UMEÅ UNIVERSITY

Human Papillomavirus in Recurrent Respiratory Papilloma, Sinonasal Inverted Papilloma, and Non-Malignant Tonsils

Anna M. Holm

Department of Clinical Sciences
Umeå 2019

Responsible publisher under Swedish law: The Dean of the Medical Faculty

Dissertation for PhD Anna Holm
ISBN: 978-91-7601-865-1
ISSN: 0346-6612
Information about cover design / cover photo / composition
Electronic version available at: http://umu.diva-portal.org/
anna.holm@umu.se
Printed by: Cityprint i Norr AB
Umeå, Sweden 2019
Front cover illustration: Illustration by the author

Success is not final, failure is not fatal. It is the courage to continue that counts.

Attributed to Sir Winston Churchill

In loving memory of Stig E. Holm

Table of Contents

Abstract

Background: Human papillomavirus (HPV) is known to cause recurrent respiratory papilloma (RRP) and certain types of oropharyngeal cancer. HPV has also been associated with sinonasal inverted papilloma (SIP). HPV transmission routes are under investigation and the conviction is that the infection occurs sexually at an adult stage, however, vertical transmission at birth with a dormant viral condition until disease eruption/co-activation has been stated as a possibility.

Purpose: The purpose of this work was to contribute to the understanding of HPV related chronic diseases in the airway. Specific aims were: 1. To increase understanding regarding changes in the immune system as well as of the glycosaminoglycan hyaluronan in patients with RRP. 2. To evaluate prevalence of HPV and its surrogate marker p16 in SIP as well as HPV, p16 and Epstein-Barr virus (EBV) in benign tonsillar disease. HPV and EBV in non-malignant tonsillar disease were studied due to the fact that incidence of HPV positive tonsillar cancer is increasing and the time of viral infection is unknown.

Methods: A phenotypic characterization of peripheral blood from 16 RRP patients and 12 age-matched controls, using immunoflow cytometry, and monoclonal antibodies against differentiation and activation markers, was performed. The cytokine mRNA profile of monocytes, T helper-, T cytotoxic-, and NK cells was assessed using RT-qPCR. 54 SIP samples were studied of which 53 were available for analyzation with PCR. Genotype screening for 18 high risk and six low risk HPV types was performed using the PapilloCheck® HPV-screening test (a PCR method). 54 samples were immunohistochemically (IHC) stained for p16. Biopsies from vocal folds (VFs) and false vocal folds (FVFs) were collected from 24 patients with RRP, 12 were randomly selected to histochemistry for Hyaluronan (HA) and IHC staining for CD44 in the epithelium, stroma and RRP lesions. The remaining 12 patients were analyzed for HA molecular mass distribution with a gas-phase electrophoretic molecular mobility analyzer (GEMMA). Eight VF samples and four FVF samples were successfully analyzed. Biopsies from 40 non-malignant tonsils were analyzed using Papillocheck® for HPV, IHC for p16 and EBER analysis for EBV.

Results: We found a dominance of cytotoxic T cells, activated NK cells, and high numbers of stressed MIC A/B (MHC class I chain-related molecule A/B) expressing lymphocytes. The HPV analysis was successful for 38 SIP samples and two (5%) were positive for HPV 11. Notably, p16 was present in the epithelia of all samples and in the papilloma portions in 37 of 38 samples. We found extensive HA staining in the stroma of both VFs and FVFs. CD44 was expressed throughout

the epithelium, stroma, and RRP lesions in both FVFs and VFs, it did however, not concur with the expression of HA. Very high mass HA was found in both VFs and FVFs, though more variation regarding amounts of HA was seen in the VFs compared to FVFs. No HPV was found in non-malignant tonsils, the p16 levels were low and the counted EBER positive cells showed great variation in numbers.

Conclusions: Our findings demonstrate an immune dysregulation with inverted CD4+/CD8+ ratio and aberrant cytokine mRNA production in RRP patients, compared to healthy controls. We concluded that p16 cannot be used as a surrogate marker for high-risk HPV-infection in SIP and that HPV incidence was low (5%). CD44 does not seem to bind to HA, which might explain the non-inflammatory response previously described in RRP. Very high mass HA possibly crosslinked was seen in both VFs and FVFs. A possibility to counteract inflammatory crosslinking of HA may be found for medical treatment options in RRP.

Abbreviations

AoRRP	Adult onset Recurrent Respiratory Papilloma
CD	Cluster of Differentiation
CDK	Cyclin Dependent Kinase
DNA	DeoxyriboNucleic Acid
EBV	Epstein-Barr Virus
EBER-ISH	Epstein-Barr Encoding Region In-Situ Hybridization
ELISA	Enzyme- Linked ImmunoSorbant Assay
ENT	Ear, Nose and Throat
FVF	False Vocal Fold (vestibular fold)
GEMMA	Gas-Phase Electrophoretic Molecular Mobility Analyzer
HA	Hyaluronan
HIV	Human Immunodeficiency Virus
HMHA	High Mass Hyaluronan
HMW-HA	High Molecular Weight Hyaluronan
HPV	Human papillomavirus
hrHPV	high risk HPV
ICD	International Classification of Diseases
IFN-γ	Interferon-γ
IHC	ImmunoHistoChemistry
ISH	In-Situ Hybridization
JoRRP	Juvenile onset Reccurent Respiratory Papilloma
LMHA	Low Mass Hyaluronan
LMW-HA	Low Molecular Weight Hyaluronan
lrHPV	low risk HPV
MHC	Major Histocompatibility Complex
MIC A	MHC class I chain-related molecule A
MIC B	MHC class I chain-related molecule B
mRNA	messenger RiboNucleic Acid
NK cell	Natural Killer cell
NKG2D	Natural Killer Group 2 member D
ORF	Open Reading Frame
OSA	Obstructive Sleep Apnea
p16	protein 16
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
pRB	Retinoblastoma Protein
RRP	Recurrent Respiratory Papilloma
RT-qPCR	Reverse-Transcription quantitative Polymerase Chain Reaction
SIP	Sinonasal Inverted Papilloma
SPSS	Statistical Pack for Social Sciences

VLP	Virus Like Particle
VF	Vocal Fold
WHO	World Health Organization

Preface

This thesis is based on the following papers, referred to in the text by their Roman numerals

Paper I Holm A, Nagaeva O, Nagaev I, Loizou C, Laurell G, Mincheva-Nilsson L, Nylander K, Olofsson K. Lymphocyte profile and cytokine mRNA expression in peripheral blood mononuclear cells of patients with recurrent respiratory papillomatosis suggest dysregulated cytokine mRNA response and impaired cytotoxic capacity. *Published* in Immunology, Inflammation and Disease, August 2017.

Paper II Holm A, Allard A, Eriksson I, Laurell G, Nylander K, Olofsson K. Absence of high-risk human papilloma virus in p16 positive inverted sinonasal papilloma. *Accepted* for publication in European Annals of Otorhinolaryngology, Head and Neck Diseases, October 2017.

Paper III Holm A, Hellman U, Laurent C, Laurell G, Nylander K, Olofsson K. Hyaluronan in vocal folds and false vocal folds in patients with recurrent respiratory papillomatosis. *Accepted* for publication in Acta Oto- Laryngologica, July 2018.

