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To Jori, Vertti, and Severi

Medicine is a science of uncertainty and an art of probability.
William Osler

ABSTRACT

Miia Ruuskanen

Nasopharyngeal Carcinoma in Finland: Epstein-Barr Virus, Human Papillomaviruses, and Toll-like Receptors as Prognostic Factors

University of Turku, Faculty of Medicine, Department of Otorhinolaryngology – Head and Neck Surgery, Doctoral Programme in Clinical Research (DPCR)

Annales Universitatis Turkuensis, Turku, Finland, 2019

Nasopharyngeal carcinoma (NPC) is a malignant tumour arising from the surface epithelium of the nasopharynx. NPC is a rare cancer type in Northern Europe, and approximately ten new cases are diagnosed in Finland yearly. NPC carcinogenesis has been linked to Epstein-Barr virus (EBV) infection, but human papillomaviruses (HPVs) have also been found in NPC tumours. Presumably, the host's innate immunological properties also have an impact on pathological processes. Toll-like receptors (TLRs) are transmembrane proteins, which recognize both microbial and host's own structures released in tissue damage in order to regulate innate immunity. The expression of TLRs has been observed in many cancer types, but their actual function is still unknown. TLR stimulation may have both pro- or anti-tumoural effects.

The aim of this nationwide study was to evaluate NPC incidence, histological subgroups, provided treatments and their adverse effects, and outcome in NPC patients diagnosed and treated in Finland from 1990 to 2009. A total of 207 patients were identified from the Finnish Cancer Registry database during the 20-year study period. In addition, we examined the samples of 150/207 patients for the presence of EBV, high-risk HPVs, and TLR expression (TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9), and compared the results with survival data. EBV was found in 62% and HPVs in 14% of the tumour cases, while 24% of the tumours were EBV/HPV-negative. The patients with EBV-positive tumours had the best 5-year survival rates, and the patients with HPV-positive tumours had a significantly better overall survival than those with EBV/HPV-negative tumours. Moreover, positive TLR7 expression was found to be an independent prognostic factor for favourable outcome.

Keywords: nasopharyngeal carcinoma, Epstein-Barr virus, human papillomavirus, toll-like receptor

TIIVISTELMÄ

Miia Ruuskanen

Nenänielukarsinooma Suomessa: Epstein-Barr-virus, ihmisen papilloomavirukset ja tollin kaltaiset reseptorit ennusteellisina tekijöinä

Turun yliopisto, Lääketieteellinen tiedekunta, Korva-, nenä- ja kurkkutaudit - pään ja kaulan kirurgia, Turun kliininen tohtoriohjelma (TKT)

Annales Universitatis Turkuensis, Turku, Finland, 2019

Nenänielukarsinooma on nenänielun limakalvon epiteelisolujen pahanlaatuinen kasvain. Se on Pohjois-Euroopassa harvinainen, ja kyseinen syöpä todetaan Suomessa vain noin kymmenellä potilaalla vuosittain. Nenänielukarsinooman syntyyn on liitetty Epstein-Barr-viruksen (EBV) aiheuttama infektio nenänielussa, mutta syöpänäytteissä on todettu myös ihmisen papilloomaviruksia (engl. human papillomavirus, HPV). Lisäksi potilaan synnynnäisillä, immunologiseen puolustukseen liittyvillä ominaisuuksilla oletetaan olevan vaikutusta sairastuvuuteen. Tollin kaltaiset reseptorit (engl. toll-like receptor, TLR) ovat solun kalvorakenteisiin kiinnittyneitä, luonnollista immuniteettia säätteleviä proteiineja, jotka tunnistavat sekä mikrobiperäisiä että elimistön omia, kudostuhossa solun sisältä vapautuneita, rakenteita. TLR:ien on todettu ilmentyvän useissa syövissä, mutta niiden merkitystä ei vielä tarkkaan tunneta. TLR:illä on havaittu olevan sekä syövän kasvua edistäviä että hidastavia vaikutuksia.

Tämän kansallisen tutkimuksen tarkoituksena on ollut selvittää nenänielukarsinooman esiintyvyys, kasvainten kudosopilliset alaryhmät, annetut hoidot ja niiden haittavaikutukset sekä hoitotulokset Suomessa vuosina 1990-2009 diagnosoiduilla ja hoidetuilla nenänielusyöpäpotilailla. Kyseiseltä 20 vuoden ajanjaksolta identifioimme Suomen Syöpärekisteristä 207 potilasta. Lisäksi tutkimme 150/207 potilaan syöpänäytteistä EBV:n ja korkean riskin HPV-virusten esiintymistä ja TLR:ien (TLR1, TLR2, TLR4, TLR5, TLR7 ja TLR9) ilmentymistä, sekä niiden yhteyttä potilaan selviytymiseen. EBV:tä esiintyi 62 %:ssa ja HPV:tä 14 %:ssa näytteistä, ja 24 % näytteistä oli virusnegatiivisia. Paras viiden vuoden eloonjäämisen prosenttiosuus oli potilailla, joiden kasvain oli EBV-positiivinen. HPV-positiivistenkin eloonjäämisosuus oli tilastollisesti merkitsevästi parempi kuin virusnegatiivisilla. Lisäksi positiivisen TLR7-ilmentymisen todettiin olevan itsenäinen, hyvää hoitotulosta ennustava tekijä.

Avainsanat: nenänielukarsinooma, Epstein-Barr-virus, ihmisen papilloomavirus, tollin kaltainen reseptori

TABLE OF CONTENTS

ABBREVIATIONS

2-D	Two-dimensional
3-D	Three-dimensional
AC	Adjuvant chemotherapy
BNCT	Boron neutron capture therapy
CI	Confidence interval
CRT	Chemoradiotherapy
CT	Computed tomography
CTV	Clinical target volume
DAB	Diaminobenzidine
DAMP	Damage-associated molecular pattern
EBER	Epstein-Barr virus-encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EBV-CTL	Epstein-Barr virus-specific cytotoxic T cell
EDTA	Ethylenediaminetetra-acetic acid
EGFR	Epidermal growth factor receptor
FDG	Fluorodeoxyglucose
FFPE	Formalin-fixed paraffin-embedded
GTV	Gross tumour volume
Gy	Gray
H&E	Hematoxylin and eosin
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	Hazard ratio
IARC	International Agency for Research on Cancer
IC	Induction chemotherapy
ICBT	Intracavitary brachytherapy
IHC	Immunohistochemistry
IMRT	Intensity-modulated radiotherapy
ISH	In situ hybridization
KSCC	Keratinizing squamous cell carcinoma
LFFR	Local failure-free rate
LMP	Latent membrane protein
MLC	Multileaf collimator
MRI	Magnetic resonance imaging
NA	Not applicable
NBI	Narrow-band imaging
NK-D	Non-keratinizing carcinoma, differentiated
NK-U	Non-keratinizing carcinoma, undifferentiated
NPC	Nasopharyngeal carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
OTSCC	Oral tongue squamous cell carcinoma
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PET	Positron emission tomography
PRR	Pattern-recognition receptor
PTV	Planning target volume
Rb	Retinoblastoma
RR	Risk ratio

RT	Radiotherapy
SCC	Squamous cell carcinoma
SD	Standard deviation
SEER	Surveillance, Epidemiology, and End Results Program
SIB	Simultaneous integrated boost
TLR	Toll-like receptor
TMA	Tissue microarray
UICC	International Union Against Cancer
VCA	Viral capsid antigen
VMAT	Volumetric arc therapy
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-III.

I Ruuskanen M, Grenman R, Leivo I, Vahlberg T, Mäkitie A, Saarilahti K, Wigren T, Korpela M, Voutilainen L, Koivunen P, Irjala H, Minn H. Outcome of nasopharyngeal carcinoma in Finland: a nationwide study. Acta Oncol 2018; 57:251-256.

II Ruuskanen M, Irjala H, Minn H, Vahlberg T, Randen-Brady R, Hagström J, Syrjänen S, Leivo I. Epstein-Barr virus and human papillomaviruses as favorable prognostic factors in nasopharyngeal carcinoma – a nationwide study in Finland. Head Neck 2019; 41:349-357.

III Ruuskanen M, Leivo M, Minn H, Vahlberg T, Haglund C, Hagström J, Irjala H. Expression of toll-like receptors in non-endemic nasopharyngeal carcinoma. [Submitted]

1 INTRODUCTION

Nasopharyngeal carcinoma (NPC) is included in head and neck cancers, even though it is a unique malignancy regarding its epidemiology, etiology, and clinical manifestation. It is rare in most parts of the world, but the geographical and racial disparities are noteworthy (Tang et al. 2016). The distribution of histological subtypes and etiological risk factors differ between regions, and presumably the interplay of genetic susceptibility, viral agents, and environmental exposures are needed for NPC carcinogenesis (Chua et al. 2016).

The nasopharynx is the uppermost part of the pharynx extending from the central skull base to the upper surface of the soft palate (Figure 1). Anteriorly, it merges with the nasal cavity. NPC arises from the epithelial lining of the nasopharynx, commonly from the pharyngeal recess (fossa Rosenmüller), which is located in the posterolateral aspect of the nasopharynx. Because of the extensive supply of lymphatics, NPC readily metastasizes to regional lymph nodes. Thus, a cervical mass is frequently the first symptom of NPC. Detecting the primary tumour in the nasopharynx often requires a nasoendoscopy, which is not available at a general practitioner's office, and the majority of patients have advanced disease at the time of diagnosis (Lee et al. 2012b). A biopsy of the nasopharyngeal tumour and an imaging study, preferably magnetic resonance imaging (MRI) and/or computed tomography (CT) scan, are necessary to definitively diagnose NPC.

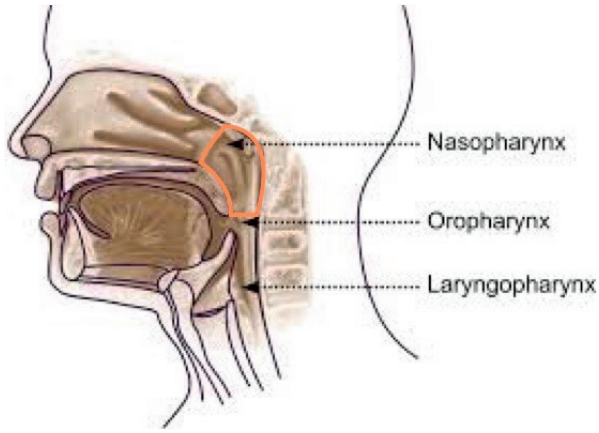

Figure 1 **Diagram of pharynx regions.** Modified from the original illustration by 'Sémhur' (reproduced under the terms of the Wikimedia Commons licence CC BY-SA 3.0).

Radiotherapy (RT) is the primary treatment for NPC because of the radiosensitive nature of the disease. Since the late 1990s, concurrent chemotherapy has been used, and a meta-analysis confirmed the benefit of this chemoradiotherapy (CRT) in NPC of stages II-IV (Baujat et al. 2006). Only stage I disease with a local nasopharyngeal tumour can be treated without combining radiotherapy and chemotherapy (Baujat et al. 2006). The evolution of irradiation techniques has improved the patient survival, and with more accurate intensity-modulated radiotherapy (IMRT) normal structures can be saved. Using IMRT with concurrent chemotherapy results in good locoregional control of NPC, but distant metastases are still a major cause of death (Lee et al. 2014). Surgery is exclusively needed for residual or recurrent NPC.

Epstein-Barr virus (EBV) infection is closely associated with NPC, but the exact role of EBV in NPC pathogenesis remains unclear (Wu et al. 2018). In addition, high-risk human papillomaviruses are supposed to be etiologically linked to NPC, especially in Caucasians (Robinson et al. 2013). In high-incidence (endemic) areas, virtually all NPC tumours are non-keratinizing, EBV-positive carcinomas, while in low-incidence (non-endemic) areas, the proportion of keratinizing squamous cell carcinomas (KSCC) is significant (Chua et al. 2016). Evidence of the prognostic value of EBV and HPV is limited, but in a study from non-endemic area, the patients with HPV-positive tumours had worse survival and local control than the patients with EBV-positive tumours, whereas distant metastases were more common in association with EBV-positive tumours (Stenmark et al. 2014). Recent studies indicate that patients' immunological responses to viruses and to precancerous lesions are important factors in the development of cancer. A failure in the innate immune response could be partly responsible for tumour progression (Chow et al. 2012).

Toll-like receptors (TLRs) are a family of transmembrane proteins involved in innate immunity, which recognize the receptor-specific pathogen-associated molecular patterns (PAMPs) of microbes and activate immune responses against them (Takeda et al. 2003). TLRs also react to endogenous damage-associated molecular patterns (DAMPs), which are released from injured tissues, and maintain tissue homeostasis (Tsan 2006). Many types of cancers are reported to express TLRs, but the actual functions of TLRs remain elusive: TLR stimulation may be tumour-suppressing or tumour-promoting depending on the TLR receptor and cancer type (Dajon et al. 2017).

The purpose of this study was to evaluate the provided treatments and the outcome of NPC patients diagnosed in Finland between 1990 and 2009. Furthermore, this study aimed at evaluating factors influencing outcome, such as EBV and high-risk HPV infections, and the expression of TLRs in NPC tumours.

A deeper understanding of tumour behaviour would be useful in developing future treatment protocols, for example, with immunomodulating therapies.

2 REVIEW OF LITERATURE

2.1 Nasopharyngeal carcinoma (NPC)

2.1.1 Epidemiology

Nasopharyngeal carcinoma (NPC) is a rare cancer in most parts of the world with an overall incidence of less than 1/100,000 persons per year. Nevertheless, NPC incidence is particularly high in southern China and among Inuits living in Greenland and Alaska (Jemal et al. 2011). The highest annual incidence rates are 26.6/100,000 in males and 10.7/100,000 in females in Chinese Zhongshan (Tang et al. 2016). Intermediate incidences of 5-15/100,000 have been reported from regions including Southeast Asia (Malaysia, Philippines, Thailand), North Africa, and the Middle East (Turkey, Saudi Arabia) (Tang et al. 2016). It has been estimated that 86,691 new cases and 50,831 deaths of NPC occurred worldwide in 2012, and for both incidence and mortality, the rates in men are 2-3 times higher than those in women (Tang et al. 2016). A significant decreasing trend in incidence has been reported from nearly all high-incidence areas (Tang et al. 2016). This decline has been remarkable in Hong Kong, where incidence has decreased 50% during the 30-year period from 1980 to 2010, most probably due to the gradual reduction of exposure to environmental risk factors (Lee et al. 2012b). A decreasing trend in NPC incidence has also been observed in several European countries: a minimal decline of 1.3% was observed in the Nordic countries between 1970-2007 (Tang et al. 2016). Based on the Finnish Cancer Registry database, the age-adjusted incidence was 0.3/100,000 per year in males and 0.1/100,000 per year in females in Finland between 1990 and 2009, and the incidence rates remained stable. In most low-incidence populations, NPC incidence increases with age, while in high-incidence populations, it peaks around 50-59 years and declines thereafter (Chang and Adami 2006). In addition, there is a minor peak observed among adolescents and young adults in endemic areas (Chang and Adami 2006).

2.1.2 Etiology

2.1.2.1 Epstein-Barr virus

Epstein-Barr virus (EBV) infection is probably the most studied etiological factor for NPC. EBV has been detected in NPC cells and in pre-invasive, dysplastic nasopharyngeal lesions but not in normal nasopharyngeal epithelium (Pathmanathan et al. 1995). Studies suggest that dysplastic nasopharyngeal epithelium becomes susceptible to EBV infection, and clonal proliferation of EBV-infected cells could be a part of a multistep process of NPC carcinogenesis (Pathmanathan et al. 1995, Fan 2006). Presumably, the carcinogenic potential of EBV strains differ, as well as the host's immunological properties, and a specific combination of these variants leads proliferating cells to escape immune control (Raab-Traub 2012, Tsai et al. 2013). In high-incidence areas, EBV is found in 99% of NPC tumours (Lin et al. 2014). In contrast, in low-incidence areas, the reported EBV positivity rates vary approximately from 32% to 85%, with an average of 42% in Caucasian patients (Svajdler et al. 2016). Table 2 in chapter 2.3.1 summarizes the results of essential studies assessing viral status in NPC.

2.1.2.2 Human papillomaviruses

High-risk human papillomaviruses (HPVs) are important etiological factors in oropharyngeal carcinoma, but in NPC evidence is limited. Nevertheless, many studies, especially from low-incidence regions but also from endemic Taiwan, have reported HPV in 1% to 47% of NPC tumours with or without EBV co-existence (Table 2) (Tyan et al. 1993, Lo et al. 2010, Laantri et al. 2011, Huang et al. 2011, Singhi et al. 2012, Robinson et al. 2013, Dogan et al. 2014, Lin et al. 2014, Stenmark et al. 2014, Jiang et al. 2016, Dogan et al. 2016, Kano et al. 2017). Interestingly, in one Taiwanese study, HPV DNA was found in non-malignant nasopharyngeal control samples more often than in NPC (in 35% vs. 31%, respectively) (Huang et al. 2011). However, the study used a sensitive polymerase chain reaction (PCR) method, and consequently, false positive findings are possible (Huang et al. 2011). On the other hand, the same study examined another set of NPC samples with a different method, in situ hybridization, and found HPV positivity in 41% of the samples (Huang et al. 2011).

2.1.2.3 Non-viral environmental risk factors

The consumption of salt-preserved food, especially salted fish, during childhood is firmly associated with at least two-fold NPC risk in endemic areas (Ward et al. 2000, Yong et al. 2017). Salt-preserved food contains nitrosamines, which are believed to be NPC associated carcinogens (Chua et al. 2016). A recent case-control study in Singapore found a significant increase in NPC risk also when an adult consumed salted vegetables at least once a week (Yong et al. 2017). In addition, they found that current tobacco smokers and ex-smokers had a higher risk of NPC than never-smoked patients. Two other studies, conducted in endemic regions of China, confirmed that both active and passive smoking were associated with a modest increase in NPC risk (Xie et al. 2015, Chang et al. 2017). Abundant alcohol consumption seems not to be associated with NPC risk (Chen et al. 2016). In some studies, occupational exposures to fumes, dusts, or chemicals have been associated with higher NPC risk, but the results are inconsistent. A study in Hong Kong suggested that exposure to chemical and welding fumes and to cotton dust might be associated with an increased risk of NPC (Xie et al. 2017). In contrast, frequent consumption of fresh fruits and vegetables may lower the risk (Liu et al. 2012).

