chronic infection <sup>110</sup>. Examples of this relationship include hepatitis B virus and hepatocellular carcinoma, *Helicobacter pylori* and gastric carcinoma, condylomata accuminata and genital cancer, Human papilloma virus (HPV) and oropharyngeal cancer, and polyoma virus and Merkel cell carcinoma <sup>111-115</sup>.

In addition to providing signals crucial to the resolution of inflammation, TLRs also have a distinct role in cancer. They act as double-edged swords, as they possess both pro- and antitumor effects. <sup>104</sup> The role of TLRs in cancer is complex and under active research. TLR activation in tumor cells may promote the survival of these cells, as well as chemoresistance, ultimately leading to tumor progression. Many tumor cells or cell lines express one, or more commonly several TLRs. Each TLR is thought to have a particular effect on cancer, and this effect depends on both the receptor and tumor type. <sup>24, 104, 105</sup> TLRs can have antitumor effects by activating host immune responses. Bacterial components, such as DNA and endo- or exotoxins, exhibit antitumor effects partly through enhanced innate immunity. In immunotherapy, microbe-derived therapeutics stimulate TLR signaling and activate innate and adaptive immune responses. For example, OK-432, a TLR4 agonist used as an immunotherapeutic agent in many cancers, is a penicillin-killed and lyophilized preparation of a low-virulence *Streptococcus pyogenes* strain. <sup>104</sup> *Mycobacterium bovis* bacillus Calmette-Guerin, which is used against superficial bladder tumors, is also a TLR2/4 agonist <sup>24</sup>. Targeting TLRs with TLR agonists might be a future weapon in the fight against cancer. However, not all TLR agonists and signaling pathways have antitumor activity. It is important to elucidate the function and regulation of each TLR in different cancers to gain more information on tumor progression and initiation. <sup>24, 104</sup>

### **2.3.2 TLRs IN HEAD AND NECK CANCERS**

Different TLRs have been studied in oral squamous cell carcinoma. TLR5 is expressed in oral tongue squamous cell carcinoma (OTSCC), and was shown to be an independent predictor of mortality and disease recurrence <sup>116</sup>. In another study on early-stage OTSCC, negative or low TLR5 expression predicted poor DSS, and expression of TLRs 2, 4, and 9 was associated with the invasive potential of the tumor <sup>117</sup>. These TLRs are suggested to have a role in both the development and progression of OTSCC, as they were expressed in primary and recurrent tumors, as well as in neck metastases <sup>118</sup>. TLR3 is overexpressed in oral squamous cell

tumors with high-risk histopathological features <sup>119</sup>. TLR2 is expressed in oral squamous cell cancer, and its activation leads to growth-promoting effects. Blocking the TLR2 receptor with a monoclonal antibody leads to the inhibition of tumor growth in both *in vitro* and *in vivo* studies. <sup>120</sup> In another study on oral squamous cell carcinoma, low tumor TLR7 expression and high stromal TLR7 expression predicted a good outcome <sup>121</sup>. Jouhi et al. <sup>122</sup> observed low TLR5 expression and high TLR7 expression in p16-positive oropharyngeal squamous cell carcinoma (OPSCC) both *in vivo* and *in vitro*. TLR7 and 9 staining also correlated with HPV status *in vivo* <sup>122</sup>. In addition, they showed that high TLR5 and low TLR7 expression may be related to a poor prognosis among HPV-positive OPSCC patients <sup>123</sup>.

In recurrent or metastatic head and neck squamous cell carcinoma (HNSCC), the TLR8 agonist motolimod combined with cetuximab was used in a Phase Ib clinical trial with encouraging antitumor activity <sup>124</sup>. In addition, the TLR9 agonist IMO-2055 was tested in combination with 5-fluorouracil, cisplatin, and cetuximab in similar patients as a first-line palliative treatment in a Phase Ib trial. This trial was, however, discontinued due to severe side effects <sup>125</sup>.

In a study on laryngeal papillomas transforming into squamous cell carcinoma, TLR4 expression was significantly lower in papillomas transforming into cancer than in papillomas without malignant transformation. In laryngeal squamous cell cancer, high TLR4 expression correlated with the tumor grade and the T class, and in this regard, possibly with tumor aggressiveness. <sup>126</sup> TLRs have also been investigated in juvenile nasopharyngeal angiofibroma, where TLR3 negativity correlated with a high tumor stage <sup>127</sup>.

### **2.3.3 TLRs IN SALIVARY GLAND CARCINOMAS**

There have only been a few studies on TLRs in SGCs. Korvala et al. <sup>128</sup> examined TLR9 expression in mucoepidermoid carcinoma. Low expression was associated with a high disease stage and a poor prognosis <sup>128</sup>. In a study by Park et al. <sup>129</sup>, TLR5 activation by flagellin, a TLR5 agonist, promoted tumor migration and invasion in salivary gland adenocarcinoma.

## **2.4 SECOND PRIMARY CANCER**

### **2.4.1 GENERAL CONSIDERATIONS**

Survival after cancer treatment has significantly improved over several decades due to improved early detection, treatment, and supportive care. In the United States, 5-year relative cancer survival has increased from under 50% in 1975–1979 to 67% in 2005–2009 (including all cancers). Survival is over 80% in childhood cancer.<sup>25</sup> The same trend is seen in Finland, where the 5-year relative cancer survival for men and women, respectively, was 16% and 25% in 1961–63, 34% and 46% in 1982–1984, and 66% and 69% in 2012–2014 ([www.cancer.fi](http://www.cancer.fi)). This fact, together with the aging population, has led to the current situation where approximately one out of six cancers appear among cancer survivors in the United States<sup>25</sup>. In Finland during 1975–2014 ([www.cancer.fi](http://www.cancer.fi)), the risk for SPCs was approximately 38% higher compared with the risk of developing a first cancer (SIR 1.38, 95% CI: 1.37-1.39).

SPCs are a significant cause of death among many cancer survivors, for example for survivors of Hodgkin's lymphoma<sup>130</sup>. SPCs can be treatment-related or caused by the influence of lifestyle factors, environmental exposures, or syndromes. Combinations of different influences, such as gene–environment and treatment and lifestyle interactions, may also play a role.<sup>131</sup> For survivors of childhood cancer, the most likely etiologic causes are primary cancer treatments and genetic factors, whereas for adults, in addition to these factors, environmental and lifestyle factors may also be etiologic causes<sup>132</sup>.

RT, certain chemotherapy regimens, and hormone treatment have the potential to cause second cancers in patients. Radiation exposure has been found to increase the SPC risk materially in brain, thyroid, female breasts, skin, bone, and soft tissue cancers. A modestly increased risk is seen in cancers of the lungs, bladder, and gastrointestinal tract, and in myeloid leukemias. The type of tissue, radiation dose, and the time period over which the radiation occurred, as well as the time delay since exposure, all play roles in the potential risk.<sup>133</sup>

Tobacco use is one of the major risk factors for SPC. Known tobacco-related cancer sites for second primary cases are the lungs and upper aerodigestive tract<sup>134</sup>. Smoking and alcohol consumption are estimated to account for more than 35% of the SPC risk<sup>135</sup>.

#### **2.4.2 SPCS IN PATIENTS WITH HEAD AND NECK CANCERS**

SPCs following HNSCCs most commonly express the same histology and appear in the upper and lower aerodigestive tract, but also in other tobacco-related sites<sup>136, 137</sup>. In a cohort of 190,468 head and neck cancer patients, RT did not increase the SPC risk, indicating that environmental and lifestyle factors play a larger role in these sites<sup>138</sup>. Field cancerization, described by Slaughter in 1956<sup>139</sup>, is often cited in describing the development of SPCs in squamous cell carcinoma. It is assumed that after the tumor is radically removed, genetically altered field could be the origin for a new cancer<sup>140</sup>.

#### **2.4.3 SPCS IN PATIENTS WITH SALIVARY GLAND CARCINOMAS**

Studies on the SPC risk among SGC patients are scarce. The association between SGC and second primary breast cancer has been the most studied. Some studies have found a positive relationship between these two cancers, with standardized incidence ratio (SIR) values ranging from 2.3–4.8<sup>141-144</sup>. However, other studies have not found an increased breast cancer risk after SGC<sup>145, 146</sup>. In a series of 782 SGC patients, Biggar et al.<sup>145</sup> detected an increased ovarian SPC risk in women and respiratory SPC risk in men. There have been two previous studies on MaSGC and SPC risk. Spitz et al.<sup>147, 148</sup> studied MaSGC patients from 1960–1981 and compared them with the general population. They found a significantly elevated risk of skin cancer both as an SPC as well as prior to SGC. Megwalu and Shin<sup>146</sup> used a database of 15,572 MaSGC patients in the United States and found an elevated risk of SPCs of the oral cavity, salivary glands, thyroid, lungs, and kidney. The risk remained elevated even 10 years after treatment of the primary cancer. RT treatment increased the risk for thyroid, laryngeal and lung/bronchus cancers, while oral SPC was not associated with RT<sup>146</sup>.