Paper IV Holm A & Schindele A, Sandström K, Laurell G, Nylander K, Allard A, Olofsson K. Mapping of human papilloma virus, p16 and Epstein-Barr virus in non-malignant tonsillar disease. Manuscript *re-submitted* to Laryngoscope Investigative Otolaryngology after minor revision, February 2019.

Sammanfattning på Svenska

Syftet med avhandlingsarbetet var att studera humant papillomvirus (HPV), dess surrogatmarkör p16 i respiratoriska papillom (RRP), sinonasala inverterade papillom (SIP) och benign halsmandelssjukdom. I halsmandel studerades även förekomst av Epstein-Barr virus (EBV) i relation till HPV och p16.

Under en livstid kommer de flesta människor i kontakt med olika genetiska varianter av HPV men endast ett fåtal individer insjuknar i RRP. Att leva med RRP innebär upprepade kirurgiska behandlingar i narkos och konstanta återverkningar på röst och andningsfunktion. Varför vissa insjuknar och andra förblir friska är inte klarlagt men den enskilda individens förmåga att immunologiskt processa HPV anses ha betydelse. Vi har studerat immunsystemet hos 16 RRP patienter i jämförelse med 12 matchade kontroller. Hos RRP patienterna hittade vi ett uppreglerat uttryck av cytotoxiska T celler, ett ökat antal NK celler samt ett stort antal stressade MIC A/B lymfocyter. Produktionen av cytokin mRNA var avvikande. Patienterna med RRP hade ett otillräckligt T hjälparcellsvar och ett uppreglerat antal cytotoxiska T celler vilket resulterade i ett omvänt förhållande mellan dessa T celler. Den inverterade kvoten mellan cytotoxiska T celler och hjälpar T celler anses vara en markör för immundysfunktion hos ex HIV patienter.

RRP är associerade med HPV 6 och 11 och uppstår oftast på de äkta stämvecken (stämbanden) men HPV mRNA kan även påvisas i de falska stämvecken i 50% av fallen. Vår avsikt var, genom att studera skillnaden mellan falska och äkta stämveck i relation till HPV på molekylär nivå, att kunna förklara varför RRP vegetationer är mindre vanliga på de falska stämvecken trots närvaro av mRNA. Hyaluronan (HA) är ett protein med viskoelastiska egenskaper vilket krävs för en fungerade röst. Storleken på HA molekylen är avgörande för dess funktion. Högmolekylär HA har anti-inflammatoriska, anti-angiogena och immunsupprimerande egenskaper medan låg-molekylär HA har motsatt effekt. 24 patienter med RRP undersöktes. Under en diagnostisk/terapeutisk operation togs biopsier från falska och äkta stämveck. 12 prover undersöktes med GEMMA (gas-phase electrophoretic mobility molecular analysis) i syfte att bestämma HA molekylens storlek. 12 prover analyserades histologiskt för att tydliggöra distributionen av HA och dess receptor CD44. Både äkta och falska stämveck uttryckte låg- och högmolekylärt HA. Vår studie visade även uttryck av väldigt hög-molekylär HA (vHMHA). vHMHA har tidigare påvisats i nakenråttan och anses vara en av orsakerna till att den sällan utvecklar cancer. Förekomsten av vHMHA var mera varierande i de äkta stämvecken vilket skulle kunna vara en förklaring till varför RRP lesioner fördelar sig olika mellan äkta och falska stämveck och att sjukdomen har ett individuellt förlopp. HA och CD44 uttrycktes

inte på samma plats i vävnaden vilket kan vara en av förklaringen till varför man inte sett någon inflammatorisk reaktion i RRP. Ett intressant bifynd är att vi kunde se papillomvävnad i hälften av alla makroskopisk friska falska stämband.

Eftersom vissa genetiska varianter av HPV är associerade med mun och svalgcancer och andra med respiratoriska papillom, ville vi studera sambandet mellan HPV och SIP. Tidigare har man visat att 37,8% av SIP var HPV positiva. I vår rapport undersöktes förekomst av HPV och dess surrogatmarkör p16 i SIP. Målet var att om möjligt reproducera tidigare resultat samt att studera uttrycket av p16 i relation till HPV i SIP. Vi fann två av 38 SIP biopsier positiva för HPV 11. Biopsierna var negativa för övriga genetiska HPV varianter som studerats. p16 var positiv i epitelet i alla biopsier och i de inverterade papillomvegetationen i 37 av 38 prover. Vi fann ett betydligt lägre uttryck av HPV i SIP jämfört med tidigare resultat, delvis betingat av metodologiska skillnader. p16 kan inte användas som surrogatmarkör för HPV i SIP.

Cancer i halsmandeln är den vanligaste formen av mun och svalgcancer i Sverige. Rökning och alkohol är välkända riskfaktorer. De senaste åren har konsumtionen av cigaretter och alkohol minskat men insjuknandet i halsmandelcancer har fortsatt att öka. HPV anses vara den bakomliggande orsaken. Runt 200 olika HPV varianter har klassificerats, varav minst 13 är onkogena enligt WHO. Två av dessa onkogena genotyper är HPV 16 och 18, båda associerade med cancer i tungbas och halsmandel. HPV anses smitta via sexuell kontakt men även i samband med graviditet/förlossning. Förekomsten av HPV, EBV och p16 i halsmandlar som opererats bort på grund av förstoring eller infektion hos både barn och vuxna har studerats. Ingen av de studerade halsmandlarna uttryckte HPV. EBV uttrycktes i 65% av fallen. p16 var svagt positivt i allt studerat epitel och i 92,5% av de lymfatiska delarna i preparaten. Vi konkluderar att ingen av de studerade godartade halsmandlarna var infekterad med onkogent HPV och att vi inte vet när patienten blir smittad.

1. Introduction

1.1 Human papillomavirus

Papillomaviridiae are icosahedral, non-enveloped DNA viruses, approximately 8000 base pairs in size, they infect epithelia in various hosts such as birds, fish and mammals. Human Papillomavirus (HPV), specifically infect humans.

When Harald zur Hausen initiated his research in the 1970s, it was assumed that only one type of papillomavirus existed and that Herpes Simplex Virus type 2 was involved in the genesis of cervical cancer [1]. As of today, 5 different genera of HPV, alpha, beta, gamma, mu and nu have been described [2]. More than 200 different types of papillomavirus have been identified, of which at least 150 can infect humans. These 150 genotypes of HPV are not equally dangerous for the human host. Some are classified as high risk (hrHPV, capable of causing cancer), whereas others are classified as low risk (lrHPV, commonly causing diseases other than cancer).

Some lrHPV lead to cutaneous warts (e.g. 4 and 5) that commonly disappear on their own, while others (6 and 11) lead to more troublesome diseases such as condylomata accuminata (benign warts in the anogenital area) or RRP (warts in the respiratory tract affecting airway and voice). The most dangerous types (e.g. 16 and 18) are capable of causing anogenital and oropharyngeal cancer, a discovery for which Harald zur Hausen was awarded with the Nobel Prize in Medicine in 2008.