2.1.2.4 Familial clustering and genetic susceptibility

Distinct ethnicity and familial clustering indicate the contribution of genetic susceptibility in NPC pathogenesis. Population-based analyses from high-incidence areas (China, Taiwan, Greenland), intermediate-incidence areas (Israel with North African immigrants), and a low-incidence area (Sweden) have reported that the relatives of NPC patients have an increased risk of NPC (Jia et al. 2005, Huang et al. 2017, Friborg et al. 2005, Rottenberg et al. 2017, Liu et al. 2015). The reported risks ratios (RRs) for first-degree relatives ranged from 4.29 in low-incidence area to 9.23 in high-incidence area. In a Taiwanese study, the RR for twins of NPC patients was as high as 34.46 (Huang et al. 2017). Such familial clustering can result from genetic susceptibility, shared environmental risk factors, or both. Thus, an extensive number of studies have been conducted to evaluate the genetic predisposition factors for NPC. Several small-scale studies have reported the associations of genes involved in immune responses, DNA repair, cell-cycle checkpoint regulation, and cell adhesion and migration, but potential causal pathways remain to be confirmed (Chua et al. 2016). In genome-wide association studies, the most consistent findings concern genes within the major histocompatibility complex region on chromosome 6p21, where the human leukocyte antigen (HLA) genes are located (Hildesheim 2012). These

genes encode the proteins needed for foreign-antigen presentation to the immune system. Because the majority of NPC tumours contain EBV, it is assumed that individuals who have inherited deficiency in HLAs to present EBV antigens may have an increased risk of developing NPC (Hildesheim et al. 2002).

In a Taiwanese population-based study with the data on 23 million individuals, the contribution of heredity was estimated to be 61%, while shared environmental factors accounted for 14% and non-shared environmental factors for 25% (Huang et al. 2017). These findings are in line with the previously reported declining trend for NPC risk related to immigration from a high-incidence region to a low-incidence region (Luo et al. 2007). The NPC incidence among immigrants and their descendants still remains higher than that among local residents (Mousavi et al. 2010, Liu et al. 2015, Rottenberg et al. 2017).

2.1.3 Histopathological classification

The World Health Organization (WHO) classification of head and neck tumours recognizes three types of NPC: non-keratinizing (squamous cell) carcinoma, keratinizing squamous cell carcinoma (KSCC), and basaloid squamous cell carcinoma (El-Naggar et al. 2017). The non-keratinizing carcinoma group can be further divided into undifferentiated (NK-U) and differentiated (NK-D) subtypes. NK-U has a typical morphology with dense infiltration of lymphocytes and plasma cells, which explains the formerly used term ‘lymphoepithelioma’ (Figure 2) (Lo 2004). Historically, it has also been called Schmincke’s tumour or Regaud’s tumour (Burman and Burman 1943). In addition to the infiltration of lymphocytes and plasma cells, NK-D subtype exhibits cellular stratification and pavementing (Figure 3) (El-Naggar et al. 2017). The histological features of KSCC are similar to SCCs arising from other sites of head and neck regions with well-differentiated histology including intercellular bridges, keratinization, and keratin pearl formation (Figure 4). Basaloid SCC is the most unusual variant of NPC with only a few cases reported in the literature, and it is histologically similar to basaloid SCCs occurring elsewhere in the upper aerodigestive tract (Müller and Beleites 2000).

Differential diagnostics are performed between NPC and lymphoma, adenoid cystic carcinoma, extramedullary plasmacytoma, melanoma, rhabdomyosarcoma, and tuberculosis (Chua et al. 2016).

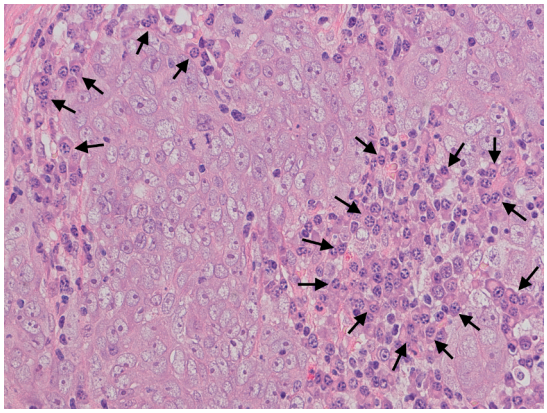

Figure 2 **Non-keratinizing undifferentiated carcinoma (NK-U) of the nasopharynx with infiltration of plasma cells (arrows).** H&E staining, magnification x 400. Photo by Ilmo Leivo.

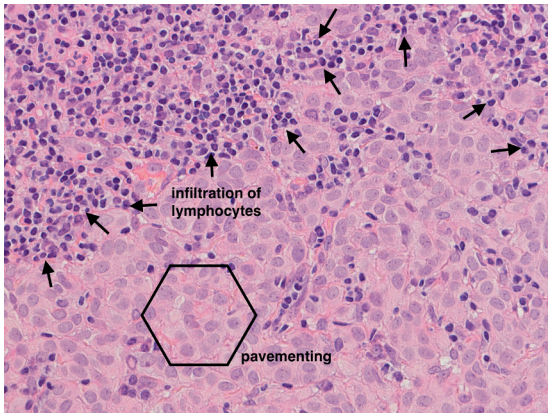

Figure 3 **Non-keratinizing differentiated carcinoma (NK-D) of the nasopharynx with infiltration of lymphocytes (arrows). An example of cellular pavementing inside the hexagon.** H&E staining, magnification x 400. Photo by Ilmo Leivo.

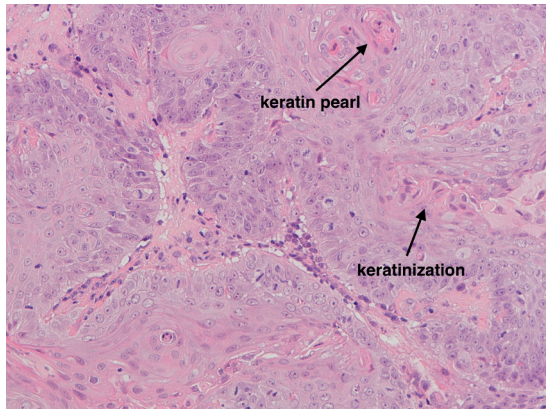

Figure 4 **Keratinizing squamous cell carcinoma (KSCC) of the nasopharynx with keratinization and keratin pearl formation.** H&E staining, magnification x 250. Photo by Ilmo Leivo.

2.1.4 Symptoms and diagnosis

The clinical manifestation of NPC can often be confusing and its symptoms minimal until the disease progresses into an advanced stage. Additionally, it is relatively difficult to examine the nasopharynx, and diagnosis can be delayed if the patient is not referred to an otorhinolaryngologist. The possible symptoms of NPC have been divided into four categories (Wei and Sham 2005): (1) presence of tumour mass in the nasopharynx and symptoms related to it, for example nasal bleeding, obstruction, and discharge; (2) dysfunction of the eustachian tube associated with the extension of the tumour to the parapharyngeal space leading to chronic otitis media, hearing impairment, and/or tinnitus; (3) skull-base erosion and paresis of the trigeminus and abducens nerves causing headache, diplopia, facial pain and numbness; and (4) neck mass usually appearing in the upper neck. Nodal metastasis in the neck is frequently part of the first findings, but retropharyngeal nodal involvements are also common. The possible routes for primary tumour invasion are anterior spread into the nasal cavity, pterygoid fossa, and maxillary sinuses; lateral spread beyond the pharyngobasilar fascia into the parapharyngeal and infratemporal spaces; and posterior/superior spread to the base of the skull, clivus, and intracranial structures (Chua et al. 2016).

NPC is routinely diagnosed via a nasoendoscopy and nasopharyngeal biopsy under local anesthesia at an outpatient clinic. However, to obtain more representative samples from the nasopharynx, it is sometimes necessary to perform the biopsy under general anesthesia. Nasoendoscopy involves passing a

flexible or rigid endoscope through the nasal cavity to permit direct visualization of the tumour (Figure 5). Early tumours might be difficult to differentiate from normal nasopharyngeal mucosa, and thus the value of narrow-band imaging (NBI) has been studied for detecting NPC. NBI is an imaging technique integrated with the endoscopy system to enhance the visualization of mucosal microvascular patterns. It is used to identify potentially malignant lesions for instance in the larynx and hypopharynx (Ni and Wang 2016). However, a recent meta-analysis did not show any significant difference in NPC detection rate when NBI was compared to white light endoscopy (Yeung et al. 2018). One possible reason for this is that the nasopharynx is rich in adenoidal tissue, which hinder the exposure of the superficial microvasculature (Ni and Wang 2016).

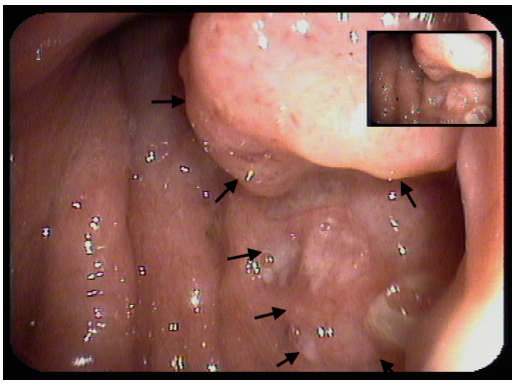

Figure 5 **Endoscopic image of the posterior wall of the nasopharynx with a tumour (arrows) originating from the right pharyngeal recess.** Photo by Jaakko Pulkkinen.

During staging and planning radiotherapy, it is crucial that the primary tumour area and the neck are accurately imaged. MRI provides a better resolution than CT in assessing the invasion of the carcinoma to the parapharyngeal spaces, intracranially, or to deep cervical lymph nodes, but the bony erosions of the skull base can be more easily detected with a CT scan (Figure 6) (Chung et al. 2004). To evaluate patients with cervical metastases of unknown origin, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET)/CT has primarily been used. However, it is also useful for assessing regional nodal and distant metastases in diagnosed NPC patients, and PET/MRI offers even better accuracy in distinguishing, for example, retropharyngeal nodal metastasis from an adjacent nasopharyngeal tumour (Chan et al. 2018b).

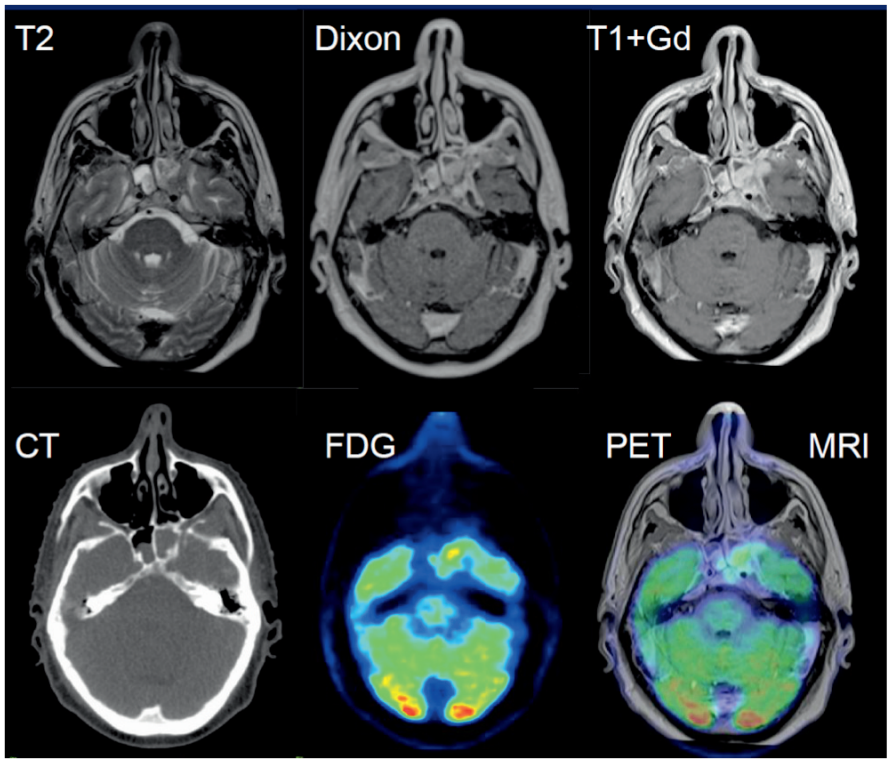

Figure 6 **Imaging of the nasopharynx.**
Upper row: MRI pictures with different image weightings.
Lower row from the left: CT, ^{18}F-FDG PET, and PET/MRI.
Picture by Heikki Minn.

2.1.5 Staging

Clinical TNM classification, based on the spread of the primary tumour (T), regional lymph node metastasis (N), and distant metastasis (M), as well as stage grouping based on TNM classification, is used to describe the extent of the disease. Table 1 presents the TNM classification and corresponding stage classification for NPC according to the International Union Against Cancer (UICC) staging system, 7th edition (Sobin et al. 2009). This update introduced changes in both the T and N categories compared to the 6th edition: the T1 category was modified to also include tumours classified as T2a in the previous edition, and the former T2b category was renamed T2. Accordingly, stage IIA was included to stage I, and the former stage IIB was renamed stage II. In addition, the mention of unilateral or bilateral retropharyngeal lymph node metastasis was added to the N1 category.

Table 1 TNM and stage classification for nasopharyngeal carcinoma according to UICC 7th edition. Modified from Sobin et al 2009.

T- Primary tumour			
TX	Primary tumour cannot be assessed		
T0	No evidence of primary tumour		
Tis	Carcinoma in situ		
T1	Tumour confined to nasopharynx, or extends to oropharynx and/or nasal cavity		
T2	Tumour with parapharyngeal extension		
T3	Tumour invades bony structures of skull base and/or paranasal sinuses		
T4	Tumour with intracranial extension and/or involvement of cranial nerves, infratemporal fossa, hypopharynx, orbit, or masticator space		
N – Regional lymph nodes			
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Unilateral metastasis in cervical lymph node(s), and/or unilateral or bilateral metastasis in retropharyngeal lymph nodes, ≤ 6 cm in greatest dimension, above the supraclavicular fossa		
N2	Bilateral metastasis in cervical lymph nodes, ≤ 6 cm in greatest dimension, above the supraclavicular fossa		
N3	Metastasis in cervical lymph node(s), > 6 cm and/or in the supraclavicular fossa		
N3a	> 6 cm in greatest dimension		
N3b	In the supraclavicular fossa		
M – Distant metastasis			
M0	No distant metastasis		
M1	Distant metastasis		
Stage grouping			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T1	N1	M0
	T2	N0-1	M0
Stage III	T1-2	N2	M0
	T3	N0-2	M0
Stage IVA	T4	N0-2	M0
Stage IVB	Any T	N3	M0
Stage IVC	Any T	Any N	M1

After the completion of our data collection, the 8th edition of the UICC classification was published in 2017 (Brierley et al. 2017). It contains minor adjustments to the T and N categories. In the T2 category, besides parapharyngeal extension, a tumour may infiltrate to the medial or lateral pterygoid muscles, and/or prevertebral muscles. In the T3 category, 'bony structures' are specified as the skull base, cervical vertebrae, pterygoid structures, and paranasal sinuses. In the T4 category, the term 'masticator space' is changed to 'infiltration to the parotid gland and/or beyond the lateral surface of the lateral pterygoid muscle'. In the N categories, the level of N2-N3 category boundary is

changed to the caudal border of cricoid cartilage instead of 'above or in the supraclavicular fossa'. The N3a and N3b subcategories were also suppressed.

2.1.6 Treatment

2.1.6.1 Radiotherapy

Radiotherapy (RT) is the mainstay of NPC treatment not only because of the extensive infiltration and lymphatic spread tendency of the disease, but also because NPC is highly sensitive to ionizing radiation. During decades, NPC was staged using planar X-ray radiography and treated with a two-dimensional (2-D) technique (Lee et al. 2014). From the early 1990s, the introduction of cross-sectional imaging enabled the evolution of the irradiation technique first from 2-D RT to three-dimensional (3-D) conformal RT. Ten years later, intensity-modulated RT (IMRT) was introduced to clinical practice and was soon followed by its modification, volumetric arc therapy (VMAT). 2-D and 3-D techniques utilized opposed lateral fields with a supplementary anterior field focused on the primary tumour. This shrinking-field technique combined high-energy photons, and during the later phase, electron portals for the dorsal neck to avoid overdosing the spinal cord. The proximity of the primary disease to critical structures such as the optic chiasm, spinal cord, cochleae, and brainstem complicated delineation with conventional techniques, and some parts of the targets were at risk of an underdose of irradiation (Zhang et al. 2015).

The use of IMRT has led to increasing conformity of tumour coverage with better sparing of normal structures (Lee et al. 2014). It uses up to nine coplanar radiation beams to cover the entire region, and the dynamic, multileaf, intensity-modulating collimators enable accurate irradiation (Tang et al. 2015). VMAT, in turn, delivers the desired dose distribution during the rotation of the gantry over 360 degrees using one isocentre (Teoh et al. 2011). With these methods, all target volumes are irradiated during every radiation session, but a higher dose is delivered to the tumour simultaneously with lower doses to adjacent tissues.

Modern planning of RT implies co-registration of CT, MRI, and PET/CT images in treatment position, which enables the accurate delineation of target and organs at risk in 3D (Minn et al. 2010). For delineating clinical target volumes in IMRT/VMAT, the same principles are used as in 3-D RT (Lee et al. 2014). First, the original gross tumour volume (GTV) including the enlarged regional lymph nodes is outlined based on imaging, MRI and/or CT. The clinical target volume 1 (CTV1) for 70 Gray (Gy) of radiation encompasses GTV plus the surrounding

areas at risk for microscopic disease (2-5 mm margin). The CTV2 for 60-61 Gy covers high-risk local structures, for example the parapharyngeal spaces, the pterygoid processes, and the base of skull, and in addition the lymph nodes bilaterally in the retropharyngeal space and in the upper neck. The CTV3 for 50-52 Gy covers the remaining potential sites of local infiltration, and lower lymphatics. The planning target volume (PTV) provides a margin around the CTV to allow variation in the treatment setup and other anatomic motion during treatment (Zhang et al. 2015). See Figure 7 for an example of the treatment plan. The most commonly used RT fractionation schedule for NPC is 1.8-2.0 Gy once daily for 5 days per week, but accelerated hyperfractionation with 1.6 Gy twice daily dosing and a planned interim break for about 11 days has also been used in 1990s (Lindholm et al. 2006).