### **3. AIMS OF THE STUDY**

The main objective of this study was to assess the factors that determine the long-term outcome of ACC patients. As finding new prognostic markers for these carcinomas is important, the intention was to clarify the role of TLR5 and 7 in MaACC. The aim was also to determine whether there is a risk for certain SPCs, because some SGC patients have long survival after treatment. Knowledge of such a risk could be considered in planning of the follow up for SGC patients.

The specific aims of the present study were:

1. To evaluate and compare the clinical and histological characteristics of both major and minor salivary gland ACC during the past 35 years at our department in order to clarify their influence on patient survival (Studies I and III).
2. To examine the expression of TLR5 and 7 in major salivary gland ACC and their association with clinical characteristics, including survival (Study II).
3. To investigate the risk of developing an SPC during a long-term follow up of MaSGC patients (Study IV).

## **4. MATERIALS AND METHODS**

All patients in Studies I, II, and III were diagnosed and treated at the Helsinki University Hospital, which is a tertiary care hospital covering an area of approximately 1.6 million inhabitants. In Study IV, the patient cohort consisted of a nationwide series in Finland (population 5.5 million).

### **4.1 STUDY I**

In this study, the characteristics, treatment, and outcome of 54 MaACCs diagnosed between January 1974 and September 2009 in the Helsinki University Hospital district area were described. All patient records were reviewed and the tumor sample histopathology was re-evaluated by two experienced head and neck pathologists (Drs Jaana Hagström and Ilmo Leivo). The diagnostic criteria were validated according to the WHO classification (2005). Tumor staging was performed according to the TNM classification (UICC 2009, 7<sup>th</sup> edition). Causes and dates of death were retrieved from Statistics Finland. All patients had a minimum follow up of five years or until death. OS, DFS, and DSS were calculated from the last day of treatment to the last day of follow up or death (OS), to any sign of tumor recurrence or death (DFS), or to any sign of tumor recurrence or death due to disease (DSS).

### **4.2 STUDY II (IMMUNOHISTOCHEMISTRY)**

For the 54 MaACC patients, 34 primary tumor samples and six metastases were identified. All of these samples were used for immunohistochemical evaluation. For the remaining 20 primary tumors, no sufficient tumor samples existed. The Helsinki Head and Neck Research Group has earlier studied TLR5 and 7 in OPSCC, yielding results on their impact on the prognosis and the viral status. Therefore, these same TLRs were chosen to examine their role in this non-viral carcinoma.

Tissue samples were sliced into 4- $\mu$ m sections. These sections were deparaffinized in xylene and rehydrated in a series of graded alcohol. The slides were heated in a Pretreatment Module (Lab Vision Corp., UK Ltd, UK) in Tris-HCl buffer for TLR7 (pH 8.5) and Tris-EDTA buffer for TLR5 (pH 9.0), followed by endogenous

peroxidase blocking with 0.3% Dako REAL Peroxidase-Blocking Solution (Agilent, Santa Clara, CA, USA). For immunostaining, monoclonal mouse anti-human antibody was used for TLR5 (1:200, IMG-664A Imgenex, Cruz Biotechnology, Dallas, Texas, USA) and polyclonal rabbit anti-human antibody was used for TLR7 (1:300, IMG-581A Imgenex). This was followed by incubation with Dako REAL Antibody Diluent S2022 (Agilent) and staining, which was visualized with Dako REAL DAB+ Chromogen (Agilent). Between each phase, slides were washed with PBS 0.04%-Tween20. Counterstaining was performed with Meyer's hematoxylin and slide mounting with PERTEX (Histolab, Askim, Göteborg). The positive control was tonsillar squamous cell carcinoma tissue. Slides in which the primary antibody was replaced with PBS served as negative controls.

### **Immunoscore (Study II)**

The slides were scored by two independent researchers (J.H. and K.H.) who were blinded to the clinical data. The location (cytoplasm, nucleus, or cell membrane) of TLR5 and 7 expression in the specimen was determined, and the percentage of positively-stained cells was then estimated. Immunoscores were grouped as follows: 0 for 0–10% (negative or very mild), 1 for 11–40% (mild), 2 for 41–70% (moderate), and 3 for 71–100% positivity (strong). Normal salivary gland tissue, which was found in most slides, was used to validate the expression in normal tissue.

## **4.3 STUDY III**

The characteristics, treatment, and outcome of 68 patients with MiACC diagnosed between 1974 and 2012 were described. Out of the records of 86 patients, the diagnosis was correct and medical records were available for 68 patients. The results were compared with those of MaACC. Histopathological diagnoses were confirmed and updated according to the WHO classification (2005) by an experienced head and neck pathologist (J.H.). Statistics Finland provided causes and dates of death. OS, DSS, and DFS were defined as in Study I. All patients had a minimum follow up of three years or until death.

## **4.4 STUDY IV**

FCR data were used to collect MaSGCs that were registered in Finland during 1953–2014. Selection of the patient cohort is illustrated in Figure 4. First, all

MaSGC topographies (n = 3055) were collected. The WHO classification (2005) was then applied to define the tumor morphology and non-salivary gland morphologies were excluded (n = 1328). Squamous cell carcinomas were also excluded, even though they can rarely have salivary gland morphologies. However, most of these carcinomas were metastases of other head and neck carcinomas. Considering mucoepidermoid carcinoma, this was coded under mucoepidermoid tumor (low or high grade) in the WHO tumor classification until 1991. In the FCR, it was coded as squamous cell carcinoma until 2007, when the ICDO-3 coding became available. A search was conducted for the term “mucoepidermoid” from the original pathology reports, and 155 cases were found to be counted as SGCs. As mixed tumors were not included in the WHO 2005 tumor classification, 30 pathology reports were randomly selected from a cohort of 195 mixed tumor samples found in the FCR data. These tumors were ex-pleomorphic adenomas, as well as malignant myoepitheliomas; these were kept in the SGC cohort. Altogether, 1727 MaSGCs were used for SPC analyses. SPC was defined as any new cancer after SGC with histology. The follow-up time was counted from the date of SGC diagnosis to the SPC diagnosis, emigration from Finland, death, or December 31, 2014. In addition to FCR data on RT, a nationwide hospital inquiry was used to complete the information.

TOPOGRAPHIES INCLUDED		MORPHOLOGIES EXCLUDED		STUDY COHORT	
	n		n		n
Co7.9 Parotid Gland	2238	8000 Neoplasma NOS	216	Co7.9 Parotid Gland	1258
Co8.0 Submandibular Gland	120	8010 Carcinoma NOS	306	Co8.0 Submandibular Gland	72
Co8.1 Sublingual Gland	4	8070 Squamous Cell Carcinoma	337	Co8.1 Sublingual Gland	3
Co8.9 Major Salivary Gland NOS	693	9591 Lymphoma	249	Co8.9 Major Salivary Gland NOS	394
		Other 40 morphologies (n between 1 and 55)	220		
TOTAL	3055	TOTAL	1328	TOTAL	1727

**Figure 4.** Morphologic and histologic inclusion and exclusion criteria used in Study IV

## **4.5 STATISTICAL METHODS**

SPSS software was used for statistical analyses in Studies I, II, and III (Chicago, IL, USA, version 19.0 in Study I, 20.0 in Study II, 23.0 in Study III). Survival rates were calculated with the Kaplan-Meier method and survival functions were compared with the log-rank test. A *p*-value less than 0.05 was considered to be statistically significant.

In Study II, the chi-squared test with exact *p*-values was used in the evaluation of differences in TLR staining in accordance with clinicopathological variables. For statistical purposes, tumors were divided into low (0 and 1) and high (2 and 3), and negative (0) and positive (1–3) categories.

In Study III, Cox regression analysis was utilized in univariate associations of risk factors with OS, DSS, and DFS. The risk factors that were associated with OS, DSS, and DFS ( $p < 0.05$  in univariate analysis) were used in a multivariable Cox regression model. To avoid multicollinearity problems, the stage was not included in the multivariable model with T class and neck metastases. Hazard ratios with 95% confidence intervals were used in reporting the results.