HPV is a small, double stranded, encapsulated, DNA virus that belongs to the family of *Papillomaviridiae*. HPVs genome contains three regions, the early region (E), late region (L) and a non-coding region (also known as long control region). Open reading frames (ORF) in the early region encode proteins E1, E2, E4, E5, E6 and E7 that are known to be involved in viral regulation, whereas ORF in the late region encode capsular proteins L1 and L2.

1.2 Viral infection

In principle, HPV infects humans via sexual contact [3], though other routes of infection such as vertical (via birth) or via surfaces and objects, have also been discussed [4]. It is an epitheliotropic virus, which implies that HPV only infects the epithelial cells [5] (Fig. 1.A.). Infection occurs in the basal layer of the host's squamous cell epithelium that has been exposed due to microtears (Fig.1.B.). Commonly this occurs in squamocolumnar junction zones, where squamous epithelium meets columnar epithelium, such as in the cervix. HPVs genome

establishes itself as episome and relies on the host cells own cell cycle for its replication [6].

HPVs capsid protein L1 binds to a Heparin Sulfate Proteoglycan (HSPG) receptor on basal membrane or epithelial cell surface which later on leads to conformational changes and HPV capsid protein L2 binding the annexin A2 heterotetramer [7]. Subsequently, HPV is endocytosed and the viral DNA is placed in the nucleus, where it establishes itself as an episome. HPV is unable to replicate on its own, instead, it multiplies together with the host's keratinocytes by using the host cells proteins and replication factors.

The first genes activated are E1 and E2, closely followed by low levels of oncoproteins E6 and E7. This can be explained by their functions. Upon infection E1, the largest and most conserved protein, increases number of viral episomes in the cells of the basal layer. E1, thereafter, maintains constant numbers of viral episomes as host cells differentiate and lastly promotes amplification of HPVs genome during the productive phase of the virus in the uppermost epithelial layer [8] (Fig.1.C.).

Meanwhile, E2 regulates transcription of HPV and can thus either activate or repress transcription. The importance of E2s function becomes obvious when it is disrupted, such as seen when HPV integrates in the host's genome, and alleviation of E2 mediated repression leads to increased expression of viral oncoproteins E6 and E7 [9] (Fig. 1.C.).

E6 and E7 promote cell cycle progression and thus cell division in differentiating cells, in which cell division normally does not take place [10]. This is achieved by E6s ability to degrade p53, allowing HPV infected cells to escape apoptosis and instead to continue to divide in upper epithelial layers [11]. E7 is the second major oncoprotein. It acts by binding Retinoblastoma protein pRb and thus disrupts pRb from binding transcription factor E2F. As a result, infected cells proceed from G1 to S phase [12] (Fig. 1.C.).

E4 is more diverse since it has adjusted itself to the type of epithelium infected, in order to be able to mediate virus release and transmission. E4 is also suggested to be biomarker of active HPV disease and a marker of disease severity in the cervix. [13] E4 is sometimes regarded as a 'late early' protein since L1 and L2 late proteins are expressed just after E4 (Fig. 1.C.).

The E5 protein has mainly been studied in HPV 16 and bovine papillomavirus 1 [14] and it is therefore difficult to predict function in other HPV types. Additionally, it is absent in the genome of some HPV [15] which suggests that it is not essential to the life cycle of the virus. However, E5 seems to induce loss of

expression of surface MHC I which implies that viral antigens cannot be presented to T-cells.

Late proteins L1 and L2 assemble the viral capsid and are expressed last in order to avoid activation of the host's humoral immune response. Major capsid protein L1 has been chosen for HPV classification. To be regarded as a distinct HPV genotype at least 10% difference in L1 nucleotide sequence must be present [16]. L1 forms majority of virus capsid and can self- assembly into virus like particles (VLPs). Minor capsid protein L2 co-assembles with L1 into VLPs [17].

Fig. 1. A-D. Illustrations made by the author

Fig. 1.A. Normal squamous cell epithelium

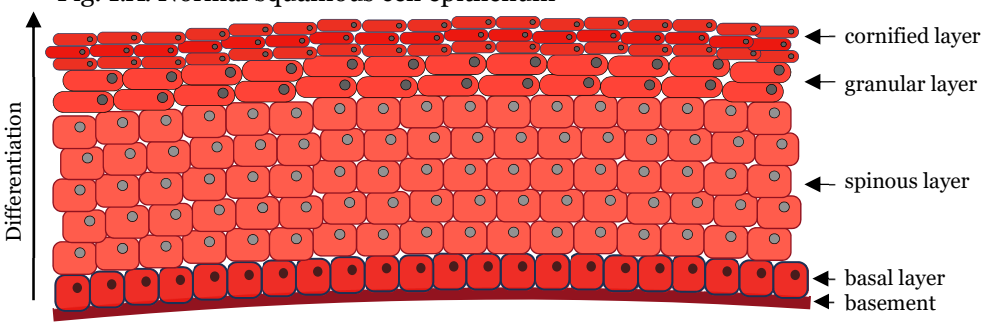


Fig. 1.B. HPV enters epithelium via microtear, attaches to basal membrane and is endocytosed into basal layer cell

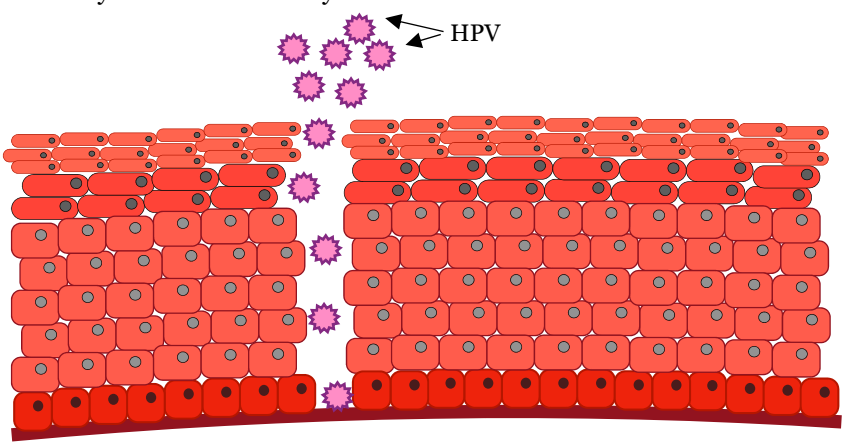


Fig. 1. C. HPV infection in the basal layer and viral replication, amplification, assembly and release in the differentiating epithelium