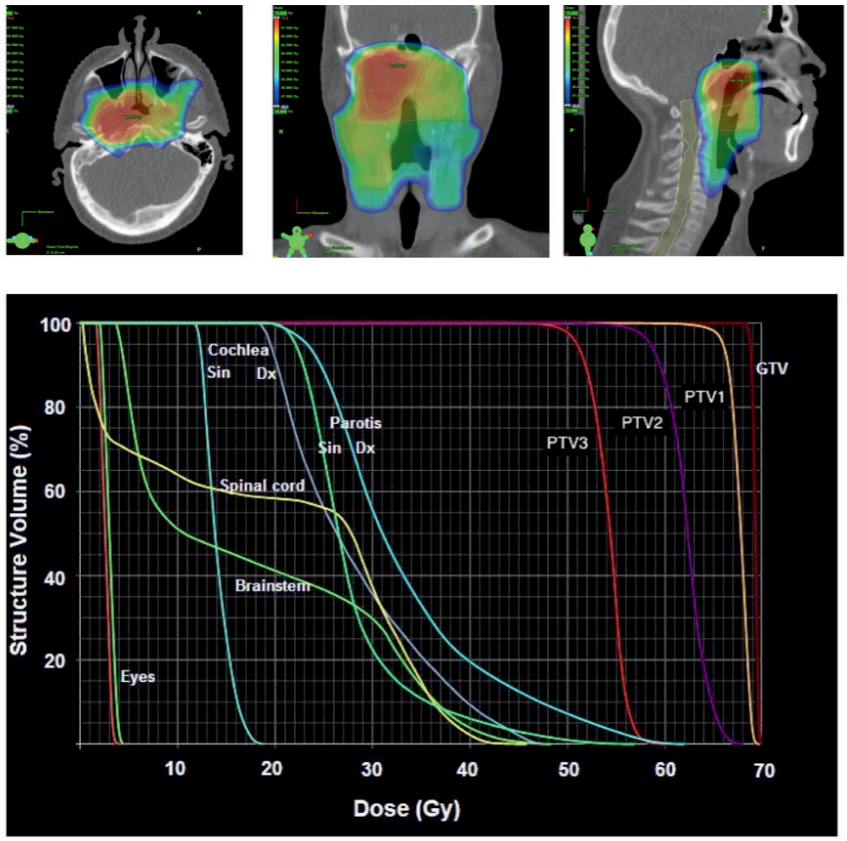

Figure 7 **Target volumes and radiation dose distribution of a patient with T2N0M0 nasopharyngeal carcinoma.**

Pictures by Heikki Minn.

Several studies have reported better survival, especially loco-regional survival, in NPC patients treated with IMRT compared to patients treated with 2-D or 3-D RT (Lee et al. 2014, Tang et al. 2015, Zhang et al. 2015). However, it has been estimated that distant metastasis-free survival has not improved considerably with the evolution of irradiation techniques, which emphasizes the role of concurrent chemotherapy in NPC treatment (Lee et al. 2014).

Intracavitary brachytherapy (ICBT) is one option to boost the primary site after definitive RT in early T category disease - residual or recurrence. Technical procedures vary, but the idea is to temporarily place a small radiation source, for example an applicator loaded with radioactive ^{192}Iridium, into the nasopharynx for local irradiation. The most frequently used protocols consist of ICBT given as 6-10 Gy in 2-3 fractions within 1-2 weeks. ICBT was a common treatment method during the 2-D RT and 3-D RT era, but it can still be used in some centres to escalate the irradiation dose after IMRT (Chao et al. 2017). However, with the modern IMRT technique, a simultaneous integrated boost (SIB) can serve a similar purpose (Chao et al. 2017).

A locally recurrent disease can also be treated with radiosurgery, i.e. stereotactic radiotherapy with cyber-knife or linear accelerator-based technologies. Stereotactic radiotherapy has been widely used already with the 3-D RT technique, but with IMRT it operates with even higher geometric precision by delivering a conformal and homogenous dose to the PTV while sparing adjacent tissues (Kung et al. 2011). In stereotactic radiotherapy, 7-15 Gy in a single fraction or 12-15 Gy in 3-5 fractions are applied to the tumour with narrow margins.

Boron neutron capture therapy (BNCT) is a treatment modality, which is used in a few centres worldwide for locally recurrent, inoperable head and neck cancer. BNCT produces a strong local RT effect based on the neutron capture reaction that occurs when non-radioactive boron is irradiated with neutrons (Barth et al. 2018). Boron is selectively accumulated into cancer cells following the intravenous injection of a boronated compound, such as L-boronophenylalanine, and once or twice delivered neutron irradiation causes a nuclear decay yielding high-energy particles with short path length (Kankaanranta et al. 2012). Thus, most radiation effect is local, and even heavily pre-treated patients can tolerate BNCT. A Finnish study group has conducted a phase II trial with 30 patients with HNSCC of whom 9 had NPC (Kankaanranta et al. 2012). They found that 76% of all patients responded to BNCT, and median progression-free survival time was 7.5 months (Kankaanranta et al. 2012).

2.1.6.2 Chemotherapy

Many chemotherapeutic agents have anti-tumour activity in NPC, and are most effective when used as radiosensitizers during RT. The effect of the most used drug cisplatin is based on repair inhibition of radiation-induced DNA damage (Wang et al. 2002). Since the late 1990s, chemotherapy has been used routinely in concurrent setting for locally or locoregionally advanced stages. Several trials and a meta-analysis published in 2006 have substantiated the benefits of chemoradiotherapy (CRT) protocols in disease control and survival in stages II-IV (Baujat et al. 2006). In CRT, cisplatin can be given intravenously either 30-40 mg/m^2 once a week or 100 mg/m^2 every 3 weeks during RT (Chua et al. 2016).

If concurrent CRT is currently the standard of care in at least locally advanced NPC, the roles of induction and adjuvant chemotherapies remain elusive. For induction chemotherapy (IC), which means initial treatment with cytotoxic drugs prior to RT, many different chemotherapeutic regimens have been used. These include doublets such as cisplatin plus fluorouracil, cisplatin plus docetaxel, and cisplatin plus capecitabine, and triplets such as carboplatin plus paclitaxel plus gemcitabine. A recent meta-analysis of four randomized trials found that additional IC was superior to CRT alone in locoregionally advanced NPC (Chen et al. 2018). In that study, the survival benefit was mainly associated with improved distant control. The compliance was satisfactory, because about 90% of the patients completed their planned IC cycles.

In adjuvant chemotherapy (AC), cytotoxic drugs are given for a pre-defined period after RT. AC typically consists of cisplatin (20 mg/m^2 daily for 4 days) and fluorouracil (1 g/m^2 daily for 4 days) given every 4 weeks in three cycles (Chua et al. 2016). Trends seen in several trials show that this additional chemotherapy after RT is poorly tolerated, and compliance is 55-75% at best. In addition, these trials, specifically designed to investigate the benefits of AC, did not show a survival advantage (Kwong et al. 2006, Chen et al. 2012). Nevertheless, there are recent positive results with metronomic AC, which consists of daily oral tegafur-uracil treatment for 12 months starting 3 months after RT (Twu et al. 2014). In that study, continuous low-dose treatment caused minor toxicity but reduced the occurrence of distant metastasis and prolonged survival among high-risk patients.

In recurrent and/or metastatic NPC, cisplatin plus 5-fluorouracil has been the standard treatment for a long time, but due to the results of recent multicentre trials, a doublet regimen consisting of cisplatin plus gemcitabine has also been introduced (Zhang et al. 2016).

Cetuximab, a monoclonal antibody for inhibiting the epidermal growth factor receptor (EGFR), has also been tested in NPC. A phase II study integrated cetuximab with CRT, and reported encouraging 2-year progression-free survival of 87% in the patients with stage III-IV disease (Ma et al. 2012). However, further studies are warranted to establish the role of EGFR inhibitors in NPC.

2.1.6.3 Surgery

Surgery plays a very limited role in the initial management of NPC. However, it is an important salvage treatment option for residual or recurrent NPC after definite RT or CRT. Persistent or recurrent disease in the neck nodes is usually treated with a neck dissection but elective post-RT neck dissections should be avoided (Wang et al. 2016). In other words, surgery on the neck should be reserved only for patients with a histologically confirmed nodal disease after RT. PET/CT has also proved useful in the evaluation of enlarged lymph nodes after primary treatment (Mehanna et al. 2016, Peng et al. 2017). Salvage surgery for neck node metastasis is effective, and in most cases, selective neck dissection is sufficient. Although the most probable site for nodal metastasis is level II, the extent of surgical resection depends on the pathological features of the tumour, and additionally the lymph nodes in levels III, IV, and V are often removed (Figure 8) (Wang et al. 2016).

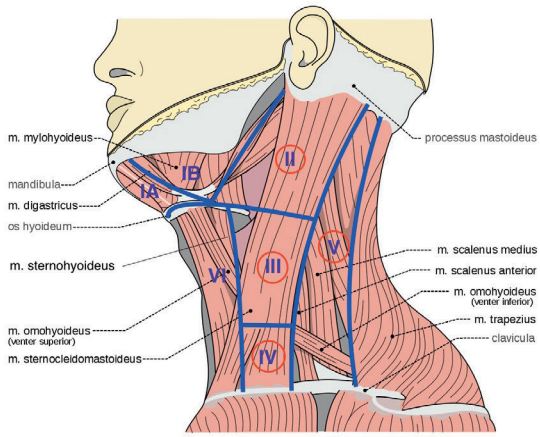

Figure 8 **Neck levels I-VI.** Modified from the original illustration by Olek Remesz (reproduced under the terms of the Creative Commons licence CC BY-SA 2.5).

Persistent or recurrent nasopharyngeal tumours can be treated surgically, if additional radiation therapy is not possible or fails, and if surgical resection is feasible with curative intent. This means that the main bulk of the tumour has to be localized in the nasopharynx and there should not be erosion at the skull base (Wei et al. 2011). Various surgical approaches have been used for nasopharyngectomy, such as anterior midfacial deglove procedure, anterolateral maxillary swing, lateral infratemporal approach, and transpalatal approach from below. In high-incidence areas, routinely used maxillary swing operation with anterolateral approach to the nasopharynx has led to good results: reported 5-year disease-free survival has been 56% (Wei et al. 2011). However, such surgery may cause important functional disabilities and compromise the quality of life. In recent years, endoscopic endonasal surgery has emerged as a viable alternative. Local control rates have been comparable to those of conventional operations (Castelnuovo et al. 2013, Emanuelli et al. 2014).

2.1.6.4 Immunotherapy

The occurrence and growth of a tumour can be considered as an escape from the anti-tumoural immune system. In recent years, immunomodulative treatments have shown promising result in various cancers, including head and neck squamous cell carcinomas (HNSCC) (Outh-Gauer et al. 2018). In the healthy immune system, activated T cells express programmed death 1 (PD-1) receptors, which interact with their ligands, programmed death ligand 1 (PD-L1) and PD-L2, to protect healthy tissues from excessive inflammatory or autoimmune responses (Keir et al. 2008). It is suggested that malignant tumours expressing PD-L1 can escape immune surveillance by reducing T-cell effector activity by the same mechanism (Pardoll 2012). Immune checkpoint inhibitors are antibodies designed to block this interaction between PD-1 and PD-L1. Most clinical studies have used pembrolizumab and nivolumab, which are PD-1 targeting antibodies (Outh-Gauer et al. 2018).

PD-L1 is highly expressed by NPC cells and tumour-infiltrating macrophages, and the expression has been higher in EBV-positive NPC tumours than in EBV-negative tumours (Chen et al. 2013a, Outh-Gauer et al. 2018). Additionally, in other HNSCCs, PD-L1 expression has been higher in HPV-positive tumours and PD-1 expression has been higher on tumour-infiltrating lymphocytes in HPV-positive tumours compared to HPV-negative tumours (Badoual et al. 2013). Recent clinical studies, conducted in patients with recurrent NPC, demonstrated that pembrolizumab and nivolumab have anti-tumour activity in NPC: partial responses were documented in approximately 20% of the patients (Hsu et al.

2017, Ma et al. 2018). However, more research is needed to validate biomarkers for better patient selection. These small-scale studies did not evaluate whether treatment response related to the histological subtype or the viral status of the tumour (Hsu et al. 2017, Ma et al. 2018). In addition, immune checkpoint inhibitors have been studied only in heavily-treated, recurrent cases, and the possible benefits of concurrent use remain to be uncovered. Other immunological therapies, such as cancer vaccines for treatment or prevention, are discussed later in EBV and HPV chapters 2.2.4 and 2.3.4.

2.1.6.5 Treatment protocol in Finland in 2019

Standard primary treatment for NPC consists of chemoradiotherapy with IMRT/ VMAT techniques and concurrent cisplatin given as a 40 mg/m^2 weekly dose. Neoadjuvant or adjuvant chemotherapy is generally not used. A neck dissection is performed for patients with residual neck metastasis seen in post-treatment ^{18}F-FDG PET/CT or PET/MRI typically obtained 12 weeks from the end of RT (Mehanna et al. 2016). Local failures can be treated with stereotactic RT or surgery depending on the extent of the tumour and dose distribution in previous RT.

2.1.7 Adverse effects of the treatment

Acute toxicity related to radiotherapy includes mucositis, dysphagia, dermatitis, and xerostomia. Clinical symptoms are usually alleviated within 4-8 weeks after the cessation of RT, but more severe grade 3 or 4 reactions may persist longer. Late xerostomia has diminished with IMRT, since much of the salivary function can be spared with the more accurate irradiation technique (Saarilahti et al. 2005). By contrast, more effective irradiation can cause additional soft tissue fibrosis to the pharynx and to the esophagus leading to exacerbated dysphagia (Ruetten et al. 2011). Soft palate fibrosis may lead to nasal speech, and fibrosis in mastication muscles or temporomandibular joints to trismus. Also the use of chemotherapy adds to the side effects, for example in the form of cisplatin-induced acute hearing impairment or decreased renal function (Lee et al. 2014). Radiation-related sensorineural hearing loss typically occurs several months after RT, and is often progressive and irreversible. Other, even more debilitating neurological complications include temporal lobe necrosis, cranial neuropathy, and cognitive dysfunction, but the rates of all these have decreased with IMRT (Lee et al. 2014). Moreover, bone necrosis, chronic suppurative otitis media, or

chronic rhinosinusitis are no longer common complications of NPC treatment in the IMRT era (Hsin et al. 2016, Lee et al. 2014).

2.1.8 Follow up

Initial post-treatment assessment is to be performed 12 weeks after the end of RT/CRT. It should be done by clinical examination, endoscopic examination with or without biopsy, and imaging studies. ^{18}F-FDG-PET/CT, or if available, PET/MRI is the best imaging modality for detection of residual and recurrent tumours (Liu et al. 2007). In high-incidence endemic countries, the measurement of plasma EBV DNA titer is part of the follow up, and it will be discussed in detail later in the EBV chapter 2.2.3.

2.1.9 Prognosis of NPC

The most important predictor of prognosis for NPC is the volume of the primary tumour and its eventual extension to the parapharyngeal space (Lee et al. 2012a, Tang et al. 2014). The involvement of the parapharyngeal space predicts distant metastases, especially in patients who already have lymph node metastasis (Tang et al. 2014). With modern oncological treatment, excellent local control and locoregional survival can be achieved in most patients, but distal failures have become a major cause of death (Lee et al. 2012b, Au et al. 2018).

The survival of patients with NPC has improved significantly over recent decades. Probably the best survival worldwide has been achieved in Hong Kong, where the 5-year overall survival (OS) peaked at 78% in Chinese patients treated during a 10-year period from 2001 to 2010 (Au et al. 2018). Most of these patients (94%) had undifferentiated carcinoma. In Western countries, the reported OS rates are markedly inferior. For example, in the Netherlands, OS was 55% in all NPC patients treated in 2004-2009 (Arnold et al. 2013). However, 35% of these patients presented with KSCC and had worse survival than the patients with non-keratinizing carcinomas (Arnold et al. 2013). Histology has been identified as an important prognostic factor for NPC survival, but racial differences have also been studied. A large study with 5,549 patients from the United States used SEER (Surveillance, Epidemiology, and End Results Program) databases to analyze the relation between histology, race, and survival in NPC (Wang et al. 2013). They found that patient characteristics and survival rates differed significantly between races, but in all groups, survival was the worst in KSCC, intermediate in NK-D, and the best in NK-U (Wang et al. 2013).

In multivariable analysis adjusted for histology and stage, Asians had a significant survival advantage compared to other ethnic groups (Wang et al. 2013). In that study, the reasons for such survival differences remained unclear, and the authors did not evaluate the viral status of the tumours.

Several studies indicate that NK-U is linked to Epstein-Barr virus and a favourable outcome both in endemic and non-endemic populations (Lin et al. 2014, Chua et al. 2016, Robinson et al. 2013, Dogan et al. 2014, Stenmark et al. 2014, Jiang et al. 2016, Dogan et al. 2016). In addition, the studies conducted in non-endemic regions have found HPVs in KSCCs and in NK-Ds with confounding survival results (Robinson et al. 2013, Dogan et al. 2014, Stenmark et al. 2014, Jiang et al. 2016, Dogan et al. 2016). In general, HPV-positive cases seemed to have similar or slightly inferior survival compared to EBV-positive cases, while the patients with EBV/HPV-negative tumours have had the worst prognosis (Robinson et al. 2013, Dogan et al. 2014, Stenmark et al. 2014, Jiang et al. 2016, Dogan et al. 2016).

Smoking can modestly increase the risk of developing NPC, but it also has an impact on treatment efficacy (Xie et al. 2015, Chang et al. 2017, Chen et al. 2013b). Alcohol consumption is found to be an adverse prognostic factor if more than 14 doses are consumed per week during and after treatment (Chen et al. 2016).