SIRs were counted in Study IV to determine the excess SPC risk for MaSGC patients. MaSGC patients were compared with the general Finnish population according to age, sex, and calendar period. This information was obtained from the Finnish cancer registry. SIRs were estimated based on the primary carcinoma age group, calendar period, tumor histology, patient RT status, and SPC site. Statistically smoothed SIRs for the SPCs as a function of the follow up after the diagnosis were assessed. Statistical language R (version 3.2.0) and the popEpi package (version 0.4.1) were utilized in all analyses.

## **4.6 RESEARCH ETHICAL CONSIDERATIONS**

The protocols used in Studies I, II, and III were approved by the Institutional Research Ethics Board (Dnro 31/13/03/02/2010, 01 February 2010). The protocol in Study IV was approved by the National Institute for Health and Welfare, Finland (Dnro THL/263/5.05.00/2015). A research permit was obtained for each study from the Department of Otorhinolaryngology – Head and Neck Surgery at the Helsinki University Hospital. All studies were retrospective and consisted of examining hospital charts, histological specimens, or registry data, and had therefore no effect on the treatment of the patients included in these series. Thus, informed consent was not warranted.

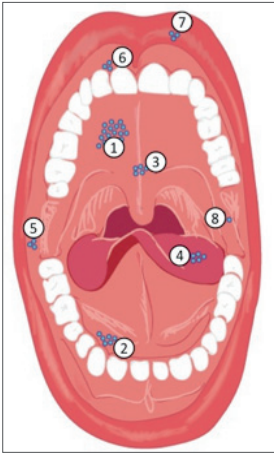
## 5. RESULTS

### 5.1 CHARACTERISTICS AND OUTCOME OF MAJOR AND MINOR SALIVARY GLAND ACC (STUDIES I AND III)

The patient and tumor characteristics of 54 MaACC and 68 MiACC patients are described in Table 4. Most MaACCs (30/54, 56%) were of parotid origin and most MiACCs (41/68, 60%) were located in the oral cavity. Figure 5 illustrates the different locations of the MiACCs in the oral cavity.

**Table 4.**  
*Patient and tumor characteristics of 54 major salivary gland ACCs and 68 minor salivary gland ACCs. Six tracheal tumors were not covered in the current TNM classification.*

		MaACC		MiACC	
		n	%	n	%
Sex	Male	28	52	29	43
	Female	26	48	39	57
Median age	Years (range)	51 (22–80)		58 (24–88)	
Tumor site	Parotid	30	56		
	Submandibular	22	41		
	Sublingual	2	4		
	Oral cavity			41	60
	Paranasal cavities			6	9
	Trachea			6	9
	Nasopharynx			5	7
	Oropharynx			3	4
	Ear			4	6
	Larynx			2	3
Esophagus			1	2	
T class	T1	16	30	18	27
	T2	12	22	12	18
	T3	13	24	6	9
	T4a	11	20	18	27
	T4b	2	4	4	6
	N/A	0	0	4	6
N class	No	47	87	54	79
	N1	5	9	1	2
	N2a	0	0	1	2
	N2b	1	2	1	2
	N2c	0	0	1	2
	N3	0	0	0	0
	N/A	1	2	4	6
Stage	I	16	30	16	24
	II	12	22	12	18
	III	12	22	7	10
	IVA	12	22	17	25
	IVB	2	4	4	6
	IVC	0	0	2	3
N/A	0	0	4	6	
Tumor size	Median, mm (range)	23 (3–70)		21 (3–100)	
Growth pattern	Cribriform	18	33	23	34
	Tubular	6	11	15	22
	Solid	6	11	4	6
	Combination of the above	22	41	8	12
	Not known	0	0	4	6



**Figure 5.** Location of 41 minor salivary gland ACCs in the oral cavity: 1. Hard palate ( $n = 15$ ), 2. Floor of the mouth ( $n = 6$ ), 3. Junction of the hard and soft palate ( $n = 5$ ), 4. Mobile tongue ( $n = 5$ ), 5. Buccal mucosa ( $n = 3$ ), 6. Gum ( $n = 3$ ), 7. Upper lip ( $n = 3$ ), 8. Mucous membrane of the oral cavity, not otherwise specified ( $n = 1$ ). Courtesy of Ms. Terhi Liuhto.

A lump at the tumor site was the most common first symptom in both MaACC (82%) and MiACC (28%) patients, followed by facial paresis (7.4%) and pain (7.4%) in MaACC and pain (18%) in MiACC patients. The duration of symptoms varied between 1–240 months in both MaACC (median 12 months) and MiACC (median 6 months) patients. Six MaACC and three MiACC patients had a previous malignancy at the time of diagnosis (stomach carcinoma, laryngeal carcinoma, renal cell carcinoma, and myeloma in one case each, and prostate and thyroid carcinoma in two cases each).

At diagnosis, ultrasound was performed on 70% and 16%, a neck CT scan on 24% and 58%, a chest CT scan on 22% and 31%, and neck MRI on 48% and 46% of the MaACC and MiACC patients, respectively. Fine needle aspiration had been performed on 77% and 12% of the MaACC and MiACC patients, respectively.

The median tumor size was 23 mm for MaACCs and 21 mm for MiACCs. The tumor size could not be identified for nine MaACC and for 19 MiACC patients either from the clinical data or by re-evaluating the tumor samples. Cribriform was the most common growth pattern in both MaACC (33%) and MiACC (34%). In these studies, a minimum of 80% of the sample was required to consist of one growth pattern, or otherwise it was classified under a combination growth pattern.

## Treatment

Most patients (96% of MaACC and 94% of MiACC) were treated with curative intent. The primary treatment modality was surgery in 98% (MaACC) and 92% (MiACC) of the cases. In both patient groups, one patient received definitive RT with curative intent. Thirty-seven percent (MaACC) and 27% (MiACC) of the patients had neck dissection. For most patients (79% of MaACC and 82% of MiACC), neck dissection was prophylactic, i.e. they had no clinical evidence of locoregional metastases during diagnosis. Histologically confirmed neck metastases at the time of diagnosis were observed in 12% (MaACC) and 18% (MiACC) of the patients.

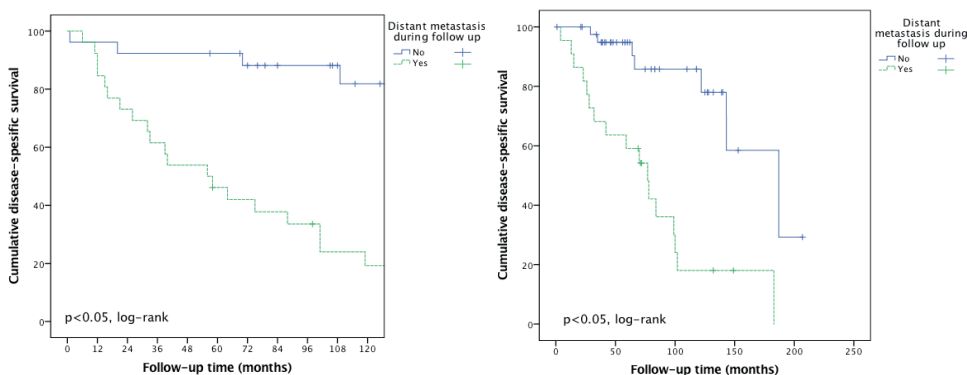
Postoperative RT for the primary tumor area was administered to 56% (MaACC) and 38% (MiACC) of the patients. The median dose was 60 Gy (range 50–66 in MaACC and 32–70 in MiACC). Within their oncological treatment, chemotherapy was offered to 4% (MaACC) and 8% (MiACC) of the patients. Two MaACC patients received palliative treatment, which was RT for the primary tumor area with doses of 30 and 55 Gy. Four MiACC patients received palliative treatment; three had palliative surgery and one had no treatment.