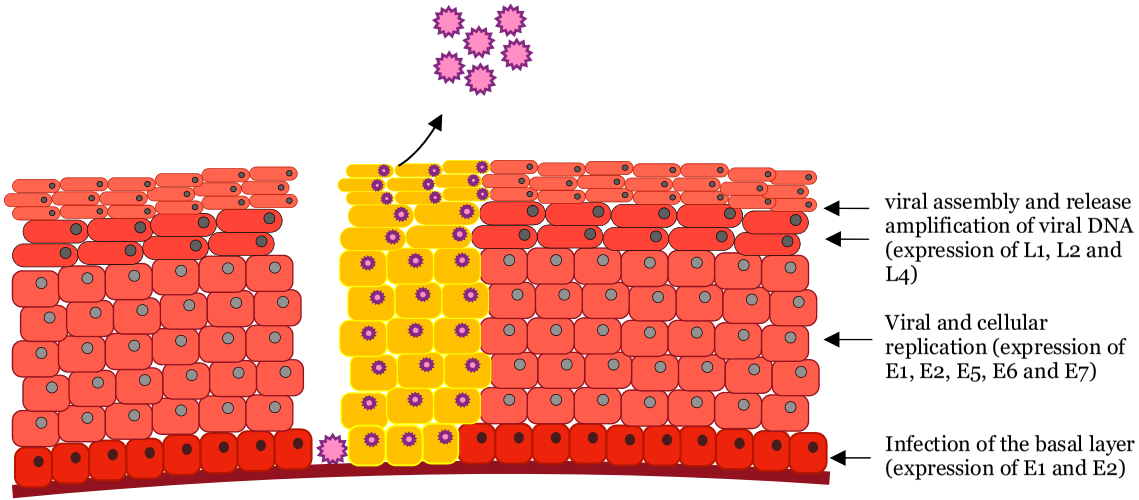
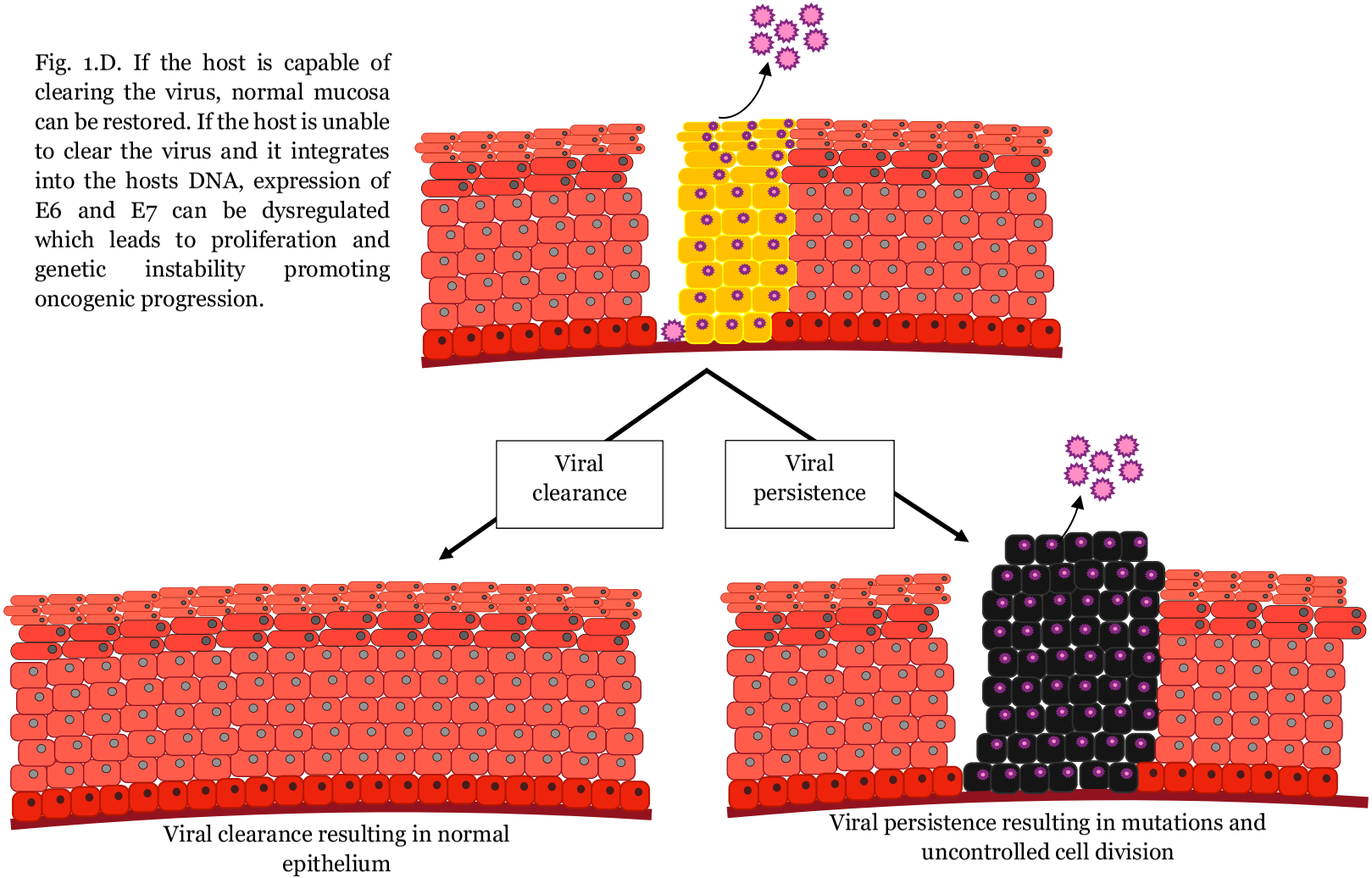


Fig. 1.D. If the host is capable of clearing the virus, normal mucosa can be restored. If the host is unable to clear the virus and it integrates into the hosts DNA, expression of E6 and E7 can be dysregulated which leads to proliferation and genetic instability promoting oncogenic progression.



1.3 Episomal and integrated HPV DNA

When HPV infects epithelial cells, it arranges itself as episome, which implies that the viral DNA enters the nucleus but does not integrate into the host's chromosomes [5]. HPV episomes replicate parallelly to the chromosomal DNA. In most cases, the host immune system eventually clears (episomal) HPV infection, in some cases however, HPV DNA becomes integrated into the host's DNA [18]. It is thought that integration of HPV leads to disruption of E2 which leads to increased expression of viral oncogenes E6 and E7 [19] (Fig. 1.D).

A recent study by Nulton et al. comparing tonsillar cancer with episomal HPV DNA with tonsillar cancer in which HPV DNA was integrated in the genome showed poor clinical outcome in the latter group [20].

1.4 HPV immune response

The healthy human immune system is generally capable of taking care of HPV infection. According to Sasagawa et al. 80% of all women become infected with HPV during their life time, however HPV is not detectable in 90% after 3 years [21]. Cancer development from HPV infection takes at least 10 years since accumulation of mutations due to accelerated cell proliferation related to E6 degrading p53 and E7 inhibiting pRb takes time [22]. When enough mutations have accumulated, the lesions progress into malignancy. Both the unspecific innate immune system and the virus specific adaptive immune system appear to be disturbed in prolonged HPV infection.

1.4.1 The innate immune system

The innate immune system is responsible for a general, quick defense against pathogens. HPV exhibits various strategies for evasion of the innate immune system. HPVs lifecycle is entirely confined to keratinocytes; thus, no viremia is induced which implies that immune cells from the blood stream do not come into contact with the virus [23]. The release of HPV particles only takes place after complete viral assembly in the superficial layers of the epithelium as far away as possible from immune cells in the submucosa. Keratinocytes are left intact during infection which implies that no inflammatory response is elicited [24]. Type 1 interferons (e.g. alpha and beta) are normally released by virus infected cells as part of initial defense [24]. Cells infected by HPV 16 and 18, however, suppress expression of interferons through E6 and E7 proteins [10]. NK cells have the capacity to kill virus infected cells. This is regulated by various receptors, one of which is the immune surveillance NKG2D receptor. This receptor mediates cytotoxicity through binding major histocompatibility complex (MHC) class I chain- related A and B (MICA/B) proteins on stressed cells [25]. Both MIC A and

B, as well their receptor NKG2D, are affected in HPV infection [26]. These invasion strategies delay activation of the adaptive immune system [27].