2.2 Epstein-Barr virus (EBV)

2.2.1 Cancers related to EBV

The International Agency for Research on Cancer (IARC) has classified EBV as Group 1 (well-established) carcinogenic agent in humans (Bouvard et al. 2009). Sufficient evidence is available for the IARC to conclude that EBV can cause NPC, Burkitt's lymphoma, immune-suppression-related non-Hodgkin lymphoma, extranodal NK/T-cell lymphoma (nasal type), and Hodgkin's lymphoma. The IARC has also concluded that there is evidence of EBV causing part of gastric carcinomas and lymphoepithelioma-like carcinoma (Bouvard et al. 2009, zur Hausen 2009). In a more detailed analysis of relative risks worldwide, it has been estimated that strong data exists on the relation between EBV and high/intermediate-incidence NPC, as well as between EBV and high-incidence Burkitt's lymphoma, but data is limited for these diseases in low-incidence regions (de Martel 2012). Approximately 100,000-200,000 new cancer cases

worldwide are attributed to EBV annually, and 1.8% of all cancer deaths are due to EBV-related malignancies (de Martel 2012).

In addition to NPC, EBV has also been detected in other HNSCCs (Turunen et al. 2017). These carcinomas in the larynx, oropharynx, hypopharynx, and tongue were all co-infected with HPV, and thus the role of EBV in carcinogenesis remains unclear. However, EBV seemed to locate at the invading front of the tumours, and prognosis was the poorest in the patients with EBV-positive tumours suggesting that an EBV/HPV co-infection might be a reason for unfavourable prognosis (Turunen et al. 2017).

2.2.2 EBV biology in NPC

EBV is one of eight known human herpesviruses. It was identified for the first time more than 50 years ago in a cell culture from Burkitt's lymphoma, and it was then the first carcinogenic virus discovered in humans (Epstein et al. 1965, Young 2014). In adolescents, EBV causes infectious mononucleosis as a primary infection, and subsequently colonizes the host's B lymphocyte pool to cause a chronic, asymptomatic infection (Rickinson 2014). Over 90% of the world's population is infected, and globally, the primary infection occurs most often during early childhood with minimal symptoms (Raab-Traub 2002). It is uncertain, how EBV gains entry from B lymphocytes into the epithelial cells, but the observations of precancerous lesions suggest that low-grade dyplasia in nasopharyngeal epithelium makes cells susceptible to EBV infection (Rickinson 2014). Underlying dysplasia can be result from exposure to the environmental risk factors discussed in chapter 2.1.2.3. In any case, EBV infection is supposed to be a critical step for the progression of a dysplastic, precancerous lesion to cancer (Pathmanathan et al. 1995, Fan 2006). Observations of latent EBV infection in normal nasopharyngeal epithelium are rare, and it has been suggested that only 0-3% of individuals even in high NPC-incidence populations carry EBV in their non-neoplastic nasopharyngeal epithelium (Pathmanathan et al. 1995, Fan 2006, Rickinson 2014).

The EBV genome encodes almost 100 open reading frames, and complete sequences of 71 different EBV strains were published a few years ago (Palser et al. 2015). Many of the genes, for example EBV nuclear antigens (EBNAs) and latent membrane proteins (LMPs), are consistently expressed in human cancers (Lieberman 2014). EBV strains have been classified into type 1 and type 2 based primarily on the sequence of their EBNA2 and EBNA3 genes (Palser et al. 2015). Strains vary between different parts of the world, and many points of genomic variation have been found between EBV strains in endemic Asian

region and other regions (Palser et al. 2015, Feng et al. 2015). The endemic EBV strain or strains in Southern China might be inherently more capable of initiating NPC carcinogenesis than other strains, which could partly explain the remarkable geographical differences in NPC incidence (Palser et al. 2015).

Once nasopharyngeal epithelial cells are infected, EBV latent genes provide cells with deregulated proliferation and survival leading in the worse cases to the development of cancer. Additional genetic and epigenetic changes occur after the EBV infection, for example, to inactivate the host's tumour suppressor genes, such as cyclin-dependent kinase inhibitors p16(INK4) and p27(KIP1), and to overexpress p53 protein (Fan 2006, Mäkitie et al. 2003). In latent infection, the viral DNA is usually episomal, i.e. not integrated into the host's chromosomes (Raab-Traub 2012). In addition to episomal DNA, tumour cells contain viral RNA. The first identified RNAs were entitled EBERs (EBV-encoded RNA) (Arrand and Rymo 1982). These non-coding RNAs can be present in tumour cells at a million copies, which make them an excellent marker for a latent EBV infection (Raab-Traub 2012).

2.2.3 EBV detection

The presence of EBER transcripts in the tumour tissue is one of the most reliable criterions to determine EBV positivity (Raab-Traub 2012). EBERs are detected with in situ hybridization (ISH), which is regarded as the gold standard for the detection of latent EBV infection in clinical practise (Fan 2006). ISH has the advantage to precisely localize EBV in tumour cells (Fan 2006).

PCR is a technique used to exponentially amplify a single copy or a few copies of a specific segment of DNA to detect it using specified primers. To examine tumour tissue for EBV, a PCR-based method (DNA PCR) can be used, but the specificity is poor because of EBV-positive tumour-infiltrating B lymphocytes (Turunen et al. 2017). However, the level of circulating cancer-derived EBV DNA in plasma has been established as a reliable tumour marker for NPC screening, with a sensitivity of 97% and a specificity of 99% (Chan et al. 2017). Post-treatment plasma EBV DNA levels can also accurately reflect the residual tumour load and predict prognosis (Chan et al. 2018a).

High EBV antibody titers are often observed in patients with EBV-positive NPC (Lo 2004). Assays detecting EBV-related antibodies, such as IgA antibodies against viral capsid antigen (VCA), EBNA, and early antigen, have been used in clinical practice (Lo 2004). Serologic tests for anti-EBV IgA have been performed to evaluate patients in whom NPC is suspected, but because of

relatively low sensitivity and specificity, their use in screening has been limited (Coghill et al. 2014).

2.2.4 EBV-specific treatments

Adoptive T cell therapy or therapeutic EBV vaccines have been tested in small-scale clinical trials with evidence of immune boosting (Louis et al. 2010). Therapeutic vaccines consist of autologous EBV-specific cytotoxic T lymphocytes (EBV-CTLs) prepared *in vitro* for infusion back into the patient (Louis et al. 2010, Jain et al. 2016). EBV-CTLs have had potent anti-tumour activity in patients with locoregional disease but not in patients with a metastatic disease (Louis et al. 2010). A prophylactic vaccine against EBV waits for its invention (Taylor and Steven 2016).

2.3 High-risk human papillomaviruses (HPVs)

2.3.1 Cancers related to high-risk HPVs

In 1977, it was proposed for the first time that HPV is related to cervical carcinogenesis (zur Hausen 2002). Indeed, it is now known that 100% of cervical carcinomas are caused by high-risk HPVs, and strong evidence exists also on the causal relationship between HPVs and other anogenital cancers, such as anal, vaginal, penile, and vulvar carcinomas (de Martel 2012). First evidence suggesting HPV involvement in oral squamous cell carcinoma (OSCC) pathogenesis was published in the 1980s by a Finnish study group (Syrjänen et al. 1988). However, the role of high-risk HPVs in HNSCCs other than oropharyngeal carcinoma (OPSCC) remains controversial (Syrjänen 2010). A strong body of evidence supports the etiological link between HPV infection and OPSCC occurring in the tonsillar region or in the base of tongue (Ang et al. 2010, Attner et al. 2010). Furthermore, the prognosis has been significantly better in HPV-positive than in HPV-negative OPSCCs, and this finding has led to modifications in the recently published 8th edition of UICC staging (Ang et al. 2010, Brierley et al. 2017). In the Western world, the incidence of OPSCC, especially HPV-positive OPSCC, has been increasing during the last three decades (Syrjänen 2010). A recent meta-analysis showed that in Europe 50% and in North America 65% of the OPSCC cases are attributed to high-risk HPVs (Stein et al. 2015).

Globally, more than 600,000 new cancer cases are associated with HPV annually, and 30% of all infection-related cancers are caused by high-risk HPVs (de Martel 2012). HPV is classified in Group 1 carcinogens by IARC, but classification depends on HPV genotype (Bouvard et al. 2009). The IARC report notifies that HPV16 is known to cause cancer at several sites, whereas many other high-risk HPV genotypes (HPV18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) have limited evidence for other than cervical carcinogenesis (Bouvard et al. 2009).

The interest in HPV as an etiological factor of NPC has increased in recent years. Evidence is limited, because HPV is believed to be associated more with the non-endemic, and thus rare, type of NPC (Chua et al. 2016). Nevertheless, numerous studies from both endemic and non-endemic countries have found HPV positivity in NPC samples with a wide percentage range from 1% to 47% (Tyan et al. 1993, Giannoudis et al. 1995, Lo et al. 2010, Laantri et al. 2011, Huang et al. 2011, Singhi et al. 2012, Robinson et al. 2013, Dogan et al. 2014, Lin et al. 2014, Stenmark et al. 2014, Jiang et al. 2016, Svajdler et al. 2016, Dogan et al. 2016, Kano et al. 2017). In most of the studies, EBV and HPV were mutually exclusive, but co-infections have also been reported. Table 2 assembles the results of HPV and EBV prevalence in previous studies. Only studies with at least 30 samples examined were taken to the summary. Data is scarce on the prognostic significance of HPV in NPC, and only one study from the United States has noted similar favourable trends in survival in HPV-positive NPC patients as in EBV-positive patients (Dogan et al. 2014).

Table 2 Summary of studies assessing the presence of Epstein-Barr virus and high-risk human papillomaviruses in nasopharyngeal carcinoma

First author, year	*Study population*	*EBV+ (%)*	*HPV+ (%)*	*EBV+ and HPV+ (%)*	*Detection method*	
					EBV	*HPV*
Tyan 1993	Taiwan	30/30 (100%)	14/30 (47%)	14/30 (47%)	PCR	PCR
Giannoudis 1995	Greece	20/63 (32%)	12/63 (19%)	0	PCR	PCR
Lo 2010	US	15/30 (50%)	5/30 (17%)	0	ISH	ISH
Laantri 2011	Marocco	70/70 (100%)	24/70 (34%)	24/70 (34%)	PCR	PCR
Huang 2011	Taiwan	43/43 (100%)	15/43 (35%)	15/43 (35%)	PCR	PCR
		43/46 (94%)	19/46 (41%)	18/46 (39%)	ISH	ISH
Singhi 2012	US	34/45 (76%)	4/45 (9%)	0	ISH	ISH
Robinson 2013	UK	47/67 (70%)	11/67 (16%)	0	ISH	ISH
Dogan 2014	US	38/63 (60%)	6/63 (10%)	0	ISH	ISH
Lin 2014	US	84/103 (82%)	5/103 (5%)	0	ISH	ISH
	China	83/86 (96%)	0	0	ISH	ISH
Stenmark 2014	US	26/61 (43%)	18/61 (30%)	0	ISH	PCR
Kano 2016	Japan	49/59 (83%)	2/59 (3%)	2/59 (3%)	ISH	PCR
Jiang 2016	US	44/86 (51%)	23/86 (27%)	13/86 (15%)	ISH	ISH
Svajdler 2016	Slovakia	53/62 (85%)	1/62 (2%)	0	ISH	ISH
Dogan 2016	Turkey	72/82 (88%)	1/82 (1%)	0	ISH	ISH

2.3.2 HPV biology in head and neck cancers

HPVs form a large family of non-enveloped, double-stranded DNA viruses called *Papillomaviridae*. HPVs are small viruses, and their genomes encode only 8 viral proteins regulating the viral life cycle (Figure 9). Classified on the basis of their L1 protein, there are currently more than 180 different HPV genotypes (Bernard et al. 2010). HPVs can be further separated into low-risk and high-risk types according to their ability to cause cancer.

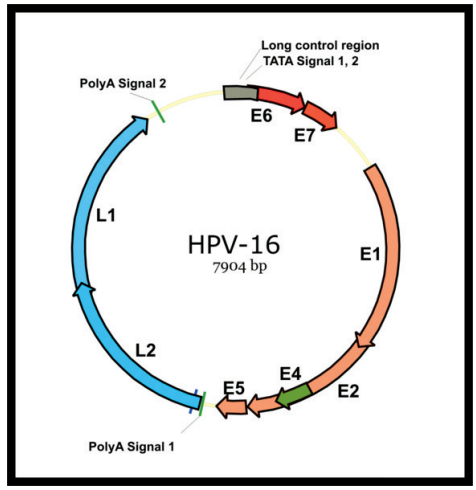

Figure 9 Organization of the HPV16 genome.

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The HPV genome comprises early genes (E1, E2, E4, E5, E6, and E7), which function at the level of viral replication and transcriptional regulation, and late genes (L1 and L2), which encode structural proteins (Stanley 2012). HPVs are exclusively intraepithelial pathogens, and a cellular differentation process is needed for viral amplification (Stanley 2012). First, the virus infects a keratinocyte in the basal layer of the mucosal epithelium exposed due to a microtrauma, and then starts to increase the viral genome number when the host cell begins to differentiate (Stanley 2012). HPV oncogenes E5, E6, and E7 promote uncontrolled cellular proliferation allowing viral amplification and thus contribute to carcinogenesis (Blitzer et al. 2014). There are several mechanisms leading to this process. For example, E6 causes degradation of tumour suppressor protein p53, which leads to inhibition of apoptosis (Blitzer et al. 2014). E7 binds to retinoblastoma (Rb), which prevents Rb's function to inhibit the cell cycle progression (Blitzer et al. 2014). E6 and E7 can also independently cause genomic instability (Fan et al. 2013). When E7 down-regulates Rb, Rb releases its regulatory partner E2F, which in turn up-regulates the production of p16(INK4) (Blitzer et al. 2014). The detection of this tumour suppressor protein p16(INK4), later called simply p16, can be used for HPV assessment (see the chapter 2.3.3).

Normal tonsillar tissues, especially tonsillar crypts, are reported to contain HPVs (Syrjänen 2004). How HPVs enter the crypts is not known, but the prevalence of HPVs in the oral cavity is highly correlated with sexual activity – the number of partners and oral sex (Pickard 2012). HPV could also be transmitted vertically during vaginal delivery or through exposure to amniotic fluid from mothers with cervical HPV infection (Chan 2014). Presently, there is no data available on HPV transmission route to the nasopharyngeal epithelium.

2.3.3 HPV detection

Several techniques are available to detect the presence of HPV in tumour tissue. In situ hybridization (ISH) is a routinely used method to detect a panel of HPV genotypes. HPV DNA ISH allows the direct identification of viral DNA within tumours cells, and thus provides a histological context to HPV infection (Blitzer et al. 2014). It is specific (88%) and can be performed on formalin-fixed paraffin-embedded (FFPE) samples, but sensitivity is low for tumours with a low HPV copy number (Blitzer et al. 2014). Furthermore, HPV DNA ISH does not confirm the transcriptional activity of the virus, since it detects HPV DNA and not mRNA (Qureishi et al. 2017).

Testing HPV E6/E7 mRNA by ISH is an ideal method for HPV detection, because it confirms the presence of the transcriptionally active virus in the tumour tissue (Wang et al. 2012, Bishop et al. 2012). HPV E6/E7 mRNA ISH is more sensitive (97% vs. 88%) and specific (93% vs. 88%) compared to HPV DNA ISH, and correlates strongly with p16 expression (Bishop et al. 2012, Qureishi et al. 2017). The disadvantages consist of technical issues, such as the lack of an automated platform, and the technique is considered too laborious for routine clinical use (Qureishi et al. 2017).

Immunohistochemistry (IHC) typically utilizes antibody-mediated staining of a protein in FFPE samples. In many laboratories, the standard test for HPV involvement is the detection of p16 by IHC (Blitzer et al. 2014). It is inexpensive and has a high sensitivity (94%) for HPV (Qureishi et al. 2017). Although interpretation is subjective, particularly when staining is weak, agreement between pathologists is reported to be almost complete (97%) (Thavaraj et al. 2011). Several studies have supported the hypothesis that overexpression of p16 can be used as a surrogate marker for oncogenic HPV infection, but the risk of false HPV-positive findings should be taken into account (Thavaraj et al. 2011, Jouhi et al. 2017a). For the optimal result, p16 IHC should be supplemented with an additional test, either HPV DNA/RNA ISH or HPV PCR (Qureishi et al. 2017, Thavaraj et al. 2011).

HPV DNA PCR is probably the most widely used detection and genotyping method for HPV, because it is relatively easy to conduct cost-effectively (Qureishi et al. 2017). PCR is a highly sensitive method but can give false-positive results due to environmental contamination. In addition, the HPV-infected cell type in the sample cannot be estimated, and detected HPV DNA can be derived from adjacent tissues or tumour-infiltrating lymphocytes (Blitzer et al. 2014, Qureishi et al. 2017). The detection of HPV E6/E7 mRNA by reverse transcriptase PCR indicates the presence of transcriptionally active HPV, but until recently, the technique required the use of fresh frozen tissue, and it is not in routine use (Qureishi et al. 2017).

2.3.4 HPV vaccines

At present, there are two prophylactic HPV vaccines available: bivalent vaccine (Cervarix®) preventing high-risk HPV genotypes 16 and 18, and 9-valent vaccine (Gardasil 9®) preventing HPV genotypes 6, 11, 16, 18, 31, 33, 45, 52, and 58. Vaccines do not contain HPV DNA, but they utilize virus-like particles composed of the capsid protein L1 derived from the targeted HPV genotypes (Blitzer et al. 2014). They have been developed against HPV in the cervix, but they will most likely also prevent oral and pharyngeal HPV infection (Syrjänen 2010, Whang et al. 2015). It is not probable that the use of these prophylactic vaccines provide clinical benefit after the development of cancer, since the expression of the capsid proteins usually stops during the carcinogenic process (Blitzer et al. 2014).

Therapeutic vaccines against HPV-related malignancies are under investigation in early phase trials (Whang et al. 2015). The aim of the therapeutic vaccines would be the activation of the T cell-mediated immune system to destroy the existing HPV-infected cells, and HPV16 oncoproteins E6 and E7 have become popular viral targets in the vaccine design (Whang et al. 2015). In addition, a patient's own dendritic cells can be transfected with the specific antigen and matured ex-vivo for subsequent transfer back into the patient (Lin et al. 2010).