## **Outcome**

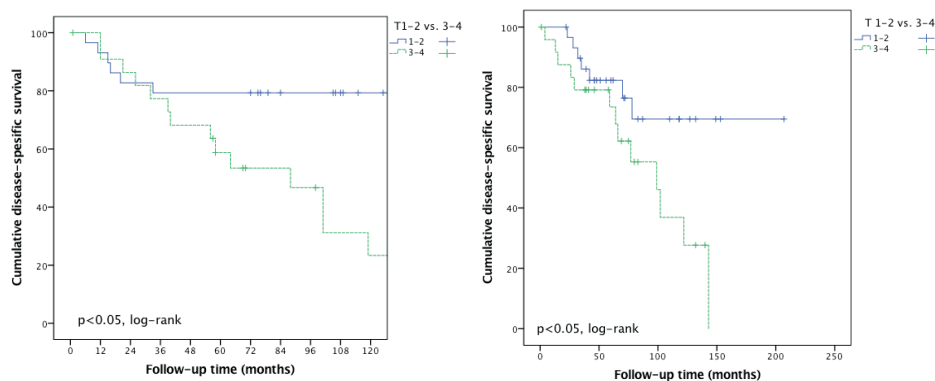
Sixty-two percent (MaACC) and 48% (MiACC) of the patients treated with curative intent had either local or distant disease recurrence during follow up. For 35% (MaACC) and 30% (MiACC) of the patients, the recurrence was in the primary tumor area. Regional metastases were observed in 8% (MaACC) and 13% (MiACC) of the patients, while distant metastases were observed in 50% (MaACC) and 34% (MiACC) of the patients. In most cases (48% in MaACC and 67% in MiACC), distant metastases appeared in the lungs, either alone or combined with another location. Other locations included the kidney, brain, liver, bone, and skin. For eight MaACC and three MiACC patients, information on the location of distant metastases was absent. The time point for the appearance of distant metastases varied between 1–156 months (median 46 months for MaACC and median 43 months for MiACC patients) after the beginning of treatment. Most patients (72% in MaACC and 67% in MiACC) had disease recurrence (either locoregional or distant) within five years. For 28% (MaACC) and 27% (MiACC) of the patients, this occurred at least five years after the primary diagnosis. All MaACC patients (except one) and all MiACC patients (except two) had distant metastases within 10 years after diagnosis.

For patients treated with curative intent, the 5-year OS and DSS rates were, respectively, 69% and 71% (MaACC) and 70% and 79% (MiACC). The 10-year OS and DSS rates were, respectively, 54% and 62% (MaACC) and 42% and 52% (MiACC). At the end of follow up, 37% (MaACC) and 38% (MiACC) of the patients were alive with no evidence of disease, 6% (MaACC) and 9% (MiACC) were alive with disease, and 43% (MaACC) and 38% (MiACC) of the patients died of the disease. In both MaACC and MiACC, advanced T class, N class, and advanced stage had a negative effect on survival (Figure 6). Age over 45 years had a negative effect on survival in MaACC patients, while age under 65 years was associated with better OS in MiACC patients, as was female gender. For MiACC patients, positive neural invasion also had a negative effect on DFS. However, in multivariate Cox regression analysis, positive neural invasion was not an independent prognostic variable. In MaACC, neither perineural invasion nor gender affected survival. In

both groups, the appearance of distant metastases during follow up negatively affected OS and DSS (Figure 7). In contrast, locoregional metastases did not affect survival for MaACC patients, but were associated with poorer DSS in MiACC patients. Stage I MiACC patients had significantly better survival than patients with other disease stages. No stage I patients died due to disease, regardless of disease failures. In this patient group, there were three disease recurrences; two were local and both were located in the ear canal. The survival of stage II MiACC patients was significantly poorer than stage I patients, even though there were no significant differences in treatment. Regarding the patients who had distant metastases, 85% (MaACC) and 77% (MiACC) of the patients died due to disease, and this occurred within 12.6 (MaACC) and 15.3 (MiACC) years. In both groups, three patients with distant metastases were alive (follow-up time 58–149 months).



**Figure 6.** Kaplan-Meier plot showing a significant difference in the disease-specific survival in major salivary gland carcinoma (A) and minor salivary gland carcinoma (B) patients with or without distant metastases.



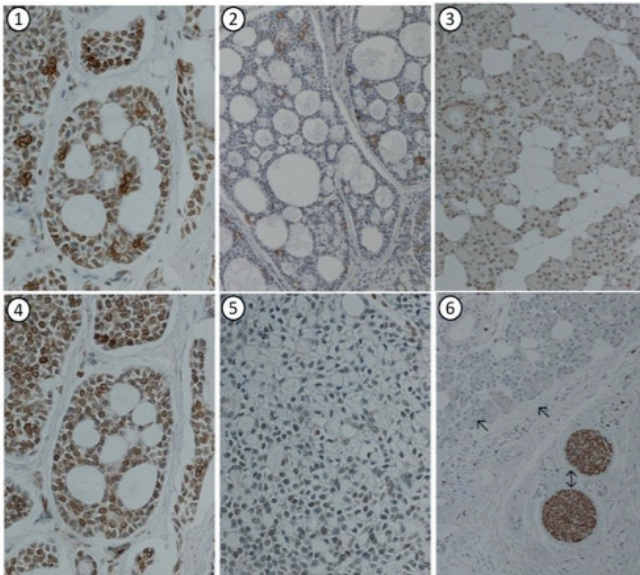
**Figure 7.** Kaplan-Meier plot showing a significant difference in the disease-specific survival of major salivary gland carcinoma (A) and minor salivary gland carcinoma (B) patients with T class 1 and 2 compared with T class 3 and 4.

## 5.2 TLR5 AND 7 EXPRESSION IN MAJOR SALIVARY GLAND ACC (STUDY II)

TLR5 and 7 expression patterns in MaACC were analyzed. TLR5 expression was mostly negative in normal salivary gland tissue. TLR5 was expressed in most primary tumor specimens (90%) and on cell membranes in all metastases. Nuclear and cytoplasmic expression was also seen. Areas with a cribriform growth pattern mostly showed cell membranous staining, whereas expression was more cytoplasmic in areas with solid and tubular growth patterns. Expression was mostly mild in both primary tumors (35%) and metastases (67%).

TLR7 expression was usually negative in normal salivary gland tissue. It was mostly (50%) moderate in primary tumors and mostly mild (33%) or moderate (33%) in metastases. TLR7 expression was very heterogeneous. All tumors included strong positive areas and negative areas. Expression was detected on cell membranes in all primary tumors and metastases. In addition, primary tumors had some nuclear and cytoplasmic expression.

TLR5 expression was lower in males (mean expression 0.89) than females (mean expression 1.69) ( $p = 0.022$ ). TLR7 expression was lower in tumor samples with a solid growth pattern (mean expression 1.25) than in those with tubular and cribriform growth patterns (mean expression 2.21) ( $p = 0.003$ ). There were no other correlations between clinical features and patient survival. Figure 8 illustrates TLR5 and 7 expression in different growth patterns.



**Figure 8.** Immunohistochemical expression of TLR5 and 7 in MaACC. TLR5 staining in ACC; cribriform area of the tumor (1, 2) and normal salivary gland tissue (3). TLR7 staining in ACC; cribriform area (4), solid area (5), and normal salivary gland tissue (6). Arrows indicate salivary gland tissue and the double arrow indicates nerves (c). Magnification  $\times 200$ .

### 5.3 SPC IN MAJOR SALIVARY GLAND CANCERS (STUDY IV)

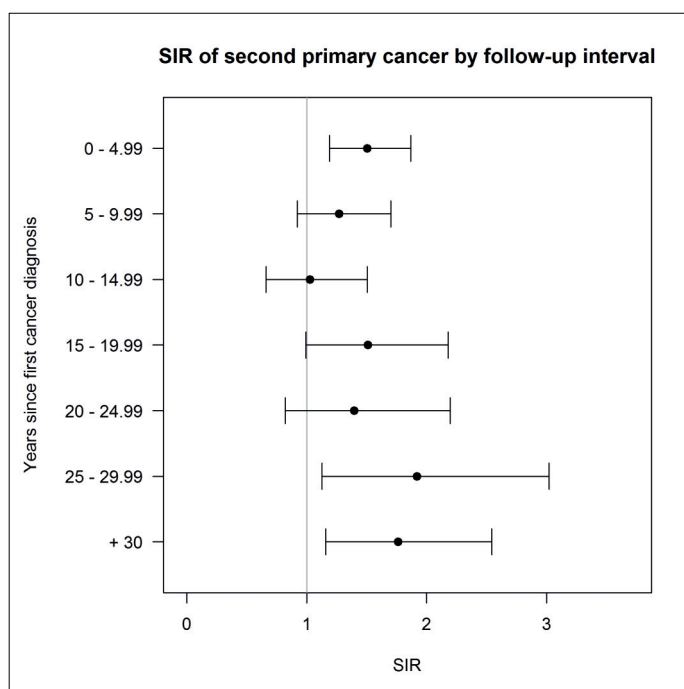
Patient characteristics and the SPC risk are described in Table 5. Altogether, 1727 MaSGC patients were registered in the FCR during 1953–2014. Most (73%) of the tumors were of parotid origin. The tumors were divided into 17 different morphologies, with ACC being the most common morphology (26%). SGC was the first cancer in most cases (92%). In total, 272 SPCs were detected, with a median follow up of 9 years (range 0–47). There was a 43% higher risk of developing SPC compared with the normal population (SIR 1.43, 95% CI: 1.24–1.63). The risk was similar in men and women.