1.4.2 The adaptive immune system

The adaptive immune system is specialized and consists of humoral immunity (B-cell mediated) and cellular immunity (T-cell mediated) [28]. There are two types of T cells, T helper cells ($CD4^+$ cells) and cytotoxic T cells ($CD8^+$ cells). T cells are activated by binding cell surface receptors called major histocompatibility complex (MHC). MHC class I proteins are recognized by $CD8^+$ cells, whereas MHC class II proteins are recognized by $CD4^+$cells. T helper cells are further differentiated into Th1 and Th2 cells, Th1 cells produce proinflammatory cytokines (such as interferon-γ (IFN-γ)) and mediate cellular immunity whereas Th2 cells produce cytokines (e.g. interleukin 4, 5 and 10) that are involved in B-cell proliferation and antibody class- switching and thereby mediate humoral immunity [28]. In anogenital warts, regression has been associated with expression of CD4+ Th1 cells [29]. In cervical cancer [30] as well as condylomata accuminata [31] and RRP [32] on the other hand a CD4+ Th2 cell response can be seen. Th1 cells release cytokines that stimulate cellular immunity, whereas Th2 cells cytokines induce humoral immune responses [28]. Regulatory T cells, Tregs, are CD4+ cells that attenuate the immune response so that the immune system does not become overactive [33]. High numbers of Tregs have been found in cervical cancer [34] as well as cancers in the head and neck [35].

1.5 Hyaluronan

Hyaluronan (HA) is abundant in the extracellular matrix, especially in cartilage, umbilical cord, dermis, vitreous body and lamina propria of the vocal folds (VFs) [36-38]. Hyaluronan (HA) differs from other glycosaminoglycans being the only one that is not sulfated and not bound to a core protein. Due to its negative charge it can retain large amounts of water which is essential for its viscoelastic properties.

The molecular mass of HA shows great variation which is important since HA's function depends on its molecular mass [39]. High Molecular Weight HA (HMW-HA), which is commonly defined as >1000kD [40] has been attributed with anti-inflammatory [41], anti-angiogenic [42] and immunosuppressive [43] functions and is important in wound healing, whereas fragmented Low Molecular Weight HA (LMW-HA) has been found in pathological conditions such as inflammation and cancer [39, 44]. Definitions of HA size varies in literature, one definition is that LMW-HA commonly is smaller than 200kDa, whereas HMW-HA ranges from 1000 to 8000kDa [38].

Hyaluronan interacts with a number of cell surface receptors such as CD44, RHAMM (receptor for hyaluronan-mediated motility expressed protein) and LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1) of which CD44 is the main receptor [45]. CD44 is expressed on most cells [46] and apart from hyaluronan, it interacts with other molecules such as collagen, fibronectin, fibrinogen, laminin, chondroitin sulfate and osteopontin [47]. Binding is commonly preceded by an external stimulus [47]. Three states of CD44 activation have been described: 1. Active CD44, that binds HA, 2. Inducible CD44, that does not bind HA unless external stimuli are present and 3. Inactive CD44, that does not bind HA despite presence of inducing factors [48]. LMW-HA binding to CD44 is associated with inflammation, angiogenesis and cancer [49].

1.6 Diseases studied in this thesis

1.6.1 Recurrent respiratory papilloma

RRP is a benign disease characterized by wart-like lesions in the upper airway that most commonly arise on the vocal folds (VFs) (Fig. 2), thus hoarseness is the most common presenting symptom [50]. Lesions can, however, even expand to supraglottic and subglottic regions [51-53]. Human papillomavirus genotypes 6 and 11 are commonly associated with RRP [50].

Fig. 2.A. Normal anatomy of the larynx. Fig. B. RRP on the vocal folds and false vocal folds as well as on the mucosa over the arytenoid cartilage

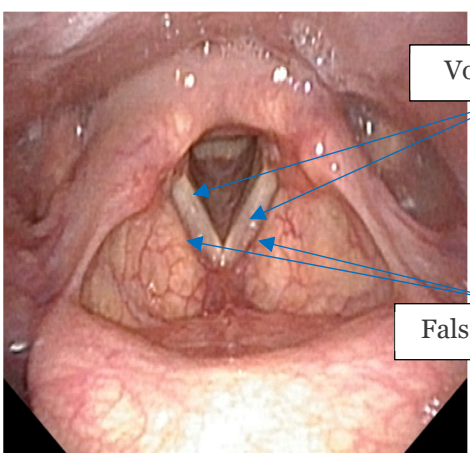


Image courtesy of Anders Erlandsson, MD with permission of the author whose larynx is depicted

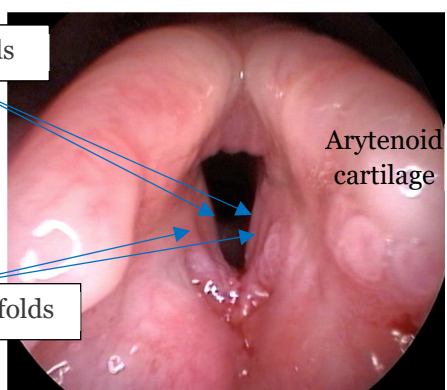


Image courtesy of Katarina Olofsson, MD,PhD with kind permission of the patients guardian (granted 190123)

Epidemiology
There are two different forms of RRP that are separated by age of onset, juvenile onset RRP (JoRRP) and adult onset RRP (AoRPP). AoRRP affects adults at a rate of 0.54-1.8/100.000 whereas JoRRP affects children at a rate of 0.17-4.3/100.000 [54, 55]. When studying these numbers, it has to be kept in mind that cut-off ages between JoRRP and AoRRP differ in literature, Omland et al. defined JoRRP as age up to 17 years [55], whereas Lawson et al. and Derkay et al. set their cut- off at 12 years [56]. AoRRP patients are reported to have more lifetime sexpartners and a higher frequency of oral sex than controls, which could imply that transmission of HPV occurs via oral-genital contact [57]. Prevalence of condylomata accuminata has been found to be the largest risk factor for developing JoRRP, Caesarian section, however, was not shown to be protective against JoRRP [58].

Clinical characteristics
Due to the location in the upper airway and most predominantly on the VF, patients present with hoarseness and in severe cases airway obstruction [50]. The course of the disease is variable, with some patients' lesions undergoing spontaneous regression, whereas other patients need repetitive surgical debulking. Presence of HPV genotype 11 rather than 6 has been associated with more aggressive disease [59, 60]. Dedo et al. described occurrence of malignant conversion in 1,6% of cases [53], whereas Karatayli-Ozgurzoy et al. found dysplasia or carcinoma in situ in 10% of AoRRP cases and invasive carcinoma-ex-papilloma in 5% of both AoRRP and JoRRP cases [61].