2.4 Toll-like receptors (TLRs)

2.4.1 TLRs in immune defence

The aim of the immune defence is to detect and eliminate invading pathogenic microorganisms. In mammals, the immune system can be divided into innate and adaptive immunity. Innate immunity constitutes the first line of defence acting promptly against pathogens, whereas adaptive immunity takes care of the later phases, like generating immunological memory (Akira et al. 2006). Innate immune cells, but also other tissues, express pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) (Akira et al. 2006). PAMPs can be components of the bacterial cell wall, viral envelope proteins, viral DNA/RNA, fungal components, or protozoal proteins (Kumar et al. 2009). Toll-like receptors (TLRs) are one example of PRRs. TLRs form a family of transmembrane proteins, which activate immune responses against pathogens after being bound to PAMPs (Takeda et al. 2003). The human TLR family is known to consist of 10 members from TLR1 to TLR10 (Takeda et al. 2003, Bourke et al. 2003). Originally, studies on the localization of TLRs in the cell were performed on leukocytes, and results indicated that TLR1, TLR2, TLR4, TLR5, and TLR6 were expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 were found in intracellular compartments, such as endosomes (Akira et al. 2006). The localization of TLR10 has not yet been well characterized (Hamonic et al. 2018). However, a growing body of evidence indicates that the expression of TLRs is not static but rather rapidly modulated by pathogens and other environmental stress factors (Akira et al. 2006). Practically all TLRs, except TLR10 with no known ligand, have been discovered to have several exogenous ligands, which trigger the cascade of signaling pathways leading to various inflammatory responses (Ioannou and Voulgarelis 2010).

2.4.2 TLRs in tissue repair

TLRs also react to endogenous damage-associated molecular patterns (DAMPs) released from host's injured tissues (Tsan 2006). Thus, TLR-mediated cascades can be activated in the absence of microbes leading to non-infectious inflammatory response (Ioannou and Voulgarelis 2010). TLR activation occurs in the tissues due to cell necrosis, which releases the cell contents, for example the host's own RNA (Matzinger 2002). Activation of TLRs can modify the tissue injury process in a negative or positive fashion, either by recruiting inflammatory cells to release cytotoxic mediators or by activating cytoprotective cascades

(Ioannou and Voulgarelis 2010). TLRs are involved in the regulation of epithelial proliferation and angiogenesis after injury (Ioannou and Voulgarelis 2010). In addition, there is evidence that TLRs can directly promote pro-inflammatory and anti-apoptotic signals in fibroblasts (Ni et al. 2015).

2.4.3 TLRs in cancer

Many types of cancers are reported to express TLRs, but their actual functions are unclear: TLR stimulation may be tumour suppressing or promoting depending on the TLR receptor and cancer type (Dajon et al. 2017). It has been suggested that pro-tumorigenic effects are due to the direct activation of TLRs in the tumour cells, whereas anti-tumour functions rely on the activation of host immune responses (Yu et al. 2013). Indeed, the outcomes of TLR involvement are challenging to investigate when one ligand is able to activate several different TLRs and multiple ligands can stimulate a single TLR (Yu et al. 2013).

Chronic infection or inflammation is recognized as a risk factor for tumorigenesis. For example, *Helicobacter pylori (H. pylori)* bacteria causes chronic gastritis, which consequently increases the risk for gastric carcinoma (Parsonnet et al. 1991). Studies on gastric lesions have shown that *H. pylori* infection augments TLR expression, and infection-related dysplastic lesions express TLRs (TLR2, TLR4, TLR5) more than normal mucosa (Schmaußer et al. 2005, Pimentel-Nunes et al. 2011). Pimentel-Nunes et al. have also found changing TLR localizations in gastric carcinogenesis. In the normal gastric mucosa, TLR2, TLR4, and TLR5 are expressed in a polarized manner on the apical and particularly on the basolateral membrane (Pimentel−Nunes et al. 2013). By contrast, in metaplasia, dysplasia, and adenocarcinoma, these TLRs are expressed throughout the cytoplasm with no apparent polarization (Pimentel−Nunes et al. 2013). Causality in carcinogenesis may be difficult to establish, but *in vitro* studies have also shown that TLR5 activation by flagellin promotes the proliferation of gastric cancer cells (Song et al. 2011). Another example has been described in cervical neoplasia. TLR9 expression was noted to gradually increase in accordance with the histopathological grade from low-grade CIN to invasive SCC (Lee et al. 2007). In line with this, overexpression of TLR9 has been reported in persistent cervical HPV infections, which are not cleared in few months as normally (Cannella et al. 2015).

TLRs can serve as negative regulators of cancer, which have led to the idea of TLR agonists in cancer treatment. In fact, some microbe-derived TLR-stimulating treatments were invented before the actual discovery of toll-like receptors. For example, OK-432, a lyophilized preparation of group A

streptococci stimulating TLR4, is used to treat cervical, gastric, and oral SCC (Okamoto et al. 2006). In addition, *Mycobacterium bovis* bacillus Calmette-Guérin is a potent activator of TLR2 and TLR4, and has been used for 40 years as a treatment for bladder cancer (Razack 2007). Many other TLR agonists are in clinical trials (Iribarren et al. 2016). However, targeting TLRs is not a straightforward process, because the direct effects on both the immune system and the tumour cells must be evaluated carefully. In tumour microenvironment, immune responses toward cancer should be enhanced, while general inflammatory state should be restrained (Dajon et al. 2017).

2.4.4 TLRs in NPC and other head and neck cancers

Data on TLR expression in NPC is scarce and limited to a few endemic studies focusing on the variation of certain TLR genes. These sequencing studies have shown that genetic polymorphisms or specific sequence variants of TLR2, TLR3, TLR4, TLR8, TLR9, and TLR10 are associated with increased NPC risk in endemic areas (Makni et al. 2017, He et al. 2007, Song et al. 2006, Yang et al. 2012, Wee et al. 2012, Dai et al. 2014, Zhou et al. 2006). An interesting hypothesis links East Asians' susceptibility to EBV-related NPC with resistance to malaria sequelae (Wee et al. 2012). It suggests that the Last Glacial Maximum about 30,000 years ago isolated a migrating human population in the Southern slopes of the Himalayas, where only few survived a malaria infection, which generally caused severe immunological reactions. Those who could continue to East Asia were selected with a TLR8 polymorphism leading to a controlled and milder immune response to cerebral malaria but also a decreased ability to respond to other pathogens, such as EBV (Wee et al. 2012).

To our knowledge, TLR expression patterns in NPC have been evaluated only in one small study on TLR3 expression (Vérillaud et al. 2012). Studies assessing the relation between NPC prognosis and TLR expressions are also lacking. In contrast, several studies on oropharyngeal squamous cell carcinoma (OPSCC) and oral tongue squamous cell carcinoma (OTSCC) have found that strong expression of TLR5 was associated with poor disease-specific survival (DSS) (Jouhi et al. 2017b, Kauppila et al. 2013). Additionally, low expression of TLR7 has been associated with poor DSS in OPSCC (Jouhi et al. 2017b). In OPSCC, the prognostic significance of TLR7 expression was particularly high among HPV-positive patients (Jouhi et al. 2017b). Furthermore, in an OTSCC study, strong TLR2, TLR4, and TLR9 expressions correlated with deeper tumour invasion, and strong TLR5 expression occurred in well-differentiated tumours (Mäkinen et al. 2015).

3 AIMS OF THE STUDY

The specific aims of the three studies were:

I To gather all available data on the NPC cases diagnosed and treated in Finland between 1990 and 2009. Special emphasis was given to the survival analyses in relation to histopathological NPC subtypes and treatment modalities.

II To examine the presence of EBV and HPV in Finnish NPC tumours, and to evaluate the associations between viral status, histopathological NPC subtype, and survival.

III To examine the expressions of six toll-like receptors (TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9) in NPC tumour samples, and to evaluate the expression patterns in association with clinicopathological variables and survival.

4 MATERIALS AND METHODS

4.1 Patients (I-III)

Patients were identified using the Finnish Cancer Registry database. During a 20-year period, from 1990 to 2009, 302 patients had been registered with a diagnosis of nasopharyngeal cancer, which also includes cancers arising from the minor salivary glands and other nasopharyngeal tissues. To exclusively select patients with squamous cell carcinoma (SCC), patient records were collected from treating hospitals and reviewed. The treating hospitals comprised all five university hospitals and four central hospitals in Finland. Finally, 207 patients were identified as having SCC, and thus they formed the study population for Study I.

In addition to patient records, the original histological samples were requested from the pathology units of the treating hospitals. All available slides (168/207, 82%) stained with hematoxylin and eosin (H&E), were reviewed by an experienced head and neck pathologist (Ilmo Leivo, I.L.) to verify the histopathological diagnosis and to reclassify the cases according to the same WHO classification (Barnes et al. 2005). For the remaining 39 patients with no samples available, diagnosis and classification was determined using the original histopathology reports. FFPE tissue samples for tissue microarray were obtained from 150 patients, and this cohort formed the study population for Studies II and III.

The hospital records provided us with data on patient characteristics, presentation of the disease at the time of diagnosis, treatment, and follow up. All cases were retrospectively re-staged according to the 7th edition of UICC staging system (Sobin et al. 2009). For the verification of T and N classifications, we reviewed the original reports of endoscopic examinations, computed tomography, and magnetic resonance imaging. The dates and causes of death were acquired from the Finnish Cancer Registry and Statistics Finland.

4.2 Tissue microarray (II-III)

Tissue microarrays (TMAs) were constructed with 1 mm core samples (n=324) taken from the original FFPE blocks of tumours. The pathologist (I.L.) marked the representative areas of each tumour on the original specimens, and an automated tissue microarrayer (TMA Grand Master, 3D Histech Ltd, Budapest,

Hungary) created five new paraffin blocks with the core samples. Each patient was represented in the TMAs by at least one core, usually two. When analyzing the stainings, scores from the duplicate cores were averaged to one score. Five of the original tumour blocks represented neck metastases, while the remaining 145 were from the primary nasopharyngeal tumours. Five cases with the neck node samples in the TMA did not have sufficient biopsy material from the nasopharynx for the TMA, but the correct diagnosis was ensured by reviewing the original slides with the nasopharyngeal samples. Each TMA block also included control tissue samples taken from the liver or the placenta.

4.3 In situ hybridizations (II)

4.3.1 In situ hybridization for EBV RNA and HPV DNA

For in situ hybridization (ISH), 5-µm sections were cut from the TMA blocks, transferred onto Super Frost Plus slides and incubated for 2 hours at 58ºC. Chromogenic ISH was performed using an automated Benchmark XT system (Ventana/Roche Medical Systems Inc, Tucson, AZ).

For EBV RNA detection, we used the Ventana EBER probe, which detects early RNA transcripts of EBV. In the automated process, deparaffinization and proteolytic treatment with Protease 3 for 28 min were followed by hybridization with the EBER probe at 57ºC for 1 hour, and counterstaining with Red Stain. Positive staining was defined as strong diffuse signals in the nucleus of nearly all (>90%) tumour cells.

For HPV DNA detection, we used the Ventana HPV III Family 16 probe, which detects high-risk HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. In the automated process, deparaffinization and proteolytic treatment with Protease 3 for 20 min were followed by hybridization with the HPV probe at 52ºC for 2 hours, and counterstaining with Red Stain. Positive staining was defined as dark blue granules in the nucleus of tumour cells.

EBV RNA and HPV DNA ISH stainings were interpreted by the pathologist (I.L.) who was blinded to clinical data.

4.3.2 In situ hybridization for HPV E6/E7 mRNA

For HPV RNA ISH, we used the RNAscope 2.5 HD Reagent kit (Advanced Cell Diagnostics, Inc., Hayward, CA), which detects HPV E6/E7 mRNA. The high-risk HPV 18 cocktail probe included the probes for HPV genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. ISH was performed manually according to the manufacturer´s protocol. First, 5-µm TMA sections were incubated for 1 hour at 58°C. After deparaffinization the sections were pretreated with hydrogen peroxidase for 10 minutes at room temperature, and target retrieval was performed for 15 minutes at 100°C. The protease treatment (RNAscope Protease Plus) was performed for 30 minutes followed by hybridization with the HPV cocktail probe for 2 hours at 40°C in a hybridization oven. Preamplifiers and amplifiers were hybridized consecutively accompanied with chromogenic signal detection with diaminobenzidine (DAB). The slides were counterstained with hematoxylin. For controls, an endogenous housekeeping gene HS-PPIB probe was used as a positive control, and a bacterial gene DapB probe as a negative control.

The HPV RNA expression was examined by a pathologist (Stina Syrjänen, S.S.) using a qualitative scoring system: a positive staining HPV test result was defined as punctate staining that co-localised to the cytoplasm and/or nucleus of any of the malignant cells. The positivity was graded weak when at least 10 cells were intensively positive or less than 50% of the cells were positive with low intensity, moderate when over 50% to 70% of the tumour cells were positive, and strong when strong intensity of dot-like nuclear and cytoplasmic signals were present in over 70% of the cells or nearly all tumour cells were positive with low intensity.

4.4 Polymerase chain reaction for HPV genotyping (II)

For HPV genotyping PCR, we used the Multiplex HPV Genotyping Kit (DiaMex GmbH, Germany), which detects 24 low-risk and high-risk HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82). First, 3 to 5 sections from original FFPE blocks were cut to obtain an approximately 1cm^2 total area of the NPC sample. DNA was extracted from the samples using high salt method as described previously (Miller et al. 1988). DNA was amplified with primer sets 1 and 2 from the Multiplex HPV Genotyping Kit. For DNA quality control, the primer set 2 contained primers to amplify ß-globin gene fragments in order to verify the amount of human genomic DNA. A negative control contained no genomic DNA to confirm the absence of

contamination in the amplification reactions. The labeled hybrids were analyzed with a Luminex LX-100 analyzer (Bio-Plex 200 System, Bio-Rad Laboratories, Hercules, CA). Genotyping HPV PCR was performed only for p16-positive samples, as proposed in the earlier published algorithm (Thavaraj et al. 2011).

4.5 Immunohistochemistry (II-III)

To perform immunohistochemical (IHC) stainings, 3.5-µm thick TMA sections were cut, mounted on glass slides, deparaffinized in xylene, and rehydrated in graded alcohol series.

4.5.1 Immunohistochemistry for p16

For detection of p16 protein, the staining was performed using an automated Benchmark XT system. After epitope retrieval with CC1 buffer for 60 minutes, the TMA sections were incubated for 16 minutes with a mouse monoclonal antibody against p16 protein (clone E6H4, Ventana/Roche). For visualizing the p16 antibodies, an UltraView Universal DAB Detection Kit was used, and the sections were counterstained with hematoxylin and Blueing Reagent. p16 expression was defined positive when there was strong, diffuse nuclear and cytoplasmic staining in at least 75% of the tumour cells (Schlecht et al. 2011).

4.5.2 Immunohistochemistry for TLRs

For TLR stainings, the sections were treated in a PreTreatment module (Lab Vision Corp, UK Ltd, Altrincham, UK) in Tris-HCl buffer (pH 8.5; TLR2, TLR4, and TLR7) or in Tris-EDTA buffer (pH 9.0; TLR1, TLR5, and TLR9) at 98˚C for 20 min. Endogenous peroxidase activity was blocked with 0.3% Dako REAL Peroxidase-Blocking Solution (Dako, Glostrup, Denmark) for 5 min. Immunostaining was performed in an Autostainer 480 (LabVision Corp, Fremont, CA, USA) incubating sections with primary antibodies against TLRs (Table 3) for 60 min (TLR9) or overnight (TLR1, TLR2, TLR4, TLR5, TLR7) followed by a 30 min incubation with Dako REAL EnVision Detection System (peroxidase/DAB+, rabbit/mouse). Between each step, slides were washed with PBS-0.04%-Tween20. Sections were counterstained with Dako Meyer's hematoxylin and mounted in PERTEX (Histolab Product AB, Göteborg, Sweden).

Table 3 Toll-like receptor antibodies used in Study III

Antigen	*Primary antibody*	*Dilution*
TLR1	sc-30000, polyclonal, rabbit, Santa Cruz Biotechnology Inc, CA	1:100
TLR2	sc-10739, polyclonal, rabbit, Santa Cruz Biotechnology Inc, CA	1:200
TLR4	sc-10741, polyclonal, rabbit, Santa Cruz Biotechnology Inc, CA	1:300
TLR5	NBP2-24787, monoclonal, mouse, Novus Biologicals, CO	1:100
TLR7	IMG-581A, polyclonal, rabbit, Imgenex/ Novus Biologicals, CO	1:300
TLR9	sc-25468, polyclonal, rabbit, Santa Cruz Biotechnology Inc, CA	1:100

4.5.3 Scoring of TLR immunoreactivity

The scoring of the TLR stainings was done by the pathologist (I.L.) who was blinded to clinical data. For NPC samples, TLR expression positivity was usually divided into two categories: mild and strong expression. As an exception, for TLR7, we used a previously described (Jouhi et al. 2015) scoring system with three positive categories. TLR7 staining was scored mild if some nuclear membranes were positive, moderate if all nuclear membranes and some nuclei were positive, and strong if nuclear membranes and nuclei stained substantially.

TLR1, TLR4, and TLR9 expressions were scored by the intensity of cytoplasmic staining as mild or strong. TLR5 was scored by the intensity of cytoplasmic and nuclear membranous staining also as mild or strong. TLR2 was expressed in the cytoplasm and in the nuclei, but the intensities varied independently of one another. Thus, cytoplasmic ($TLR2^{cyto}$) and nuclear ($TLR2^{nucl}$) positivities were scored separately as mild or strong. Nuclear expression was scored mild when the intensity of the staining was weaker or similar to cytoplasmic intensity, and strong when the staining was more intensive in the nuclei than in the cytoplasm.

4.6 Statistical analyses (I-III)

Statistical analyses were performed using the SAS System for Windows, release 9.4 (SAS Institute Inc., Cary, NC, USA) by a professional statistician. The mean ages in the patient subgroups were compared with 1-way analysis of variance using the Tukey's method for pairwise comparisons. The associations of categorical variables with different clinicopathological factors were compared with Pearson chi-squared test or Fisher's exact test. Univariate survival rates were analyzed using the Kaplan-Meier method. Survival analyses were conducted for patients treated with curative intent to compare the responsiveness

to treatment in different patient subgroups. The follow-up time was calculated from the last day of primary treatment, usually from the end of radiotherapy, to the end of follow up or to the death. A log-rank test was used to compare the Kaplan-Meier survival curves. Age-adjusted and multivariable Cox regression was used to test the association of viral and TLR statuses with DSS and OS. The multivariable Cox regression analysis was adjusted for the appropriate confounding factors depending on the analyzed factor. For example, age, gender, stage, RT dose, treatment (RT vs. CRT), and viral status were used for adjustment. To avoid multicollinearity problems, histology was excluded from the multivariable models due to the high correlation with EBV/HPV status. The results are expressed using hazard ratios (HRs) with 95% confidence intervals (CIs). Two-tailed P-values of less than 0.05 were considered as statistically significant.