**Table 5.** Second primary cancer risk among patients with major salivary gland carcinoma.

	First cancer	Second cancer			
	Number of MaSGCs (%)	Observed number of cancers (%)	Expected number of cancers	SIR	[95%CI]
<b>Sex</b>					
Male	788 (45.6)	106 (13.4)	75	1,42	[1.16–1.71]
Female	939 (54.4)	116 (12.4)	81	1,43	[1.19–1.72]
<b>Histology</b>					
Adenocarcinoma	366 (21.2)	33 (9.0)	35	0,94	[0.64–1.31]
Adenoid cystic carcinoma	455 (26.3)	59 (13.0)	46	1,29	[0.98–1.66]
Mucoepidermoid carcinoma	221 (12.8)	28 (12.7)	19	1,5	[1.00–2.17]
Acinic cell carcinoma	246 (14.2)	42 (17.1)	19	2,2	[1.59–2.98]
14 other histologies	439 (25.4)	60 (13.7)	36	1,68	[1.28–2.16]
<b>Extent of disease</b>					
Localized	998 (57.8)	158 (15.8)	111	1,42	[1.21–1.66]
Locoregionally advanced/ Metastasized	418 (24.2)	29 (6.9)	19	1,55	[1.04–2.22]
No information	311 (18.0)	35 (11.3)	26	1,35	[0.94–1.87]
<b>Age at diagnosis</b>					
<55	671 (38.8)	90 (13.4)	55	1,63	[1.31–2.00]
55–69	622 (36.0)	85 (13.7)	66	1,28	[1.03–1.59]
70–84	381 (22.1)	43 (11.3)	30	1,42	[1.02–1.91]
>84	53 (3.1)	4 (7.5)	3	1,41	[0.38–3.60]
<b>Year of diagnosis*</b>					
1953–69	274 (15.9)	17 (6.2)	11	1,49	[0.87–2.39]
1970–84	361 (20.9)	27 (7.5)	21	1,27	[0.84–1.85]
1985–99	471 (27.3)	41 (8.7)	27	1,49	[1.07–2.03]
2000–14	621 (36.0)	40 (6.4)	28	1,44	[1.03–1.96]

\*Follow-up time limited to 10 years to make calendar periods comparable

Figure 9 shows the SIR in follow up. The highest SPC risk was during the first 5 years from SGC diagnosis (SIR 1.50). The risk was then closer to that in the normal population up to 19 years after diagnosis (SIR 1.25), but was again elevated 20 years after diagnosis (SIR 1.81). Table 6 presents the risk for different SPC sites. The SPC risk was significantly elevated for thyroid cancer (SIR 5.12), melanoma of the skin (SIR 3.35), non-melanoma skin cancer (SIR 2.50), respiratory organ cancer (SIR 1.63), breast cancer (SIR 1.63), and cancer of the male genital organs (SIR 1.48). During the first five years after SGC diagnosis, the risk was higher for thyroid cancer (SIR 5.88, 95% CI: 1.21–17.20), melanoma (SIR 4.36, 95% CI: 1.42–10.18), and non-melanoma skin cancers (SIR 6.73, 95% CI: 3.23–12.37). After 20 years from SGC diagnosis, the risk was higher for cancers of the respiratory organs (SIR 2.71, 95% CI: 1.17–5.34), male genital organs (SIR 2.81, 95% CI: 1.03–6.12), and breasts (SIR 2.21, 95% CI: 1.01–4.19). Patients from all age groups had an elevated SPC risk. Carcinomas with all morphologies (except adenocarcinoma NOS) had an elevated SPC risk. Patients who had received RT did not have a significantly higher SPC risk compared with those who had not. Neither did extent of disease significantly affect the SPC risk.



**Figure 9.** Standardized incidence ratios of second primary cancer by follow-up interval.

**Table 6.** Standardized incidence ratios of the second primary cancer risk in major salivary gland carcinoma patients in Finland during 1950–2014.

Second cancer sites	n	SIR [95%CI]	
Brain, other central nervous system	8	2.00	[0.86–3.94]
Eye	-		
Thyroid gland	8	5.12	[2.21–10.08]
Mouth, pharynx	7	2.33	[0.94–4.81]
Respiratory organs	31	1.63	[1.11–2.31]
Lungs, trachea	26	1.45	[0.95–2.12]
Nose, sinuses	1	3.72	[0.09–20.74]
Larynx, epiglottis	3	3.29	[0.68–9.60]
Unspecified	1	2.37	[0.06–13.21]
Breasts	33	1.63	[1.12–2.29]
Digestive organs	34	0.82	[0.56–1.16]
Stomach	6	0.69	[0.25–1.50]
Small intestine	1	2.03	[0.05–11.32]
Colon	9	0.96	[0.44–1.82]
Rectum, rectosigmoid	6	0.99	[0.36–2.15]
Liver	2	0.86	[0.10–3.11]
Pancreas	4	0.62	[0.17–1.58]
Gallbladder, bile ducts	3	1.33	[0.27–3.88]
Salivary glands	1	3.25	[0.08–18.08]
Female genital organs	13	1.21	[0.64–2.07]
Male genital organs	31	1.48	[1.00–2.10]
Prostate	29	1.39	[0.93–1.99]
Testis	2	13.02	[1.58–47.02]
Urinary organs	16	1.53	[0.88–2.49]
Melanoma of the skin	12	3.35	[1.73–5.85]
Skin, non-melanoma	13	2.50	[1.33–4.28]
Other endocrine glands	-		
Bone	-		
Soft tissues	1	1.17	[0.03–6.49]
Ill-defined or unknown	3	0.66	[0.14–1.94]
Mesothelioma	-		
Autonomic nervous system	-		
Lymphoid and hematopoietic tissue	15	1.19	[0.67–1.97]

## **6. DISCUSSION**

Salivary gland malignancies are rare. The prognosis depends on the histological type and tumor characteristics. ACC is known to have a high distant metastasis rate and the prognosis is poor, especially when located in minor salivary glands. Treatment has not markedly changed over the years, with surgery and, in certain cases, postoperative RT being the standard treatment. Research on new molecular markers for diagnostics and target-specific therapies is currently active.

To gain knowledge and to have an appropriate follow up for these patients, the behavioral pattern of ACC and secondary malignancies in SGCs were investigated in a Finnish population (Studies I, III, and IV). TLRs have been studied as prognostic markers in several cancers, and an attempt was made to clarify the role of TLR5 and 7 in ACC (Study II).

### **6.1 GENERAL CHARACTERISTICS OF ACC PATIENTS (STUDIES I AND III)**

The present finding of most MaACCs being located in the parotid (56%) and most MiACCs in the oral cavity (60%), specifically in the palate (49%), is consistent with the existing literature<sup>14, 17, 18, 38, 60</sup>. In MiACCs, all TNM stages were observed in the palatal tumors, with slightly more advanced stage distribution than in the other intraoral sites. All nasopharyngeal and paranasal sinus tumors were of advanced stages. In a study by Shum et al.<sup>67</sup>, palatal ACCs were mostly of high T3–T4 classes. Other studies have also demonstrated that paranasal tumors are often found at an advanced stage and diagnosed late<sup>101, 149</sup>. Tumors in the nasopharynx and paranasal sinuses may become symptomatic relatively late, being already in an advanced stage. In addition, palatal tumors may be more difficult to detect than in other intraoral locations, possibly explaining these findings.

MaACC patients had a slight male predominance, but among MiACC patients there was a female predominance. Other studies have also shown gender variation, with a female predominance being more common<sup>16, 66, 67, 150</sup>. Neural invasion was present in most patients, as has been noticed in other studies<sup>151, 152</sup>. It appears that the clinical pattern of ACC in Finland does not significantly differ clinically or epidemiologically from that observed elsewhere.

## 6.2 TREATMENT AND SURVIVAL IN ACC (STUDIES I AND III)

Surgery has remained the main treatment modality in ACC. Neck dissection should always be performed in cases of clinically evident neck metastases. It is recommended to remove all lymph nodes in affected neck levels <sup>50</sup>. In our series of MaACCs, neck dissection was performed on 37% of patients treated with curative intent. Neck dissection was elective with no clinically evident neck metastases in 79% of patients; of these patients, 12% had histologically proven neck metastases. Of the MiACC patients treated with curative intent, 42% had neck dissection, and 82% of these were elective. The occult neck metastasis rate has varied between 15–44% in Europe and the United States <sup>84</sup>. It is not clear whether elective neck dissection should be performed in all ACC patients. Lee et al. <sup>153</sup> recommended it to achieve staging and prognostic information, even though there was no significant effect on survival in their study. Amit et al. <sup>85</sup> did not observe an effect on survival in their collaborative study, in which the occult metastasis rate was 17% and neck metastases mostly appeared in the ipsilateral neck levels I to III.