Treatment
The treatment of choice for removal of the RRP is surgery. Commonly used surgical instruments are laser, microdebrider and cold instruments. Laryngeal surgery implies risks such as laryngeal scarring, vocal fold injury or formation of synechiae that further compromise voice quality, especially when formed in the anterior commissure [62]. Adjuvant treatments, such as antivirals (e.g. Cidofovir [63]), interferons (e.g. interferon alpha [64, 65]) and monoclonal antibodies (e.g. bevacicumab [66]) have also been studied.

1.6.2 Schneiderian papilloma
The epithelial lining of the nose is called Schneiderian epithelium. This epithelium can give rise to three different types of Schneiderian papilloma: Sinonasal inverted papilloma (SIP), exophytic (fungiform) papilloma and oncocytic (cylindric cell) papilloma. The cause of these papilloma is largely unknown, though HPV has been found at a rate of 37,8%, 65,3% and 22,5%, respectively [67].

Epidemiology
SIP is a rare disease that affects men more frequently that women [68] and is most common at an age between 50 and 60 years [69, 70]. Despite its benign histology, it has a tendency to grow aggressively and to recur upon surgical removal [70]. Synchronous as well as metachronous malignancy has been reported [71].

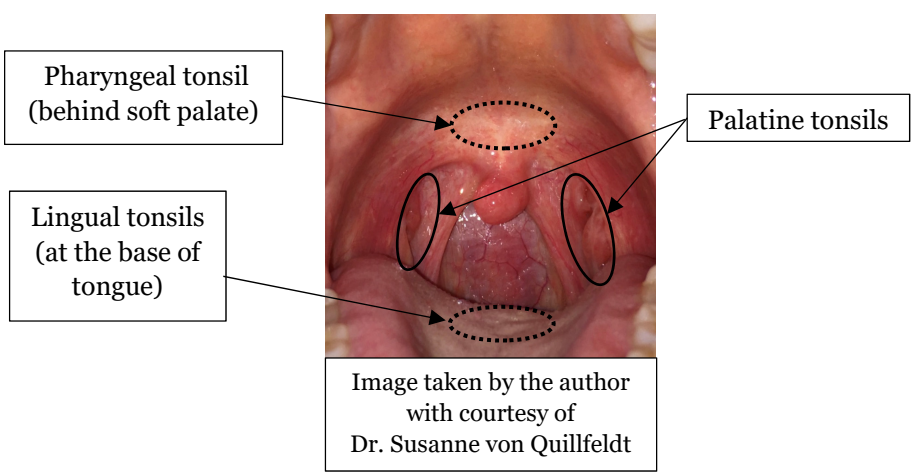
Clinical Characteristics
Patients with SIP commonly present with unilateral nasal blockage, nosebleeds [68] or epiphora [72] if the lacrimal duct is affected by the papilloma. Upon inspection, inverted papilloma often appear firm and white, in contrast to the ordinary translucent and soft nasal polyps that most commonly occur bilaterally [71].

Treatment
SIP is treated surgically, nowadays mostly with endoscopic techniques that are, depending on location and extend of the lesion, combined with open surgery if needed [70]. In order to reduce risk of recurrence or metachronous malignancy it is important to remove the complete lesion especially including the origin of the papilloma [73].

1.6.3 Tonsillar disease

Palatine tonsils are part of Waldeyers lymphatic ring in the pharynx and are as such part of the immune system [74] (Fig. 3.).

Fig. 3. Normal anatomy of the Waldeyer ring.



Benign tonsillar disease

Tonsillar hypertrophy

Tonsillar hypertrophy is especially common in children and can lead to obstructive sleep disordered breathing (SDB), dysphagia, and resonance changes. SDB is a clinical diagnosis, when confirmed with polysomnogram it is defined as obstructive sleep apnea (OSA) [75]. Adeno/tonsillotomy or tonsillectomy (partial or complete surgical removal of palatine tonsils) is recommended as first-line treatment for OSA in children and adolescents with adeno/tonsillar hypertrophy whereas continuous positive airway pressure is recommended if adeno/tonsillotomy or tonsillectomy is not performed or if OSA persists postopertively [76].

Chronic/recurrent tonsillitis

Chronic tonsillitis is defined as infection or inflammation of the palatine tonsils for at least 3 months. Various factors can lead to chronic tonsillitis, such as: viral infections, bacteria and gastroesophageal reflux. Even asthma and allergies have been discussed as possible causes [77]. It can be treated operatively or conservatively (supportive care/antibiotics). Tonsillectomy (complete surgical removal of the palatine tonsils) can be considered if symptoms recur and chronic infection is the most common indication for tonsillectomy in adults in the United States of America [78]. Different tonsillitis recurrence rates have to be met in order to qualify for surgical removal of the palatine tonsils according to various guidelines. One of which is the Paradise guideline, where recurrence rates that qualify for surgery are as follows: at least seven episodes of tonsillitis in 1 year, at least five episodes of tonsillitis in each of two preceding years or at least three episodes of tonsillitis in each of three preceding years [79]. An episode of tonsillitis is defined by the clinical symptom of sore throat that is accompanied by at least one of the following symptoms: fever (temperature >38,3°C), cervical adenopathy, tonsillar exudate and positivity of group A streptococcus [79, 80].

Tonsillar cancer

There are two major groups of tonsillar cancer: HPV negative and HPV positive tonsillar cancer. Smoking and alcohol are considered traditional predisposing factors for tonsillar cancer [81] but the WHOs international agency for research against cancer also recognized the association between HPV and oropharyngeal squamous cell cancer in 2007 [82]. There are some major differences between HPV positive and HPV negative tonsillar cancer. HPV negative cancer origins from the surface epithelium rather than the tonsillar crypts [83], affects mostly older men and survival is poorer than in the HPV positive counterpart [84]. There is, however, a subgroup of HPV-positive cancer in the head and neck in which survival is as poor as in HPV-negative cancer. In these patients, HPV has been found to be integrated into the genome instead of being located episomally [20].

1.7 Vaccine

Since 2006, two vaccines against certain HPV genotypes are available: the quadrivalent Gardasil (Gardasil/Silgard®, Merck & Co., Whitehouse Station, NJ USA) and the bivalent vaccine Cervarix (Cervarix®, GlaxoSmithKline Biologicals, Rixensart, Belgium) [85]. In 2014, a nine-valent Gardasil (Gardasil 9®) was approved by the FDA [86].

All three vaccines have the same mechanism of action in common. Virus-like-particles (VLP) of HPV 16 and 18 are included in Cervarix, whereas the quadrivalent Gardasil additionally includes HPV 6 and 11.

In Gardasil, recombinant HPV 6, 11, 16 and 18 L1 protein VLP are individually produced in a *Saccaromyces cervisiae* expression system [87]. Cervarix HPV 16 and 18 VLP are made in insect cells infected with L1 recombinant insect virus vectors [88]. Both vaccines have included aluminium salts to increase immunogenicity, Cervarix, however has a toll-like receptor 4 agonist (monophosphoryl lipid A) included as well [89].

Both vaccines rely on L1 VLP. This implies that no replicative HPV enter the body of the recipient. As a result, the recipients immune system is stimulated by the high doses of L1 VLP and antibodies are produced without risking actual HPV infection [90]. Vaccination against HPV is regarded as safe [91] and efficient [92, 93].