4.7 Ethical considerations

The study was approved by the Research Ethics Committee of the Hospital District of Southwest Finland, National Institute for Health and Welfare (THL), and National Supervisory Authority for Welfare and Health (Valvira). Due to the retrospective setting of the study, formal consent was not required.

5 RESULTS

5.1 Outcome of NPC in Finland 1990-2009 (Study I)

5.1.1 Overall characterization of the patients

In total, 207 patients were diagnosed with nasopharyngeal SCC in Finland during this 20-year period. The patients were from Finnish, Caucasian background in 96% of the cases and the majority were men (142/207, 69%). The mean (SD) age of the patients was 57 years (15) ranging from 12 to 85 years. Almost half of the patients (97/207, 47%) were smokers or ex-smokers, but smoking history was unknown for 31% of the patients. The most frequent initial symptoms were a cervical mass (44%) and/or ear problems, for instance hearing impairment due to serous otitis media, in 42% of the patients. The disease was predominantly diagnosed in advanced stage. Stage III and IV comprised 64% of the cases, and neck metastases were common. See Table 4 for the detailed stage distribution and other patient characteristics.

The histopathological subgroups were mostly (81%) determined by reviewing the original slides, and the remaining 39 cases were classified according to the original reports. Forty-two patients (20%) had KSCC, 31 patients (15%) NK-D, and 132 patients (64%) NK-U (Table 4). There were no basaloid SCCs. In two cases (1%) the exact subgroup could not be specified. The patients with KSCC were significantly older than those with NK-U ($p=0.005$); the mean (SD) ages were 63 (14) years and 55 (15) years, respectively. When we compared the original histopathological diagnoses with those diagnosed in the reviewing examination, there were few significant changes: six KSCCs changed to non-keratinizing carcinomas, and six were vice versa. In general, no viral results were mentioned on the histopathological records. Thus, it can be assumed that no EBV or HPV detections were originally made, except one sample that was diagnosed EBER-positive.

Table 4 Patient characteristics and clinicopathological features of the patients with nasopharyngeal carcinoma diagnosed in Finland between 1990 and 2009. Adopted from Publication I.

Characteristics	*No. of patients*	*Percentage (%)*
Total number	207	
Gender		
Male	142	69
Female	65	31
Mean (SD) age at diagnosis		
years; range	57 (15); 12-85	
Ethnicity		
Finnish	198	96
Other*	9	4
Smoking		
Smoker or ex-smoker	97	47
Non-smoker	46	22
Not known	64	31
Symptoms		
Cervical mass	92	44
Ear sensations (hearing impairment, otalgia)	86	42
Nasal obstruction	57	28
Bloody nasal discharge	34	16
Cranial nerve involvement (diplopia, hypoesthesia)	21	10
T class		
T1	74	36
T2	55	27
T3	40	19
T4	38	18
N class		
N0	76	37
N1	47	23
N2	71	34
N3a	10	5
N3b	3	1
Overall stage		
I	23	11
II	51	25
III	81	39
IV	52	25
Histology		
Keratinizing squamous cell carcinoma (KSCC)	42	20
Non-keratinizing differentiated carcinoma (NK-D)	31	15
Non-keratinizing undifferentiated carcinoma (NK-U)	132	64
Treatment		
Palliative	16	8
Radiotherapy	85	41
Chemoradiotherapy	106	51
Irradiation technique		
2-dimensional radiotherapy (2D)	21	11
3-dimensional radiotherapy (3D)	117	61
Intensity modulated radiotherapy (IMRT)	54	28

*Four from South-East Asia, two from Africa, three from Eastern Europe.

5.1.2 Treatment modalities

The treatment was performed with a curative intent in 191 cases (92%) with definitive RT or CRT. The remaining 16 patients (8%) received only palliative treatment because of a compromised general condition or distant metastases, and they were omitted from the survival analyses. Most patients had their definitive oncological treatment at five university hospitals (Helsinki, Tampere, Turku, Kuopio, Oulu), but 21/191 (11%) patients were treated at three central hospitals (Vaasa, Jyväskylä, Kotka). We analyzed the RT methods at different hospitals and found minor differences in the irradiation techniques and fractionation schedules between them. In addition, the evolution of RT techniques was rapid during the study period. For our patients, the irradiation techniques were 2-D in 11% of the cases, 3-D in 61%, and IMRT in 28% of the cases (Table 4). IMRT had already been used at Helsinki University Hospital since 2000, and became the method of choice at all university hospitals during subsequent years. IMRT was sometimes combined with a SIB during the last five years. The most commonly used fractionation schedule was 2 Gy daily, but 10 patients (5%) received hyperfractionated accelerated RT with 1.6 Gy b.i.d. and a planned interim break for about 11 days. The median dose was 69 Gy in the primary tumour area, 61 Gy in the involved lymph nodes, and 50 Gy in the elective neck area. The median treatment time was 7 weeks. Six (3%) patients were additionally treated with intracavitary brachytherapy to increase the radiation dose in the nasopharyngeal tumour up to 74-76 Gy. The other six (3%) patients received a second course of RT for locoregional recurrences using stereotactic RT in four cases and with an additional boost of IMRT in two cases.

Concurrent chemotherapy during RT was given for 55% (106/191) of the patients treated with curative intent. The most adopted chemotherapeutic regimen was cisplatin 100 mg/m^2 at 3-week intervals (54%), and weekly cisplatin protocol (40 mg/m^2) was used in 33% of the cases. The remaining 13% were treated with other protocols, for example using a combination of cisplatin and fluorouracil, or taxanes. Neoadjuvant chemotherapy was given to 14 patients (7%), and adjuvant chemotherapy to 43 patients (21%).

A neck dissection was performed for 28 patients (15%) as part of the primary treatment, and in eight patients, the neck dissection was bilateral. In 21/28 patients, the neck dissection was planned prior to the oncological treatment to be performed electively approximately two months after the end of RT/CRT. In 12 elective neck dissections (57%), the nodes did not contain viable malignant cells, in 6 cases (29%) they were positive for cancer, and in three cases (14%) the histopathological data was missing.

5.1.3 Adverse effects of the treatment

Due to the retrospective setting, acute toxicities were reported with various degrees of accuracy. Thus, we concentrated on major incidents representing higher than UICC grade 2 severity, in patients treated with curative intent. Six (3%) patients died during or shortly after RT/CRT, and the reason in five cases was a septic infection: pneumonia, meningitis (with pneumonia), re-activated lung tuberculosis, or sepsis of unknown origin. One patient died of a hemorrhagic complication after a neck dissection. In total, nine patients (5%) had pneumonia. In addition, an endocarditis and a neck abscess necessitating tracheostomy were reported, as well as facial nerve paresis, peripheral sensory neuropathy, acute gastrointestinal bleeding, and a rise in intracranial pressure. The termination of the treatment due to acute toxicity was slightly more common in the RT group (12%) compared to the CRT group (7%).

Data concerning late toxicities was incomplete, because in one third of the cases this information was not reported by the follow-up hospitals. The severe adverse effects that were reported included osteoradionecrosis in the mandibula in four cases, peripheral neuropathy in three cases, and cranial nerve pareses of the abducens and recurrens nerves in one case. Ear problems were frequent and reported in 33 cases (17%). Three patients even needed a mastoidectomy to treat chronic mastoiditis. Dysphagia was not uncommon in its mild form, but also severe complications occurred. One patient remained permanently dependent on gastrostomy tube feeding, and in three patients oesophageal strictures required repeated dilatations. Two patients had late-onset post-irradiation laryngeal oedema and needed a tracheostomy.

Twenty patients (10%) developed another primary cancer of which only two were detected within the irradiated field: one in the soft palate and another in the nasopharynx more than 15 years later. Other second primaries were found in prostate (n=3), breast (n=3), lung (n=2), kidney (n=2), and rectum (n=2), respectively. Single cases of liver, pancreas and ovarian cancers were also found, as well as an endocrine tumour of the trachea, a glioblastoma, and a B cell lymphoma.

5.1.4 Follow up and clinical outcome

The first follow-up visit was usually scheduled 6-8 weeks after the completion of RT/CRT. Thereafter, the patients had follow-up visits every 3-4 months during the first two years, and then every 6 months until five years. The median follow-up time was 65 months (range 0-271 months) for the patients treated with

curative intent. Statistics Finland provided us with the list of dates and causes of death in December 2014.

Altogether, 85 patients (45%) had a treatment failure within five years. The median latency for any failure was 5 months (range, 0-56). The patients treated with RT had slightly more failures than those treated with CRT: the percentages of local failure were 28% and 27% (p=0.26); nodal failure 15% and 14% (p=0.40); distant failure 20% and 18% (p=0.26); and overall failure 45% and 41% (p=0.049), respectively.

5.1.4.1 Local failure-free rate

In Kaplan-Meier analyses, we took into account only patients treated with curative intent to compare treatment results. The overall 5-year local failure-free rate (LFFR) was 72%. When analyzed according to T classification, the results were 87% for T1, 79% for T2, 63% for T3, and 35% for T4. Advanced T class (T3-4) was the only statistically significant factor ($p<0.001$) for LFFR. For instance, gender (p=0.074), age (p=0.087), or treatment modality (RT vs. CRT; p=0.233) did not show prognostic significance.

5.1.4.2 Overall survival

In Kaplan-Meier analysis, the 5-year OS was 57% for all patients treated with curative intent. The OS rates for different stages were 87% for stage I, 66% for stage II, 54% for stage III, and 33% for stage IV (Figure 10).

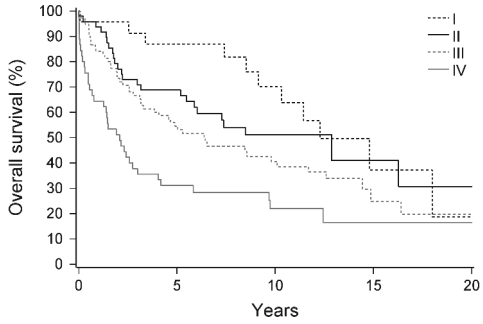

Figure 10 Overall survival of 191 NPC patients diagnosed and treated with curative intent in Finland between 1990 and 2009 according to different stages. Modified from Publication I.

In the age-adjusted Cox regression analysis, smoking, keratinizing histology, advanced T classification (T2-4 vs. T1), advanced N classification (N2 vs. N0), advanced stage (stage III-IV), and lower total RT dose were significant prognostic factors for a worse OS. In the multivariable Cox regression analysis, older age, keratinizing histology, advanced T and N classifications, and RT (vs. CRT) as a treatment modality were significant adverse prognostic factors for OS (Table 5). The best outcome was achieved in patients with NK-U followed by patients with NK-D.

Table 5 Multivariable Cox regression analyses of 191 NPC patients diagnosed and treated with curative intent in Finland between 1990 and 2009 relative to overall survival. Modified from Publication I.

	***Multivariable*[#]**	
Variable	***HR (95% CI)***	***p value***
***Age*[*]**	1.04 (1.03-1.06)	<0.0001
Gender		
Female vs male	0.91 (0.59-1.42)	0.69 NS
Smoking		
Smoker vs non-smoker	1.39 (0.79-2.44)	0.25 NS
Ex-smoker vs smoker	0.86 (0.45-1.65)	0.65 NS
Not known vs smoker	0.93 (0.52-1.66)	0.81 NS
Histology		
KSCC vs NK-U	1.84 (1.13-2.99)	0.01
NK-D vs NK-U	1.60 (0.89-2.87)	0.12 NS
T classification		
T2 vs T1	1.73 (1.04-2.87)	0.03
T3 vs T1	2.23 (1.24-4.01)	0.007
T4 vs T1	3.35 (1.89-5.96)	<0.0001
N classification		
N1 vs N0	1.07 (0.61-1.90)	0.80 NS
N2 vs N0	2.48 (1.54-3.98)	0.0002
N3 vs N0	1.32 (0.57-3.05)	0.52 NS
***Total RT dose (Gy)*[**]**	0.95 (0.93-0.97)	<0.0001
Treatment modality		
RT vs CRT	1.60 (1.05-2.44)	0.03

Abbreviations: HR, hazard ratio; CI, confidence interval; KSCC, keratinizing squamous cell carcinoma; NK-U, non-keratinizing undifferentiated carcinoma; NK-D, non-keratinizing differentiated carcinoma; RT, radiotherapy; CRT, chemoradiotherapy; Gy, Gray, NS, not significant.

[*] HR for one year increase in age
[**] HR for one Gy increase in the total RT dose
[#] Adjusted for other variables included in the multivariable model

5.1.4.3 Disease-specific survival

In Kaplan-Meier analysis, the 5-year DSS was 63%. The percentages according to the clinical stage were 91% for stage I, 78% for stage II, 60 % for stage III, and 38% for stage IV (Figure 11). In the age-adjusted Cox regression analysis, same variables were significant for DSS as for OS except smoking, which was not a significant prognostic factor for DSS. In the multivariable Cox regression analysis, older age, advanced T and N classifications, and lower total RT dose were significant prognostic factors for a worse DSS.

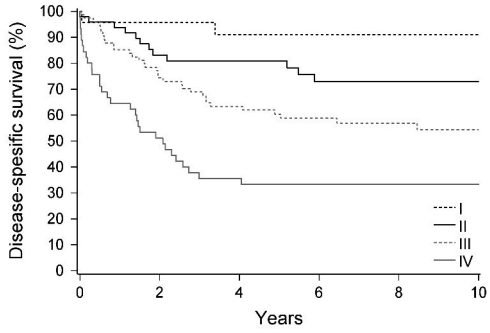

Figure 11 Disease-specific survival of 191 NPC patients diagnosed and treated with curative intent in Finland between 1990 and 2009 according to different stages. Modified from Publication I.

5.2 EBV and high-risk HPVs in NPC (Study II)

5.2.1 Characterization of the patients

The study cohort comprised 150/207 patients with FFPE samples available from treating hospitals for TMA. Thus, the patient exclusion occurred randomly, and the proportions of different patient groups remained roughly the same as in the Study I. Also in this cohort, the mean age (SD) of patients was 57.0 (15) years. A total of 145/150 (97%) were Caucasians from Finnish ethnic background, and 67% were men. The histological groups of the tumours were KSCC in 22%, NK-D in 17%, and NK-U in 61%, respectively.

5.2.2 Presence of EBV and high-risk HPVs in NPC tumours

The presence of EBV was common in Finnish NPC tumours. We found that 93/150 (62%) had positive staining in EBER ISH. HPV positivity was defined so that the sample had to be positive for both p16 IHC and HPV DNA/RNA ISH and/or HPV PCR. HPV ISH detections were performed on all samples but genotyping HPV PCR only for p16-positive samples. As a result, 21/150 (14%) tumours were positive for HPV. There were no co-infections of EBV and HPV. In 36/150 (24%) samples, all EBV and HPV detections proved negative, and thus they formed the EBV/HPV-negative group.

p16 detection seemed to be a reliable surrogate test for HPV because all p16-positive tumours also showed positivity in the specific HPV E6/E7 mRNA ISH. In contrast, only 16/21 (76%) of p16-positive tumours were positive in HPV DNA ISH. Furthermore, among p16-positive cases, HPV DNA PCR was negative in 2/18 (11%) of the available samples, and three patients did not have sufficient FFPE material for DNA extraction. Table 6 shows the correlation between different HPV detection methods. HPV16 was the most prevalent genotype presenting in 11/18 (61%) of the cases. HPV18 was found in two samples, and HPV11, HPV33, and HPV59 were present in one sample each. The incidences of EBV or HPV remained stable over the 20-year study period.

Table 6 The relationship between different methods for detecting human papillomavirus and its genotypes in cases showing positive p16 immunohistochemistry

case	*p16 IHC*	*HPV E6/E7 mRNA ISH*	*HPV DNA ISH*	*HPV DNA PCR*	*HPV genotype*
1	+	+	+	+	16
2	+	+	+	+	16
3	+	++	++	+	11
4	+	++	-	+	59
5	+	+	++	+	16
6	+	++	++	+	18
7	+	++	++	+	16
8	+	++	+	-	NA
9	+	+++	+	NA	NA
10	+	+	+	NA	NA
11	+	+	++	+	16
12	+	++	-	NA	NA
13	+	++	+	+	33
14	+	+	-	+	18
15	+	++	+	+	16
16	+	++	++	+	16
17	+	++	+	+	16
18	+	+	-	+	16
19	+	+	++	+	16
20	+	+	++	+	16
21	+	++	-	-	NA

Abbreviations: IHC, immunohistochemistry; ISH, in situ hybridization; NA, not applicable

5.2.3 Relationship between viral status and other patient or tumour characteristics

The patients with EBV-positive or HPV-positive tumours were younger than the patients with EBV/HPV-negative tumours with mean (SD) ages of 54.5 (15), 56.9 (13), and 63.5 (15) years, respectively. The age difference was statistically significant when comparing the EBV-positive patients to the EBV/HPV-negative patients ($p=0.007$). There were more women in the EBV/HPV-negative group (56% were women) compared to the EBV-positive (26%, $p=0.001$) and the HPV-positive (24%, $p=0.020$) groups. Smoking was most frequent in the HPV-positive patient group, since 57% of the patients were smokers or ex-smokers. However, the differences were not statistically significant compared to the EBV-positive and the EBV/HPV-negative groups, of which 46% and 44% of patients, respectively, had a positive smoking history.

Viral status was associated with histological subtypes ($p<0.0001$). Table 7 demonstrates the distribution of EBV-positive and HPV-positive findings in

relation to WHO histological subtypes. The majority (78%, 91/117) of non-keratinizing tumours were EBV-positive, whereas the proportion was only 6% (2/33) in KSCCs. In contrast, more than one third (36%, 12/33) of KSCC tumours were HPV-positive, while HPV was detected in 12% (3/25) of the NK-D, and in 6% (6/92) of the NK-U tumours.

Table 7 The relationship between histopathological classification and viral status of 150 tumours of NPC patients diagnosed in Finland between 1990 and 2009. Modified from Publication II.

WHO histopathological subtype	***No. of patients (%)***			
	All	***EBV+***	***HPV+***	***EBV-/HPV-***
Keratinizing SCC (KSCC)	33	2 (6)	12 (36)	19 (58)
Non-keratinizing differentiated (NK-D)	25	13 (52)	3 (12)	9 (36)
Non-keratinizing undifferentiated (NK-U)	92	78 (85)	6 (6)	8 (9)
Total	150	93 (62)	21 (14)	36 (24)

Abbreviations: EBV, Epstein-Barr virus, HPV, human papillomavirus; SCC, squamous cell carcinoma.