Postoperative RT of primary tumors was administered to 56% (MaACC) and 38% (MiACC) of the patients. In other studies, postoperative RT has been shown to increase the survival of ACC patients in all stages, and particularly in the case of positive lymph nodes and positive margins <sup>87, 88</sup>. IMRT has brought better local control and reduced side effects <sup>86</sup>. Proton-beam therapy and raster-scanned carbon ion boost have shown promising results in advanced diseases <sup>89, 90</sup>. CRT has also been shown to improve local control in some high-risk cases <sup>19, 20</sup>.

In both MaACCs and MiACCs treated with curative intent, factors that negatively affected survival included age over 45 (MaACC) and 65 (MiACC) years, advanced T class, advanced stage, and neck metastases during diagnosis. During follow up, the appearance of distant metastases negatively affected survival. In MiACCs, the appearance of locoregional metastases also negatively affected survival. Furthermore, no MiACC patients with stage I disease died due to ACC. In a study by Mücke et al. <sup>154</sup>, stage I and II tumors did not have significant differences in prognosis. Amit et al. <sup>22</sup> found that age over 70 years, the presence of nodal and distant metastases, positive or close margins, and a sinonasal origin were prognostic markers for poorer survival. Similarly to our results, others have also shown that T class, N class, and stage affect survival <sup>150, 154, 155</sup>.

In this study of MiACCs, the only stage I patients who had treatment failure had tumors located in the ear or in the oropharynx. No disease recurrences appeared in oral cavity tumors. The assumption is that disease-free surgical margins are

more easily achieved with these small tumors located in the oral cavity than in the oropharynx or in the ear, which are locations with a more challenging anatomy. In contrast, two other patients with advanced-stage tumors in the ear did not have disease failures, but were treated more aggressively with postoperative RT. ACC patients with tumors in this challenging location could possibly benefit from more aggressive treatment, even with stage I disease. This might also be feasible for MiACC patients with stage II tumors in the oral cavity, as these patients appear to have a poor outcome and a high percentage of disease failures.

In MiACCs, female gender was also associated with improved OS. In our study, there was no gender correlation with survival in MaACCs. However, while Ellington et al.<sup>81</sup> also found that females had a better prognosis, Marcinow et al.<sup>101</sup> observed that female gender predicted a poorer outcome. The finding of improved survival might also reflect the higher life expectancy of women in general.

Neural invasion did not correlate with survival in MaACC. While neural invasion negatively affected DFS in MiACC, no correlation was found with local or locoregional recurrences. Some studies have demonstrated that perineural invasion is associated with local recurrence and margin involvement, but not with survival<sup>66, 100</sup>. However, many studies have also found a correlation between perineural invasion and poorer survival<sup>23, 100, 101, 156</sup>. ACC is thought to metastasize mainly hematogenously, but neurotropism is still typical of ACC<sup>60</sup>.

In this study of patients treated with curative intent, 62% (MaACC) and 48% (MiACC) of the patients had disease recurrence or the appearance of distant metastases. Bradley et al.<sup>157</sup> also found that approximately 60% of patients will suffer from disease recurrence. Recurrence in the primary tumor area was observed in 35% (MaACC) and 23% (MiACC) of the patients in our studies. In a MiACC study by He et al.<sup>23</sup>, the primary metastasis rate was 40%. In our series, 8% (MaACC) and 9% (MiACC) of the patients had neck metastases during follow up. This is consistent with other studies on neck metastases, which have been reported to occur in 3–16% of patients<sup>23, 84</sup>.

Distant metastases are thought to be the main reason for poor survival rates. They were observed in 50% (MaACC) and 67% (MiACC) of the patients. Most distant metastases appeared within 10 years of treatment. In other studies, the median time to appearance of distant metastases has varied from 1–9 years<sup>12, 14, 152, 158, 159</sup>. In the literature, distant metastases are reported to appear in 20–55% of ACCs<sup>17, 22, 67, 91, 160</sup>. The most common location for distant metastases is the lungs, consistent with our studies. Lung metastases are often not solitary. Routine

thoracotomy is not advised, as metastases can remain relatively stable for months.<sup>17, 91</sup> According to Girelli et al.<sup>21</sup>, lung metastasectomy should be considered to achieve local control if complete metastasis removal is possible and pulmonary metastases appear more than 36 months after primary treatment. Whether incomplete lung resection of lung metastases provides symptomatic benefit is not clear<sup>17, 91</sup>.

Otherwise, the options for patients with metastatic disease are supportive care and observation, palliative systemic therapy, or participation in clinical trials<sup>60</sup>. In our series of MaACC, only two patients received palliative RT, with a subsequent survival of five and 12 months. Choi et al.<sup>151</sup> found no significant difference in survival between patients treated with palliative chemotherapy after a relapse compared with patients receiving only supportive care. The median survival after recurrence was 77 months<sup>151</sup>.

The solid growth pattern is related to a higher metastatic rate and poorer survival. According to WHO, tumors consisting of more than 30% solid pattern have more aggressive behavior<sup>10, 26</sup>. He et al.<sup>23</sup> found the solid pattern to be an independent prognostic marker in MiACC. Others have found the solid pattern to increase the distant metastasis risk<sup>12, 22, 96</sup>. In this study, a correlation between the solid type and survival was not found in MiACC. In MaACC, while the solid type had a slight negative association with survival, the results were not statistically significant.

In our studies, the 5-year OS and DSS rates were, respectively, 69% and 71% (MaACC) and 70% and 79% (MiACC). The 10-year OS and DSS rates were, respectively, 54% and 62% (MaACC) and 42% and 52% (MiACC). While the difference in 5-year survival was not significant, patients with MiACCs appear to have a poorer prognosis for long-term survival. In a study by Ellington et al.<sup>81</sup>, MiACC patients had decreased survival compared with MaACC patients. Similarly, in a Danish series, the 5-year OS and DFS rates were 80% and 72%, respectively, while the 10-year rates were 58% and 60%, respectively<sup>14</sup>. In addition, in a study by Amit et al.<sup>22</sup>, the 5-year OS and DSS rates were 76% and 80%, respectively. As most distant metastases appear within 10 years, it might be reasonable to prolong routine follow-up imaging for an extended time period.

### **6.3 TLR5 IN SALIVARY GLAND CANCERS (STUDY II)**

In this study, TLR5 and 7 expression was found in both primary ACC tumors and metastases. Expression varied from negative to strong within the same tumor. Different expression patterns (nuclear, cytoplasmic, and membranous) were also

usually observed within the same tumor sample. Both TLRs were mostly negative on normal salivary gland tissue. Low TLR5 and 7 immunopositivity correlated with male gender and a solid growth pattern, respectively. These TLRs did not correlate with disease recurrence or patient survival, and they are therefore not suitable as clinical prognostic markers in ACC.

TLR5 has previously been described in salivary gland adenocarcinomas. That study suggested that bacterial flagellin promotes invasion and migration through a TLR5-induced pathway <sup>129</sup>. TLR5 was also investigated in OTSCC, where high immunopositivity correlated with advanced age, female gender and DSS, and it was an independent predictor of disease recurrence and mortality <sup>170</sup>. Similar to our study, low TLR5 staining intensity correlated with male gender. In contradiction, in early stage OTSCC, Mäkinen et al. <sup>118</sup> found negative or mild TLR5 staining to predict poorer DSS. In HPV-positive OPSCC, high TLR5 expression was possibly related to a poor prognosis <sup>123</sup>. In our study on ACC, TLR5 was mainly expressed on plasma membranes, although some subcellular positivity was additionally seen. Other studies have also shown TLR5 expression on plasma membranes and additionally in cytoplasm <sup>116, 122, 161</sup>. Further research is necessary to understand the role of TLR5 in SGCs.