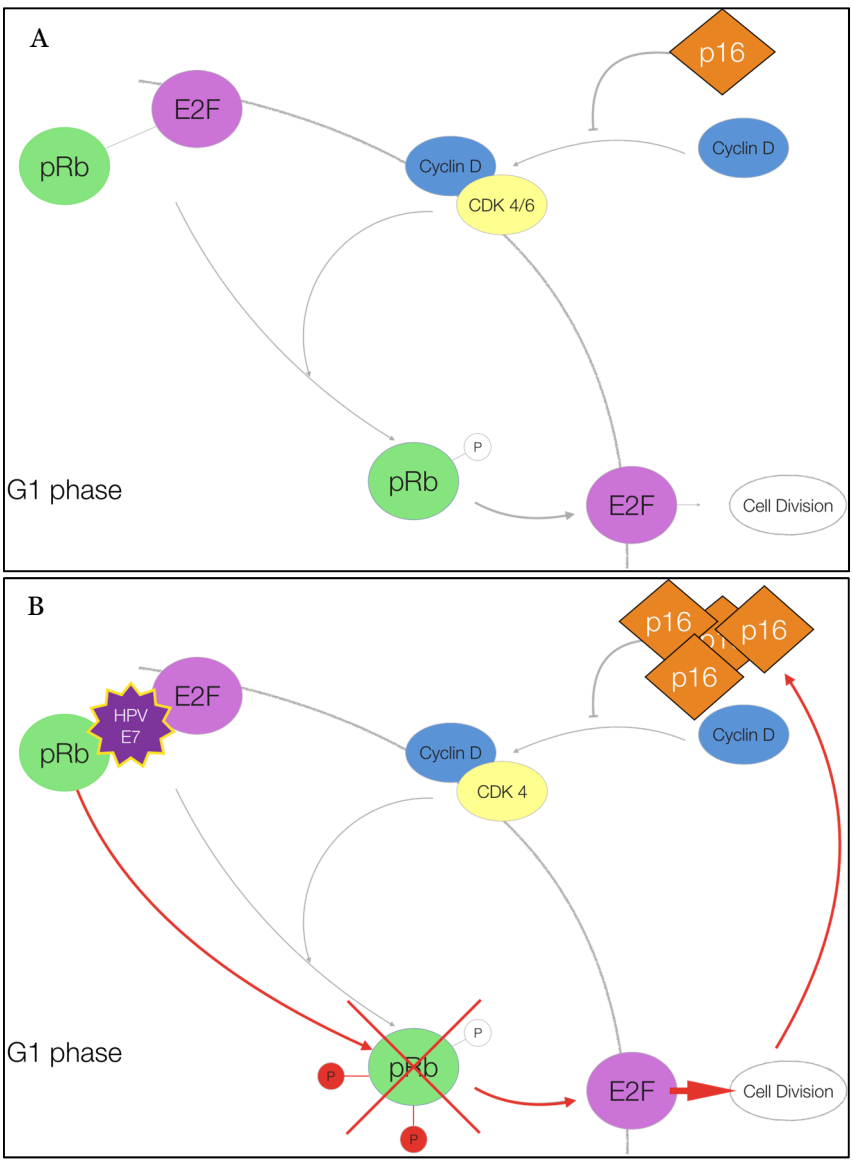
1.8 Tumor suppressor protein p16

1.8.1 p16 in normal cells

P16 is encoded by the CDKN2A gene and functions as a tumor suppressor protein in healthy cells. Progression from G1 to S phase leading to cell division takes place when Retinoblastoma protein (pRb) is phosphorylated and releases transcription factor E2F. Phosphorylation of pRb is initiated by D type Cyclins binding to their Cyclin dependent Kinases 4 and 6. P16 is capable of inhibiting Cyclin D from binding its CDK4/6 [94, 95]. As a consequence, pRb remains in its unphosphorylated state, bound to E2F, and the cell does not progress to S phase (Fig. 4.A.).

When cells are exposed to stressors such as DNA damage or oncogenic signals, cell division is inhibited via p16 pathway. When stressors prevail and p16 levels are high for longer stretches of time, cells enter cellular senescence, which implies irreversible cell cycle arrest [96].

Fig. 4.A. p16 in normal cells: here p16 is capable of suppressing abnormal cell division by inhibiting Cyclin D from binding its cyclin depending kinase. Transcriptions factor E2F stays bound to pRb and thus no cell division takes place. Fig. B. p16 in hr-HPV: here HPV E7 protein binds to pRb, which leads to phosphorylation and degradation of pRb. E2F is released, which promotes cell division. P16 tries to regulate cell division via inhibiting Cyclin D from binding the Cyclin dependent kinase but fails since this mechanism is surpassed by HPV E7. Illustration made by the author.



1.8.2 p16 in cancer

Lost p16 function due to point mutation, deletion or promotor methylation can be seen in about 1/3 of all human cancers [97]. However, a study from Gruis showed that homozygotes with absent functional p16 develop normally [98]. This suggests that p16 loss alone does not cause cancer, but other endogenous (e.g. spontaneous mutation) or exogenous (e.g. carcinogens) stimuli are needed.

In other types of cancer, P16 is overexpressed, suggesting that function of pRb is either lost or inhibited. Loss of pRb function due to e.g. point mutations or deletions can be seen in small cell lung cancers, in which p16 consequently is overexpressed [99].

In HPV positive cancer E7 binds to Retinoblastoma protein and inactivates its function. Consequently, transcription factor E2F is released, which leads to elevated cell division. p16 responds to this with overexpression as a part of a negative feedback loop in a futile attempt to inhibit cell division. It remains unsuccessful since its mechanism of action via Cyclin D is surpassed [100] (Fig. 4.B.). According to a consensus paper from 2018 at least 70% of cells in a specimen of oropharyngeal squamous cell carcinoma should be p16 positive to be regarded as a p16 positive sample [101].

1.9 Epstein-Barr Virus

1.9.1 Overview

EBV is a gamma-herpesvirus that is commonly transmitted via the saliva and persistently infects the host [102]. It targets B cells, but most virions do not enter the cell, which implies that EBV on the surface of B cells has access to both epithelial and lymphoid cells [103]. Primary infection is mostly asymptomatic in early childhood but presents as infectious mononucleosis in adolescence [102, 104]. EBV is a common infection, with a prevalence of about 90% in adults [105, 106], and it is thought to primarily infect B cells in the oropharynx [107]. The virus thereafter persists in resting memory B cells [108].

1.9.2 Diseases associated with EBV

Infectious mononucleosis

Patients with EBV associated infectious mononucleosis often present with sore throat, cervical lymphadenopathy, fatigue, upper respiratory tract symptoms, headache, deceases appetite, fever, body aches and sometimes abdominal pain [109]. EBV disease usually resolves when virus specific immunity emerges. Balfour et al. found a mean duration of symptoms of 17,3 days (range 2-66), with longer disease time in patients with higher severity of illness [109]. In rare cases EBV manifests as chronic active EBV, which is characterized by severe illness lasting more than six months with an initial EBV infection, histologic evidence of organ disease (such as hepatitis or pneumonitis) and EBV antigens/EBV DNA in analyzed tissue [106, 110].