The EBV-positive primary tumours were significantly smaller than the EBV/HPV-negative tumours (p=0.030) based on their T classifications. No significant differences were found in T classifications between the HPV-positive and the EBV/HPV-negative groups, or between any groups according to N classification, distant metastases, or stage.

5.2.4 Viral status and clinical outcome

For survival analysis, 143/150 (95%) patients treated with curative intent were included. Their median follow-up time was 63 months, and overall, 67/143 patients (47%) had a treatment failure with a median latency of 5 months. The EBV/HPV-negative patients tended to have more failures than the patients in the virus-positive groups, and they had significantly more local failures (49% of the patients) than the EBV-positive patients (25%; p=0.014). The rate of local failures in the HPV-positive patients was 32%. There were no statistically significant differences in nodal, distal, or overall failures between different viral status groups.

In Kaplan-Meier analysis, the 5-year OS was 66% for the patients with EBV-positive tumours, and 58% for the patients with HPV-positive tumours, whereas

only 27% of the patients with EBV/HPV-negative tumours survived 5 years (Figure 12A). In age-adjusted Cox regression analysis, the patients with EBV-positive tumours had significantly better OS compared to the patients with EBV/HPV-negative tumours ($p<0.0001$). For multivariable Cox regression analysis, the adjustments were made for age, gender, smoking, T and N categories, total RT dose, and treatment. Analysis was not adjusted for histology because of the strong association between viral status and histology. In multivariable Cox regression analysis, both EBV positivity and HPV positivity were significant prognostic factors for better OS compared to EBV/HPV negativity ($p=0.005$ and $p=0.034$, respectively) (Table 8a).

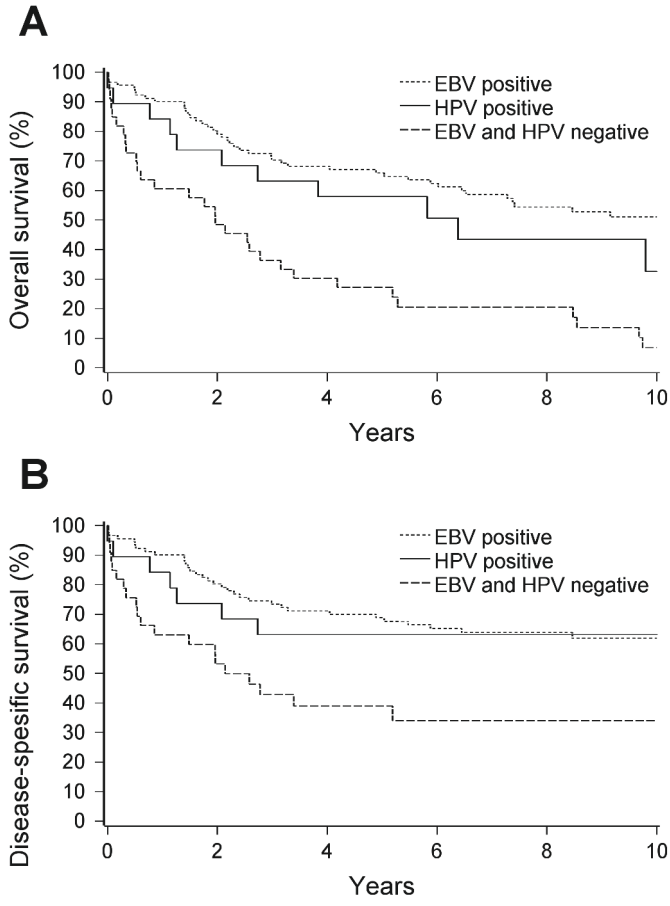

Figure 12 **Overall (A) and disease-specific survival (B) of 143 NPC patients diagnosed and treated with curative intent in Finland between 1990 and 2009 according to different viral statuses.** Adopted from Publication II.

In Kaplan-Meier analysis, the 5-year DSS was 69% for the patients with EBV-positive and 63% for the patients with HPV-positive tumours, while it was 39% for the patients with EBV/HPV-negative tumours (Figure 12B). In age-adjusted Cox regression analysis, the patients with EBV-positive tumours had significantly better DSS compared to the patients with EBV/HPV-negative tumours (p=0.007). However, it was not the case in the patients with HPV-positive tumours, probably due to the small number of patients in that group. In multivariable Cox regression analysis, viral status was not found to be an independent prognostic factor for DSS (Table 8b).

Table 8 **Age-adjusted and multivariable Cox regression analysis of 143 NPC patients diagnosed and treated with curative intent in Finland between 1990 and 2009 relative to overall and disease-specific survival.** Modified from Publication II.

Patient survival	*Age-adjusted*		*Multivariable-adjusted* [≠]	
	HR (95% CI)	*p value*	*HR (95% CI)*	*p value*
a) Overall survival				
Viral status				
EBV+ vs. EBV-/HPV-	0.38 (0.23-0.61)	<0.0001	0.44 (0.25-0.78)	0.005
HPV+ vs. EBV-/HPV-	0.61 (0.31-1.20)	0.15 NS	0.45 (0.21-0.94)	0.034
b) Disease-specific survival				
Viral status				
EBV+ vs. EBV-/HPV-	0.45 (0.25-0.80)	0.007	0.69 (0.33-1.44)	0.32 NS
HPV+ vs. EBV-/HPV-	0.50 (0.21-1.20)	0.12 NS	0.44 (0.16-1.17)	0.10 NS

Abbreviations: HR, hazard ratio; CI, confidence interval; EBV, Epstein-Barr virus; HPV, human papillomavirus; NS, not significant.

[≠]Adjusted for age, gender, smoking, T classification, N classification, total radiotherapy dose, and treatment.

5.3 Toll-like receptors in NPC (Study III)

5.3.1 TLR expression in NPC and in benign nasopharyngeal epithelium

In this study, we examined 150 NPC samples cut from the same TMA blocks as in Study II. Thus, the patient characteristics and the details on viral status are identical to those described in chapters 5.2.1 and 5.2.2. Every TLR had its specific staining pattern, and these patterns are summarized in Table 9. In addition to the NPC specimens, we also examined five control samples taken from benign adenoid tissues representing normal lymphoepithelium. These benign tissues expressed TLR1 and TLR2 similarly as NPC, but TLR4, TLR5, TLR7, and TLR9 were expressed in a different manner. A comparison between the staining patterns in NPC and in the benign epithelial cells is presented in Table 9. TLR5 was expressed in similar cell locations, but the benign epithelium expressed TLR5 exclusively in the basal cell layer, while the expression was ubiquitous in NPC.

Table 9 Toll-like receptor staining patterns in malignant nasopharyngeal carcinoma cells and in benign adenoid cells in Finland between 1990 and 2009

TLR	*Staining pattern in NPC*	*Staining pattern in benign cells*
TLR1	Cytoplasmic	Cytoplasmic
TLR2	Nuclear and cytoplasmic	Nuclear and cytoplasmic
TLR4	Cytoplasmic	Nuclear and cytoplasmic
TLR5	Nuclear membranous and cytoplasmic	Nuclear membranous and cytoplasmic
TLR7	Nuclear membranous and nuclear	Nuclear and cytoplasmic
TLR9	Cytoplasmic	Plasma membranous and cytoplasmic

All TLRs studied, i.e. TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9, were highly expressed in NPC tissues. TLR5 was expressed in 60% of the samples, while all other TLRs were expressed in 92-98% of the samples. The distribution of TLR expression intensities in NPC samples is presented in Table 10, and representative images of the most interesting TLR expressions (TLR5 and TLR7) are presented in Figure 13. TLR expressions were usually scored as negative, mild, or strong, as described in more detail in Materials and Methods in chapter 4.5.3. TLR7 was originally scored in three positive categories (mild, moderate, strong), but we combined the categories of moderate and strong expression into the single

category ‘strong’, because the distinction between these expression patterns was often challenging.

Table 10 Expression of TLR1, TLR2^{nucl}, TLR2^{cyto}, TLR4, TLR5, TLR7, and TLR9 in nasopharyngeal carcinoma tissues biopsied from Finnish NPC patients between 1990 and 2009

TLR type	*Intensity of TLR expression, n (%)*			
	negative	***mild***	***strong***	***total***
TLR1	3 (2)	24 (16)	122 (82)	149
TLR2^{nucl}	8 (6)	93 (66)	40 (28)	141
TLR2^{cyto}	8 (6)	59 (42)	74 (52)	141
TLR4	11 (8)	50 (35)	80 (57)	141
TLR5	58 (40)	54 (38)	32 (22)	143
TLR7	9 (7)	45 (31)	89 (62)	143
TLR9	4 (3)	61 (41)	84 (56)	149

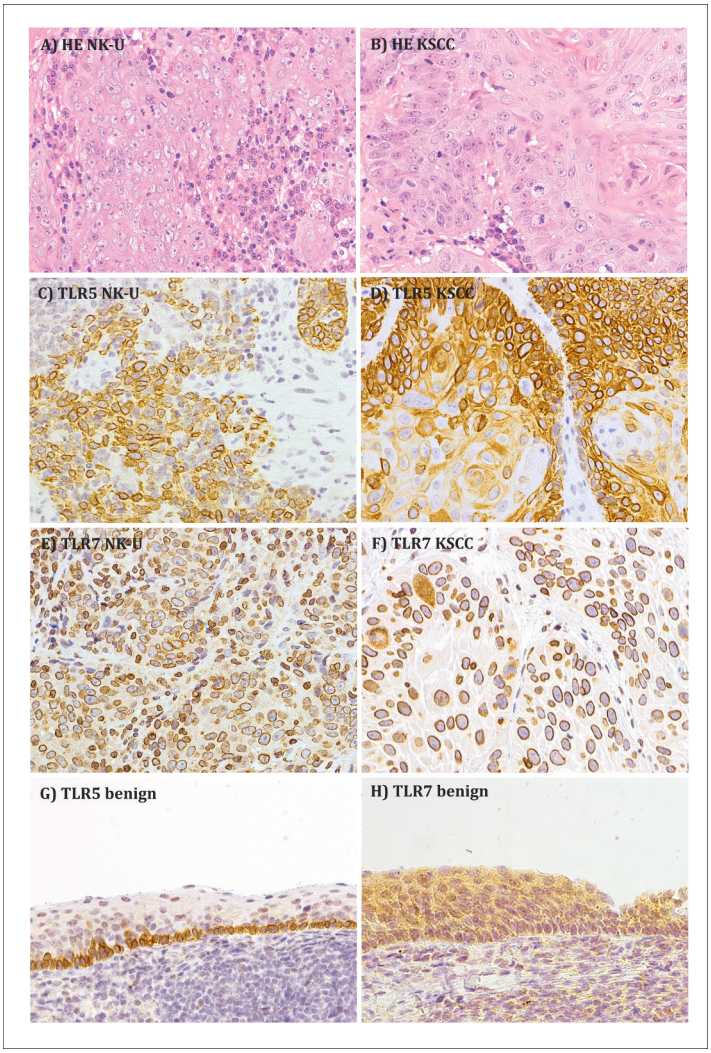

Figure 13 TLR5 and TLR7 expression in nasopharyngeal carcinoma and in benign nasopharyngeal epithelium.

A) H&E staining in non-keratinizing undifferentiated carcinoma (NK-U).

B) H&E staining in keratinizing squamous cell carcinoma (KSCC).

C) Positive TLR5 expression in NK-U. D) Positive TLR5 expression in KSCC.

E) Positive TLR7 expression in NK-U. F) Positive TLR7 expression in KSCC.

G) Positive TLR5 expression in benign nasopharyngeal epithelium.

H) Positive TLR7 expression in benign nasopharyngeal epithelium.

Magnification x 250. Photos by Ilmo Leivo, layout by Heikki Peuravuori.

5.3.2 Association between TLR expression and clinicopathological features

The expressions of TLR1, TLR4, TLR7, and TLR9 were not associated with essential patient and tumour characteristics such as age, gender, histology, or viral status. Neither was any TLR expression related to smoking or stage. By contrast, the expression of $TLR2^{nucl}$ and TLR5 associated with age, histology, and viral status.

The patients with mild $TLR2^{nucl}$ expression were significantly younger than those with strong $TLR2^{nucl}$ expression with mean (SD) ages of 54.7 (16) and 61.9 (13), respectively (p=0.036). In addition, the patients with negative or mild TLR5 expression were younger than those with strong TLR5 expression with mean (SD) ages of 54 (15), 56 (16), and 63 (13), respectively (p=0.021 and p=0.053). There were significantly more women in the group with strong TLR5 expression (50%) compared to the group with negative TLR5 expression (24%, p=0.013). Moreover, $TLR2^{nucl}$ and TLR5 expressions were associated with histology. Keratinizing tumours often presented with strong $TLR2^{nucl}$ (18/31, 58%) and strong TLR5 (19/32, 59%) expression, whereas undifferentiated tumours mostly had mild $TLR2^{nucl}$ (65/87, 75%) and negative TLR5 (48/88, 55%) expressions (p=0.0006 and p<0.0001, respectively).

The expressions of $TLR2^{nucl}$ and TLR5 were related to viral status (p<0.0001 in both). $TLR2^{nucl}$ expression was stronger in the HPV-positive and EBV/HPV-negative tumours than in the EBV-positive tumours: strong $TLR2^{nucl}$ positivity was seen in 57% and 53% vs. 12% of the tumours, respectively. The same tendency was seen in TLR5 expression, which was strong in 58% of the EBV/HPV-negative cases, strong (29%) or mild (52%) in the HPV-positive cases, and negative in 55% of the EBV-positive cases.

5.3.3 TLR expression and clinical outcome

The median follow-up time was 63 months like in Study II. In Kaplan-Meier analysis, TLR5, TLR7, and TLR9 expression patterns associated with clinical outcome. OS and DSS rates were worst in patients with strong TLR5 expression and patients with absent TLR7. In addition, absent TLR9 expression associated with worse OS. For all these three TLRs, mild expression was associated with the best survival. The 5-year OS and DSS rates according to TLR5 expression were 65% and 69% (mild), 57% and 63% (negative), and 40% and 48% (strong), respectively (Figure 14 a,b). The 5-year OS and DSS rates according to TLR7 expression were 66% and 70% (mild), 52% and 59% (moderate or strong), respectively, and 22% (negative) for both OS and DSS (Figure 14 c,d). As

mentioned before, negative TLR9 expression associated with worse OS, but the significance of this is limited by the presence of only four TLR9-negative cases. The 5-year OS rate according to TLR9 expression was 63% (mild), 52% (strong), and 25% (negative). In Kaplan-Meier analysis, we did not find survival differences related to TLR1, TLR2, and TLR4 expressions.

In multivariable Cox regression analysis, positive TLR7 expression (mild, p=0.018; moderate or strong, p=0.038) compared to negative TLR7 expression was a significant prognostic factor for a better OS. For DSS, only mild TLR7 expression (p=0.046) compared to negative TLR7 expression remained as a significant factor for a favourable prognosis. The differences between different TLR5 expression groups or TLR9 expression groups were not significant when adjusted for age, gender, stage, and viral status.

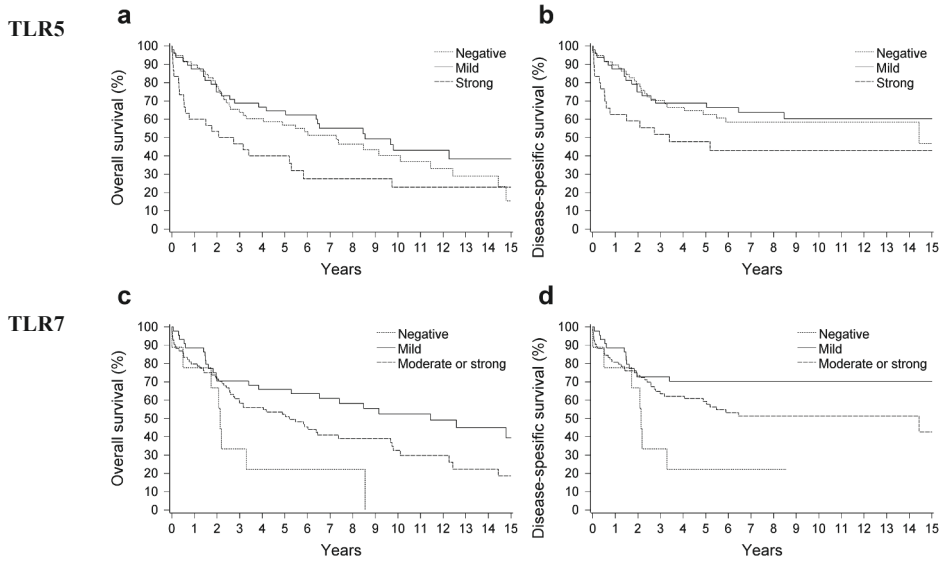

Figure 14 Overall (a,c) and disease-specific (b,d) survival of 143 NPC patients in Finland between 1990 and 2009 according to TLR5 (a,b) and TLR7 (c,d) expressions. Adopted from Manuscript III.

6 DISCUSSION

6.1 General discussion

Nasopharyngeal carcinoma (NPC) is a rare cancer in Finland with approximately 10 new cases yearly in the whole country. Little is known about its carcinogenetic process and etiology even in endemic cases, and its distinct geographical and racial characteristics makes it an intriguing target for study. The aim of this study was to investigate etiological and prognostic factors of NPC in Finland. To our knowledge, only a few nationwide NPC studies have been published to date (Kajanti et al. 1996, Carman and Strojan 2012, Arnold et al. 2013). In addition, our study was one of the largest to evaluate the prognostic significance of EBV and HPV in NPC, and the first to study TLR expression in non-endemic NPC.

6.2 Epidemiology and etiology of NPC (I-II)

Worldwide, the incidence of NPC is decreasing (Tang et al. 2016). The decline has been most rapid among the Chinese in Hong Kong and immigrant Chinese in the United States, presumably due to changes in lifestyle (Lee et al. 2012b, Yu and Hussain 2009). The number of KSCCs has decreased the most, which could be explained by a reduction of exposure to non-viral environmental risk factors, such as smoke and carcinogenic dietary elements (Tse et al. 2006). In Finland, the incidence of NPC has been stable for decades, but etiological factors may also differ from endemic populations.