To our knowledge, TLR7 has not previously been studied in salivary gland tumors. In juvenile angiofibroma, TLR7 was expressed on cell membranes but had no clinical correlations <sup>127</sup>. In OPSCC, HPV-positive cancers showed high TLR7 positivity and low TLR5 positivity. These tumors are known to have better sensitivity to RT <sup>122</sup>. Low TLR7 expression was possibly related to the poor prognosis in this patient group <sup>123</sup>. In another OPSCC study, low tumor cell TLR7 expression as well as high stromal TLR7 expression correlated with improved survival <sup>162</sup>. Another study demonstrated high TLR7 and low TLR5 expression in p16-positive OPSCC *in vivo* and *in vitro*. In addition, TLR7 and 9 expression correlated with the HPV status *in vivo* <sup>122</sup>. TLR9 has also been studied in mucoepidermoid carcinoma, where low TLR9 expression correlated with an advanced disease stage and a poor prognosis <sup>128</sup>. In lung cancer, TLR7 correlates with resistance to neoadjuvant chemotherapy and a poor clinical outcome <sup>163</sup>. In this study, TLR7 expression was detected on plasma membranes in all tumor samples. TLR7 is usually expressed intracellularly in endosomes, lysosomes, and endoplasmic reticulum, and it recognizes viral single-stranded RNA <sup>24, 104, 161</sup>. Jouhi et al. <sup>122</sup> demonstrated TLR7 expression on the nuclear membrane and in nuclei. TLR7 expression on the plasma membrane remains to be elucidated.

In this study, low TLR7 expression was found to correlate with a solid growth pattern. A solid growth pattern has a poorer prognosis and the highest metastasis

rate <sup>12</sup>. Similarly, in our series, a solid growth pattern correlated with the appearance of neck metastases, but not with survival.

#### **6.4 SPCS IN SALIVARY GLAND CANCERS (STUDY IV)**

The long-term outcome of SGC patients depends on histology, grade, and location, among other factors. Many patients live long after SGC treatment, and knowing the risk of SPC is important when considering their follow up. In head and neck cancers, routine follow up has not been clearly shown to prolong survival, even though it helps to detect recurrences and SPCs earlier <sup>164</sup>. SPCs have an important impact on survival in some cancers, for example in Hodgkin's lymphoma <sup>130</sup>. The risk of SPC in any cancer patients is estimated to be approximately 14% higher than would have been expected in the general Surveillance, Epidemiology, and End Results (SEER) population in the United States <sup>25</sup>. Some SPCs are probably treatment-related (such as the late effects of RT), and some are caused by the influence of lifestyle factors (such as tobacco smoking or environmental exposures) <sup>25</sup>. Syndromes and a mixture of different influences such as gene–environment and treatment and lifestyle interactions may also be the cause of SPC <sup>131</sup>.

In patients with HNSCC, SPCs usually appear in the head and neck area, lungs, esophagus, and colorectum. Alcohol consumption and tobacco smoking are the same risk factors as for the primary cancer. <sup>137, 138, 165</sup> In a large study with 13 cancer registries, Chuang et al. <sup>165</sup> showed HNSCC patients to have a 36% increase in the 20-year cumulative risk of SPCs. The risk was highest in the lungs, oral cavity, pharynx, esophagus, and larynx.

In this study, the SPC risk for MaSGC patients was investigated over a 60-year period in Finland. It was found that these patients had a 43% higher risk for an SPC than the cancer risk in the general population. The risk is higher than in cancer patients in general in Finland ([www.cancer.fi](http://www.cancer.fi)) and in HNSCC patients in a study by Chuang et al. <sup>165</sup>. In this study, the SPC risk after SGC was found to be highest for thyroid cancer. The risk of melanoma of the skin, other skin cancers, breast cancer, cancer of the respiratory organs, and cancer of the male genital organs was also significantly increased.

Few studies exist on the SPC risk of SGC patients. Most of the existing studies have focused on the risk of second breast cancer appearing after SGC. Similar to our observations, others have shown that SGC patients have a higher risk of developing breast cancer, with SIRs ranging between 2.3–4.8 <sup>141-144</sup>. However, in

other studies, no association has been found between these two cancers <sup>145-147</sup>. There are some histological similarities between SGCs and certain breast cancers. Both cancers have an adenoid-cystic cell type and, furthermore, epithelial-myoeithelial carcinoma of the salivary gland and adenomyoepithelioma of the breasts also share similarities. It is unknown whether common etiological factors might explain this relationship. <sup>142</sup>

Similar to our findings, Biggar et al. <sup>145</sup> demonstrated an increased respiratory SPC risk in men. Contrary to our findings, however, they also found an increased ovarian SPC risk <sup>145</sup>. In a series of 825 SGC patients, Prior et al. <sup>141</sup> observed an increased breast and bronchus SPC risk in women and prostate and skin SPC risk in men. These findings are consistent with our observation of an increased risk of breast cancer, melanoma and non-melanoma skin cancer, and cancer of the respiratory organs and male genital organs. In contrast to our observations, der Maur et al. <sup>144</sup> found that the oral/oropharyngeal SPC risk is higher after SGC. However, their study also included minor salivary glands and both benign and malignant tumors <sup>144</sup>. Kwon et al. <sup>166</sup> compared the incidence of SPCs in 184 SGC patients with a number of newly developed cancers in 200 healthy controls and found no significant difference in cumulative SPC incidence between these groups. However, the sample size was quite limited.

There have only been two previous studies on the SPC risk in MaSGC patients. Megwalu and Shin <sup>146</sup> found a 24% higher overall SPC risk in their SEER database study with 15,572 MaSGC patients. The risk was significantly increased for cancers of the salivary glands (SIR 9.97), oral cavity (SIR 3.48), thyroid (SIR 2.66), lungs and bronchi (SIR 1.60), and kidney (SIR 1.68) <sup>146</sup>. Similar to the findings in this study, they observed the thyroid to be among the three sites with highest risk. In their study on 498 MaSGC patients, Spitz et al. <sup>147</sup> found an elevated risk for skin cancer in men, with a relative risk of 3.83 (95% CI: 1.28–11.49). In this study, an increased risk for melanoma and non-melanoma skin cancers in MaSGC patients was also found, and men had a higher risk than women. The risk was high only during the first five years after diagnosis. As these cancers can be visible and thus detected by clinical examination, it is assumed that they were more effectively diagnosed during the close surveillance period following cancer management. However, Spitz et al. <sup>148</sup> reported that men who had primary skin cancer had an estimated odds ratio of 13.7 (95% CI: 4.17–44.97) for developing MaSGC. Others have also shown that skin melanoma and non-melanoma skin cancer patients have an elevated risk of developing SGC. It has been hypothesized that a genetic link or shared risk factors (e.g., UV radiation) exist for these cancers. <sup>167, 168</sup> There is also a biological relationship between cutaneous and major salivary gland neoplasms, with similarities in their embryologic derivation, histogenesis, and

histological composition. They are both formed from ectoderm and manifest similar stages in embryologic development.<sup>169</sup>

In the study by Megwalu and Shin<sup>146</sup>, the SPC risk remained high up to 10 years after MaSGC diagnosis. In this study, it was found that the SPC risk was higher during the first few years after diagnosis, but between 5 and 10 years, this risk did not differ from that of the general population. The risk then started to rise again, with the highest risk being at 20 years after SGC diagnosis. An increased thyroid cancer risk was demonstrated from the start of follow up to 20 years after SGC diagnosis. It is assumed that some overdiagnosis may occur, particularly in cases of thyroid and skin cancer, when patients are under careful follow up. However, the fact that the thyroid cancer risk remained elevated up to 20 years after SGC diagnosis does not suggest surveillance bias but more likely indicates that RT in the neck region increases the risk of thyroid cancer. The finding of an elevated thyroid SPC risk among patients who received RT supports this possibility, even though RT was not observed to significantly increase the overall SPC risk compared with SGC patients who did not receive RT. In the study by Megwalu and Shin<sup>146</sup>, RT treatment increased the SPC risk for thyroid, larynx, and lungs/bronchi, but had no correlation with the development of oral SPC. In the SEER registry study with a series of 190,468 HNSCC patients, Shaaban et al.<sup>138</sup> did not observe an increase in SPC risk following RT. Our finding of an elevated SPC risk in MaSGC patients even after 20 years after diagnosis should be recognized when planning the follow up for these patients.

## **6.5 LIMITATIONS AND STRENGTHS OF THE THESIS**

In Studies I and III, the hospital records of patients diagnosed with ACC over a period of 35–38 years were retrospectively reviewed. Most of the needed data were available. However, particularly in the older charts, some information was lacking in single cases. Some parameters, such as surgical margins, were not sufficiently reported to enable relevant conclusions, and the amount of paraffin-embedded tissue was also insufficient for the corresponding cases to collect this information. Even though treatment modalities have not significantly changed, there are still variations in treatment approaches, which might affect the survival data in retrospective studies. However, follow-up data were available for all patients except one, and a minimum follow-up time of 5 years and 3 years or until death in MaACC and MiACC studies, respectively, was obtained. Studies I and II included patients from only one academic center representing approximately one third of the Finnish population. The number of patients (54 in Study I and 68 in Study III) was still large compared with previous studies.