We studied all 207 NPC cases diagnosed in Finland between 1990 and 2009, and were able to retrieve 150 samples (72%) from these patients to evaluate the histology and eventual presence of EBV and HPV in the tumours. To our surprise, EBV was found in as many as 62% of the tumours, which represented non-keratinizing carcinoma in 98% of the cases. Previously, EBV has not been routinely examined in NPC tumour samples in Finnish clinical practice, and the incidence of EBV has been supposed to be lower (Giannoudis et al. 1995, Svajdler et al. 2016). In the literature, the role of EBV in non-endemic NPC can be conceived equivocal, since the prevalence of EBV in Caucasian patients has ranged in different studies from 32% to 85% (Table 2). In contrast, in endemic areas, EBV is almost invariably associated with NPC and found in 97-100% of the cases (Laantri et al. 2011, Lin et al. 2014, Chua et al. 2016). In our study

population, in addition to EBV-positive cases, 14% of the patients presented with HPV-positive tumour, and 24% of the tumours were virus-negative.

We did not find any co-infections with EBV and HPV, which has been the case in most of the ISH-based NPC studies (Table 2). However, in a Taiwanese study, 39% of the patients had high-risk HPVs in their EBV-positive tumour cells (Huang et al. 2011). In that endemic patient cohort, most co-infected tumours (84%) were non-keratinizing carcinomas, and no survival differences were noted according to HPV positivity or EBV/HPV co-infections (Huang et al. 2011). The authors concluded that their findings do not support a contribution of high-risk HPVs to the carcinogenesis of NK-D/NK-U NPCs in Taiwan (Huang et al. 2011). In contrast, we found that the majority of HPV-positive tumours (57%) had keratinizing histology. The overall proportion of KSCCs was also higher in our patient cohort compared to high-incidence or intermediate-incidence cohorts (22% vs. 0-5%, respectively) (Mirzamani et al. 2006, Lin et al. 2014, Laantri et al. 2011, Dogan et al. 2016). Moreover, the patient characteristics in the KSCC group differed from non-keratinizing patient groups suggesting that HPV-positive and virus-negative KSCCs form a specific entity in non-endemic NPC. In fact, a study from the US, which reported the results of a comprehensive genomic profiling of different NPC subtypes, indicated that tumour mutation burden differed between KSCC and non-keratinizing carcinomas (Ali et al. 2017).

Genetic factors relating to immunological defence are supposed to have an impact on developing a latent EBV infection in the nasopharyngeal epithelium, but EBV variants may also have different carcinogenic potentials (Hildesheim et al. 2002, Palser et al. 2015). It has been postulated, that EBV infection early in childhood might predispose to NPC development, whereas delayed primary infection in adolescence causes mononucleosis but protects from NPC (Melbye et al. 1984). A Swedish study has found an increased risk of NPC related to the number of siblings supposing that older siblings transmit EBV to younger ones at an early age (Liu et al. 2016). For our retrospective study, the data on family size or other environmental factors were not available. Also smoking history was unknown for 29% of the study population. The youngest patients, those under 20 years, were EBV-positive, and overall the patients with EBV-positive tumours were significantly younger than the patients with EBV/HPV-negative tumours suggesting a different etiological background. In our patient cohort, we observed the same but unexplained male preponderance as in all other NPC studies. In contrast, the majority of the EBV/HPV-negative patient group were women. This raises the question about a higher susceptibility of men to virus-related NPC, but it unfortunately remains unanswered.

The incidence of HPV-positive oropharyngeal carcinoma (OPSCC) is rapidly increasing especially in Western countries (Chaturvedi et al. 2013). NPC is a different disease, but the distance between the nasopharynx and the oropharynx is not long, and both locations contain tonsil tissue. It has even been claimed that HPV-positive NPCs are actually expansions of OPSCC (Singhi et al. 2012). In our HPV-positive patients, all primary tumours clearly originated from the nasopharynx according to the reviewed endoscopy and imaging reports. This fact should be kept in mind when planning treatment for HPV-positive cervical metastasis from an unknown primary tumour. Direct consequences would be to irradiate the nasopharynx in addition to the orofarynx, even in the absence of apparent findings in the nasopharyngeal epithelium.

In recent years, the reported HPV prevalence in non-endemic NPC has ranged from 5% to 30% (Lo et al. 2010, Singhi et al. 2012, Robinson et al. 2013, Dogan et al. 2014, Stenmark et al. 2014, Lin et al. 2014, Jiang et al. 2016). The detection of high-risk HPVs has been often conducted with PCR, which may lead to false positive results, or with HPV DNA ISH leading to false negative results in samples with low HPV copy number (Qureishi et al. 2017, Blitzer et al. 2014). We used, for the first time in NPC studies, a sensitive and specific HPV E6/E7 mRNA ISH method detecting transcriptionally active high-risk HPVs (Bishop et al. 2012). The method is highly reliable but laborious due to lthe ack of an automated system. The good news for clinicians is that all p16-positive samples were also positive for HPV E6/E7 mRNA ISH. Thus, easily detectable p16 could serve as a reliable surrogate marker for HPV identification. Of interest was that during the 20-year study period, we did not observe an increasing trend for HPV involvement in our NPC cases.

6.3 Management and outcome of NPC (I-II)

The primary treatment for NPC is radiotherapy (RT) with or without concurrent chemotherapy. In the 1990s, most Finnish NPC patients were treated with 3-D RT without chemotherapy. Since then, the guidelines in treatment have undergone major changes, which include (1) systematic use of chemoradiotherapy (CRT) in advanced stages, (2) IMRT/VMAT as the preferred radiation technique, and (3) MRI in staging and delineation of treatment volumes. Most probably due to this intensification of the treatment, the outcome rates have improved with time. Realizing that irradiation techniques and chemotherapy protocols varied slightly across the hospitals, we dichotomized the patients in Study I into two groups: patients treated between 1990-1999 or 2000-2009. The observed 5-year DSS and OS rates according to this division in time

were 58% and 49% vs. 66% and 63%, respectively. Improvement was also seen when these figures were compared to the Finnish nationwide study from 1980-1989, which reported an actuarial survival of 52% (Kajanti et al. 1996). Our results are in line with reports from other non-endemic European countries comprising patients from the same decades: in Slovenia DSS 59% and OS 50%, in Sweden DSS 61% and OS 55%, in UK OS 60% with CRT, and relative survival of 55% in the Netherlands (Carman and Strojan 2012, Taheri-Kadkhoda et al. 2007, Colaco et al. 2013, Arnold et al. 2013). However, the recent survival rates reported from a large study in endemic Hong Kong are superior to ours (Au et al. 2018). That study analyzed the outcome of 3328 NPC patients treated at six public hospitals in Hong Kong over a 10-year period from 2001 to 2010. They found that in Asian patients having mostly (94%) undifferentiated tumours, 5-year progression-free survival was 70% and OS 78% (Au et al. 2018).

Many studies on NPC have concluded that patients with NK-U have better outcome than those with KSCC (Carman and Strojan 2012, Taheri-Kadkhoda et al. 2007, Colaco et al. 2013, Arnold et al. 2013). We also noted that non-keratinizing undifferentiated histology was a favourable prognostic factor. Nevertheless, NK-U was strongly related to EBV, which means that solely virus positivity might be one reason for the better radiosensitivity of NK-U tumours. This phenomenon has been observed in other HNSCCs among whom HPV-positive tumours are more sensitive to radiation than HPV-negative tumours (Rieckmann et al. 2013). The supposed mechanisms include the impaired DNA repair of tumour cells and increased immune mediated tumour clearance (Blitzer et al. 2014). Our results for HPV-positive tumours support this idea, because patients with HPV-positive tumours had better survival than EBV/HPV-negative patients, even if more than half of the HPV-positive tumours were of keratinizing histology. Finally, if we compare the outcome of our virus-positive cases to cases from Hong Kong, the differences are smaller. When considering the whole 20-year period of our study, the DSS for EBV-positive patients was 69% and OS 63%, and the same figures for HPV-positive patients were 63% and 58%, respectively.

NPC is often diagnosed in advanced stage. One might expect that in endemic countries the diagnosis is made earlier because of a higher awareness of the disease. However, according to recent research, this is not the case. If the stage distribution at diagnosis of our study was I 11%, II 25%, III 39%, and IV 25%, the same figures in Hong Kong were 8%, 18%, 48%, and 27%, respectively (Au et al. 2018). Also the RT doses were similar.

6.4 TLRs in NPC (III)

In Study III, we evaluated the expression patterns of TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 in 150 tumours of Finnish NPC patients, and analysed the findings according to histology, EBV and HPV status, and clinical outcome. We found that TLR1 and TLR4 expressions were not associated with any patient or disease characteristics. In contrast, strong expression of TLR2 and TLR5 was associated with features typical of non-endemic NPC, such as older age, keratinizing histology, and non-EBV-related etiology. The majority of the tumours (66%) with strong TLR5 positivity were negative for both examined viruses. Knowing that bacterial flagellin serves as an agonist for TLR5 and promotes gastric cancer (Song et al. 2011), we can only speculate that bacterial exposure may also affect the nasopharyngeal cell differentation. In addition, TLR2 has been strongly present and activated in nasal polyps of cystic fibrosis patients in response to bacteria (Muir et al. 2004).

In Kaplan-Meier analysis, the patients with strong TLR5 expression had worse DSS and OS compared to those with mild or negative expression. The same trend in survival has been observed in OTSCC and OPSCC studies, but in the latter, prognostic significance of TLR5 was present only among HPV-positive cases (Kauppila et al. 2013, Jouhi et al. 2017b). In our multivariable Cox regression analysis, TLR5 expression was not an independent prognostic factor for NPC because of strong relation to other prognostic factors, such as age, histology, and viral status. In this mixture of different prognostic factors, causal connections are difficult to establish. Infection-induced TLR stimulation and inflammation can promote tumorigenesis, but on the other hand, tumour growth can mimic tissue damage leading to TLR activation by DAMPs. It has been proposed that the initiating factor is probably not the inflammatory stimulation *per se* but its timing, duration, and intensity (Oblak and Jerala 2011).

TLR7 expression was not associated with viral status or other characteristics. Nevertheless, the patients with positive TLR7 expression had better DSS and OS than the patients with no TLR7 expression in their tumours. The differences were significant even when adjusted for other prognostic factors. The same correlation was observed in the OPSCC study, where high TLR7 expression was associated with a better DSS (Jouhi et al. 2017b). However, this was true only for patients with HPV-positive OPSCC tumours. We did not observe such a difference according to viral status, but the proportion of HPV-positive tumours was small in our study. TLR7 was highly expressed in NPC. This observation is of interest for the development of future treatments targeting TLR7. TLR7 agonist imiquimod has been used for years in the treatment of basal cell carcinoma and

other skin lesions. In preclinical studies, it has also inhibited the proliferation of OSCC cells (Ahn et al. 2012).

In our study, we examined TLR expression by IHC to determine the expression sites of TLRs in NPC. We observed differences in the localisation of the TLR expression when we compared benign tissues with malignant tissues. For example, TLR5 was expressed exclusively in the basal layer of the benign epithelium, while in NPC the expression was diffuse. Similar findings have been published in gastric carcinogenesis induced by *H. pylori* (Pimentel−Nunes et al. 2013). These findings suggest that activation of TLRs in abnormal locations may be related to the carcinogenetic processes. However, further research is needed to describe the mechanisms and causal connections between infection, TLR expression sites, and neoplastic growth.

The previous TLR studies on NPC have focused on genetic polymorphisms of toll-like receptors. They have found that certain polymorphisms of TLR2, TLR3, TLR4, TLR8, TLR9, and TLR10 are linked to increased cancer risk, and the authors suggest that impaired TLR function is the reason for carcinogenesis (Makni et al. 2017, Song et al. 2006, Yang et al. 2012, Wee et al. 2012, Dai et al. 2014, Zhou et al. 2006). These sequencing studies on genes encoding TLRs have been exclusively conducted in endemic areas reflecting the risk of developing EBV-related cancer. In conclusion, it has been estimated that polymorphism of pattern-recognition receptors in general may be associated with almost all cancer types, and particularly with those induced by carcinogenic infectious agents (Kutikhin and Yuzhalin 2012).

6.5 Methodological considerations and study limitations

Study I was a nationwide study on patients with NPC diagnosed and treated between 1990 and 2009. The patients were identified using the Finnish Cancer Registry database, which covers over 99% of all solid tumours diagnosed in Finland (Teppo et al. 1994). Thus, it is unlikely that many cases remained unidentified, and our patient cohort truly represents the NPC patients of the whole country. The patient records were studied case by case, and data on diagnostics and treatment were noted on a consistent manner. However, due to the retrospective setting, it was not possible to have complete information of cigarette smoking history, alcohol consumption, or occupational risk factors, which might have had importance in evaluating prognostic factors. Details on adverse effects of the treatment were also occasionally lacking. In addition, a few patients were withdrawn from follow up, and for their survival end points, we had to rely on data acquired from the Finnish Cancer Registry and Statistics

Finland. The original histological samples were not available for 39/207 (19%) patients, and their tumours were classified using the original pathology reports.

Patients were treated in several hospitals during the 20-year period, and as a consequence, treatment strategies were somewhat heterogenous reflecting local patterns of management. To simplify the survival data analysis, we dichotomized the treatments to RT and CRT. In CRT group, a significant number of patients had received neoadjuvant or adjuvant chemotherapy, which might have had an impact on their outcome. Nevertheless, the principal aim of the study was not to evaluate the efficacy of the treatment but the survival according to different prognostic factors, and these factors were documented uniformly in patients in both groups.

Study II and III comprised the patients with FFPE blocks available for further analysis. We were able to obtain the samples from 150/207 (72%) patients, which can be regarded as a high proportion given the high number of hospitals and long period of our study. The selection occurred randomly, since there were missing samples in all hospitals and over the entire study period. Some biopsy samples were discarded from the TMA due to their inadequate size. In addition, a few core samples detached from the TMAs during the staining processes, but in general, at least one sample from each patient remained on the slide. Although the TMA specimens were taken from the original FFPE tumour block according to the pathologist's (I.L.) markings, the small size (1 mm^2) of the core sample signifies that the specimen represents only a minor area of the tumour, and variations in protein expression in different parts of the tumour can escape the analysis. Finally, immunohistochemistry and in situ hybridization are qualitative or at most semiquantitative detection methods, which implies a risk of subjectivity in the evaluation. However, the pathologist was blinded to clinical data. Furthermore, TLR stainings were performed with antibodies available at the Department of Pathology in Helsinki University Hospital. Accordingly, due to technical reasons, we did not have a possibility to evaluate expression of TLR3 or TLR8, which might have revealed interesting aspects with respect to NPC.

NPC is a rare disease in Finland, and despite of a long study period and a nationwide setting, the number of patients was relatively small compared to study populations in high-incidence areas. We may speculate that with a larger patient cohort the differences in multivariable analyses between the viral status groups might have been more significant.

6.6 Future perspectives

Our study indicated that patients with NPC in a non-endemic area have different characteristics and survival figures than patients in endemic areas. A significant part of the patients in Finland had EBV/HPV-negative tumours, which might require intensified treatment compared to the patients with EBV-positive or HPV-positive tumours. Current international guidelines do not address viral status as a treatment-modifying factor, because NPC trials have been conducted in endemic populations with consistent EBV positivity. Nevertheless, after the completion of this study, treating Finnish hospitals will be advised to assess viral status of the NPC tumours with EBV-encoded RNA and HPV DNA ISH, and/or p16 IHC.

Preventive EBV vaccine is not available, but several ongoing clinical trials have shown encouraging results of therapeutic EBV vaccines and immune-checkpoint inhibitors in EBV-positive NPC patients (Taylor and Steven 2016, Louis et al. 2010, Hsu et al. 2017, Ma et al. 2018). In the future, the detection of PD-L1 expression of the tumour could be part of the diagnostic setup. However, the whole tumour microenvironment should be taken account. It has been suggested that high PD-L1 expression on tumour and immune cells would be of favourable prognostic value only if associated with dense infiltration of CD8-positive tumour infiltrating lymphocytes (Ono et al. 2018).

One-quarter of NPC tumours in Finnish patients did not reveal EBV or HPVs, and these cases presented with the worst survival. This emphasizes a strong need to continue studying the virus-negative cases. It is tempting to speculate that these tumours might be genetically different from the virus-positive ones. The etiology is unknown, given that their smoking history in our study was similar to the patients with EBV-positive tumours, and the largest proportion of smokers or ex-smokers was found among patients with HPV-positive tumours. Could there still be another, yet unknown microbe initiating the NPC carcinogenesis? In fact, the expression of TLR5, which responds to bacterial flagellin, was strongest in the virus-negative tumours. Further research is also needed to study NPC tumours for other viruses, such as polyomaviruses.

In our study, positive TLR7 expression seemed to be an independent prognostic marker for a favourable outcome in NPC. However, the sample size was too small to allow for firm conclusions. Larger cohorts and prospective studies are required to validate the finding before introducing TLR7 agonists to the treatment of NPC. In addition, TLR3 and TLR8 expressions would be interesting to examine in the light of the endemic findings of their polymorphisms.

Finally, from the vantage of NPC research globally, the most important consideration would be to discover how EBV enters the epithelial cells of the nasopharynx and how does the replicative life cycle of EBV function *in vivo*. By gaining an understanding of EBV-associated oncogenesis, the majority of NPC cases could most probably be prevented.

7 CONCLUSIONS

1. The survival of patients with NPC has improved over the 20-year study period. This is likely due to improvements in radiotherapy and imaging techniques, and concurrent chemotherapy. Tumours are of keratinizing histology in 20% of the Finnish NPC patients, which predicts worse outcome compared to non-keratinizing tumours.

2. The majority of NPC tumours show viral etiology in Finland. Almost two-thirds of the cases associate with Epstein-Barr virus and one out of seven associate with high-risk human papillomaviruses. Patients with these virus-positive tumours have better prognosis than those with virus-negative tumours. The patients with EBV-positive non-keratinizing undifferentiated carcinoma have the best 5-year survival, and it is the worst among the patients with EBV/HPV-negative keratinizing carcinoma.

3. Toll-like receptors are highly expressed in NPC. TLR7 expression seems to be an independent prognostic factor in non-endemic NPC, and the patients with positive TLR7 expression have better survival than the patients with no TLR7 expression. Strong expression of TLR2 and TLR5 is related to HPV-positive and EBV/HPV-negative keratinizing tumours.

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