For immunohistological evaluation (Study II), 34 MaACC samples were available, which is a limited series. However, this is a common problem with rare diseases, and multi-institutional studies are warranted in order to draw even more reliable conclusions. Some of the samples were also small, which made the assessment of the staining intensity difficult. Determining the percentage of positive staining is subjective and thus prone to bias. However, two independent researchers performed the scoring, which reduced this bias.

In Study IV, all histological types of MaSGCs were included and FCR data over 60 years were used to investigate the appearance of SPCs in a nationwide series. This long time period is a strength, and practically complete follow-up data including causes of death were available. However, there was no information on smoking or alcohol consumption. Another limitation in Study IV is that the histologic classification of SGCs has changed over the years. This might have led to the missing of some cases that would currently be classified as SGCs. In addition, diagnostic methods have significantly improved and classification has become more specific. In 1972, there were only five SGC categories, whereas the 2017 WHO classification has 22 SGC categories. An attempt was made to gather all possible cases with changed nomenclature and to exclude the cases with missing classification data to minimize this bias.

## **6.6 FUTURE PERSPECTIVES FOR ACC RESEARCH**

Finding novel treatment approaches for recurrent ACC remains an important objective for future research. ACCs are rather rare and challenging to manage, and the development of more efficient treatment protocols thus requires multicenter collaboration. Patients with stage II tumors are a specific group to be included in such trials. The present study suggests evaluating the effect of prolonged follow up on the long-term outcome in this patient population. Larger patient cohorts would enable the validation of potential prognostic and predictive markers. This would also allow the inclusion of patients in need of palliative therapy in clinical trials. As their tumors and previous treatment are heterogeneous, these factors should be carefully considered when assessing data and study conclusions.

Proton-beam therapy and raster-scanned carbon ion boost combined with IMRT have shown promising results in advanced disease. Similarly, in the Helsinki University Hospital setting, it would be interesting to examine the role of boron neutron capture therapy. Future studies might clarify the role of these treatment modalities in the management ACC.

Efforts towards the development of targeted molecular therapy are currently in progress. However, further molecular analysis studies are needed. In the future, genome sequencing may be useful in identifying critical molecular alterations and their impact on the possibilities to develop individualized treatment for ACC patients.

## 7. CONCLUSIONS

1. More than half of MaACC and MiACC patients developed distant metastases within 10 years in the present studies and therefore, an extended follow-up time is recommended for this patient population. Older age, advanced T class, advanced stage, neck metastases at diagnosis, and distant metastases during follow up negatively affected survival in both MaACC and MiACC patient groups. In addition, the occurrence of locoregional metastases had a negative effect on survival in MiACC patients. Furthermore, they also appear to have worse 10-year survival compared with MaACC patients. MiACC patients with stage I disease had a significantly better prognosis than stage II patients. Stage II patients should be considered as having clearly advanced disease, and a more aggressive treatment approach thus needs to be considered for this subgroup.

2. TLR5 and 7 expression was observed in MaACC specimens. The intensity and pattern of expression were extremely heterogeneous, reflecting the histological diversity of ACCs. Low TLR5 positivity correlated with male gender and low TLR7 positivity with a solid growth pattern. There was no correlation between expression and survival.

3. SGC patients appeared to have a significantly increased risk for the development of SPC, especially thyroid cancer, compared with the cancer risk of the general population. This risk remained high even after 20 years after diagnosis, and an extended follow-up time regarding this issue in primary health care is therefore recommended, as detecting the SPCs early may prolong long-term survival.

## 8. ACKNOWLEDGEMENTS

This study was carried out while a resident between 2009 and 2017 at the Department of Otorhinolaryngology – Head and Neck Surgery, Helsinki University Hospital, in collaboration with the Department of Pathology and the Finnish Cancer Registry.

The work was financially supported by grants from the Helsinki University Hospital Research Funds, the Finnish-Norwegian Medical Foundation, the Research Fund of the Finnish Otolaryngological Society, Finska Läkaresällskapet and the Orion Research Foundation. I gratefully acknowledge them all.

I extend my deep gratitude to all who have helped and supported me during this long and educative process.

My first and biggest thanks go to my supervisors, Professor Antti Mäkitie and Docent Leif Bäck. Antti, your encouragement, time, patience, endless help, and faith in me and this project is something I will forever be grateful for. When I have had doubts about this process, your wise and inspirational words and clear vision have always guided me back on track. Lefa, I thank you for first introducing this project to me and for your help with statistics, among other things. Your clinical expertise has been highly appreciated in this process. With both of you, we have also shared many fun moments during these years: thank you for those.

Warm thanks to the present and former chiefs of the department, Erna Kentala, Hans Ramsay, and Anne Pitkäranta, and to the current acting Head of Department, Docent Antti Aarnisalo, for providing an encouraging and positive working atmosphere for research. I am also extremely grateful to Docent Heikki Rihkanen, who acted as the Head of Department for most of my specialization, for his kindness and encouragement during those years.

I want to sincerely thank my official reviewers, Docents Jaana Rautava and Heikki Teppo, for their valuable and constructive comments and encouraging and warm attitude. I thank Docents Miikka Peltomaa and Risto Kontio for being members of my thesis committee and giving me positive feedback and good advice.

I am extremely grateful to all my coauthors. Docent Jaana Hagström, thank you for introducing me to the world of pathology. Getting to know your lively and positive personality has been a huge pleasure. Our talks about the research and

other matters have given me so much. Professors Ilmo Leivo, and Caj Haglund, and Docent Kauko Saarilahti, thank you for your kind collaboration. Hanna Hämetoja, DDS, thank you for those relaxed yet very educative moments when we have discussed research and solved practical questions. Lauri Jouhi, MD, thank you for your valuable help with statistics and for your patience in explaining these matters always so kindly. Aaro Haapaniemi, MD, PhD, in addition to being a co-author in one of my studies, you have helped me greatly with all practical matters concerning the whole defense process: I really appreciate this.

I am truly grateful to my co-authors at the Finnish Cancer Registry: Professor Nea Malila, Docent Janne Pitkaniemi, and Matti Rantala, MSc. It was a pleasure to work with you all. Nea, your expertise and wise and precise advice has helped me a lot. Matti, your contribution to the statistics was invaluable. Thank you for always being so patient and friendly in helping me and answering my questions.

I also extend my gratitude to Päivi Peltokangas and Eveliina Markkula for their invaluable technical assistance with immunostaining. Also, Antti Markkola, MD, PhD, thank you for finding MRI images for me and for the joyful conversations.

I want to extend my gratitude to all my wonderful colleagues at the Department of Otorhinolaryngology. The warm and welcoming atmosphere at the clinic was the reason I first chose ENT specialization. Special thanks to Docent Timo Atula for sharing his knowledge and discussing salivary gland tumors with me. I sincerely thank all the other residents with whom I have had the privilege to work and for the friendship and unforgettable times. The help of those who have already defended their thesis has been invaluable. Thank you for sharing all the practical tips with me. Lena, I am extremely thankful for your help in applying for the grant in Swedish: this was crucial for me to finish this project. I deeply thank the 'Lukupiiri': Laura, Maija, and Jenni. Your heartfelt friendship and peer support has meant the world to me. Thank you for sharing the joys and worries of both professional and personal life, every day. Laura, thank you for reading my thesis and discussing it with me; you have helped me more than you can imagine.

And my friends from childhood, medical school, and through our kids – you are the spice and sugar in my life, and I thank you from the bottom of my heart for sharing this journey with me. I feel unbelievably lucky to have such strong, wise, joyful and beautiful women in my life.

I want to thank my parents, Liisa and Niilo, for always supporting me and for showing a good example in life. You are the best parents one can hope for. Thank you to my brothers, Niki and Miihkali, and sister-in-law Kati for being there for

me. I am also truly grateful to my wonderful parents-in-law, Riitta and Heikki, for being a huge help and joy for my family during these busy years. Thank you for providing me the peace and the best possible atmosphere to write this thesis last summer in your cottage.

Most of all, I want to thank my dear husband, Petteri, and our three incredible kids, Olavi, Viola, and Laila, for making my life so happy and filled with love. The four of you are the true treasures of my life and number one priority. Petteri, your practical help with Word and Excel was crucial for this thesis to ever get done. Your rationale and smart perspective and sense of humor has kept me sane. You have lived through all the ups and downs with me during this process, and I have never felt alone. I love you.

Helsinki, October 2017

Karoliina Hirvonen

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