Burkitt lymphoma

Burkitt lymphoma is a B cell Non-Hodgkin lymphoma and there are three different types: endemic (occurring in areas where malaria is present), sporadic and immunodeficiency related [111]. Burkitt lymphoma is the most common childhood cancer in areas in which Malaria is present [111, 112]. EBV is detected in the endemic tumors [113] and very high EBV titers can be seen even before the development of disease [114]. However, sporadic Burkitt lymphoma is rarely associated with EBV and immunodeficiency related Burkitt lymphoma in Europeans has been shown to be EBV positive in less than 40% of cases [111]. Endemic Burkitt lymphoma commonly presents in the face or abdomen [115], whereas sporadic Burkitt lymphoma most often occurs in the lymph nodes and abdominal organs [116]. Localized Burkitt lymphoma can be surgically removed and treated with moderately intensive chemotherapy. More advanced disease is treated with more aggressive chemotherapy [111].

Hodgkin lymphoma
Twenty percent of all lymphoma in the western world are Hodgkin lymphoma [102]. EBV can be detected in about 40-60% of all Hodgkin lymphoma in the United States [106], it is however not fully elucidated if EBV plays a causative role in the pathogenesis of Hodgkin lymphoma.

Nasopharyngeal Carcinoma
There are three types of Nasopharyngeal Carcinoma. Type 1 is a keratinizing squamous cell carcinoma whereas type 2 (differentiated) and type 3 (non-differentiated) are non- keratinizing squamous cell carcinoma [117]. Pathmanathan et al. found EBV in all types of nasopharyngeal carcinoma [118] and nearly 100% of anaplastic or poorly differentiated nasopharyngeal cancer contain EBV genomes and express EBV proteins [106]. Thus, it is likely that EBV is a contributing factor in the genesis of nasopharyngeal carcinoma but the etiological relationship has to be studied further in order to be able to draw conclusions.

1.9.3 EBV & HPV co-infection and cancer
Since both HPV and EBV are prevalent, potentially carcinogenic viruses it is possible that they influence each other or that one virus's impact on the immune system facilitates the other virus establishment as a pathogen in the host.

EBV/HPV co-infection has been shown in nasopharyngeal carcinoma but it is not known if the viruses have an impact on one another in carcinogenesis [119]. A study by Atula et al. did not find HPV/EBV co-infection in epithelial tumors of the head and neck [120] whereas Jiang et al. showed co-infection rates of up to 25% and 70% for tonsillar cancer and cancer of the base of tongue, respectively [121]. It has been hypothesized that HPV's oncoproteins could interact with EBV's oncoproteins but this needs to be studied further [122].

2. Purpose and aims

2.1 Purpose

The purpose of this thesis was to contribute to the understanding of diseases associated with HPV in the upper airway, specifically in patients with recurrent respiratory papilloma, sinonasal inverted papilloma and benign tonsillar disease. The general aim was to assess the impact of the immune system, hyaluronan, possible co-infectors and surrogate markers in defined HPV related airway diseases.

2.2 Aims

The thesis addresses four interrelated aims:

1. To increase understanding regarding changes in the immune system in patients with RRP.

2. To assess prevalence of HPV and its surrogate marker p16 in SIP.

3. To investigate if changes in hyaluronan are present in RRP and where hyaluronan and its receptor CD44 are located.

4. To assess prevalence of HPV, EBV and p16 in benign tonsillar disease.

3. Material and Methods

The four studies in this thesis are descriptive, observational, quantitative studies. Patients were included due to specific diseases. In study I, patients were afflicted with and compared to controls.

Table 1. Study designs

	Study I	Study II	Study III	Study IV
Design	Descriptive, observational	Descriptive, observational	Descriptive, observational	Descriptive, observational
Controls	Yes	No	No	No
Disease studied	RRP	SIP	RRP	Benign tonsils
Study participants	16 patients 12 controls	54 patients	24 patients	40 patients
Female patients	4	16	6	19
Male patients	12	38	18	21

3.1 Study I

3.1.1 Study participants

In this study blood samples from 20 RRP patients were compared with 12 age, gender and co-morbidity matched controls. Samples were collected between January 2011 and May 2013. Patients were included as they presented to receive surgical treatment for their papilloma lesions due to breathing or voice related symptoms at the tertiary referral hospital, University Hospital in Umeå, Sweden.

Three of the 20 RRP patients' samples could not be analyzed since the yield of viable peripheral blood mononuclear cells (PMBC) was too low with expected false negative results. One sample was excluded since it belonged to a small child which implies that the immune system is not fully developed. This could have affected the results due to reasons independent of the disease itself. Additionally, a matched control was not available for the child's blood sample. 16 patients with a mean age of 44,6 years (range 12-69 years) remained, 4 patients were female and 12 were male.

3.1.2 Lymphocyte phenotype characterization and cytokine expression

PBMC were isolated from the blood by density gradient centrifugation and used in immunoflow cytometry and real-time quantitative RT-PCR to characterize their phenotypic profile and cytokine mRNA production, respectively. The $CD4^+/CD8^+$ ratio was inverted in the majority of patients, and only few had normal $CD4^+/CD8^+$ ratios. Therefore, when studying the cytokine mRNA profiles by real-time RT-qPCR, we chose to compare two groups of patients - those with with the lowest $CD4^+/CD8^+$ ratio and those with normal/highest $CD4^+/CD8^+$ ratios.

Immunoflow cytometry

Indirect immunofluorescence was used for staining NKG2D receptor and its ligands MIC A/B, all other surface molecules were labeled with direct immunofluorescent staining. Isotype matched irrelevant mononuclear antibodies served as negative controls for nonspecific fluorescence. A minimum of 10.000 events for each marker were collected for analysis.

Separation of lymphocyte subpopulations

Positive selection with immunomagnetic beads coated with specific antibodies was used to separate subpopulations of $CD14^+$, $CD4^+$, $CD8^+$ and $CD56^+$ cells from the PBMC. The separated cells, attached to the beads were washed extensively to ensure that unattached contaminating cells were removed and total RNA was extracted from each subpopulation attached to the beads.

Real time reverse transcriptase quantitative polymerase chain reaction (Real-time RT-qPCR)

A spectrophotometer was used to assess total RNA yield and purity and reverse transcription was performed. A reference gene, 18s rRNA, was included in every real-time RT-qPCR to ensure RNA integrity, efficiency of reverse transcription and cDNA stability. The gene of interest and the reference gene underwent multiplexed detection. Real-Time qPCR was performed for 40 cycles. As a negative control a non-template control was used and a template of PMA/ionomycin- stimulated PBMC was used as positive control to verify that each cytokine was detectable.

3.2 Study II

3.2.1 Study participants

Patients with sinonasal inverted papilloma (SIP) diagnosed between 1984 and 2014 were included in this study. They were identified through ICD 10 codes for inverted papilloma (80530) and exophytic/inverted urothelcellular papilloma

(81210) registered in the pathology register. The latter diagnosis was included since it is also labeled as Schneiderian papilloma which implies that inverted papilloma samples could be present in this group. All diagnoses of samples analyzed in this study were confirmed histopathologically in order to ensure that only SIP samples were included. If malignant conversion could be seen, the sample was excluded as the aim of the study was to address SIP.

54 samples were evaluated with immunohistochemistry for p16 and 53 samples for presence of HPV using PapilloCheck®. In one case, the number on the label on the PCR tube was mixed up and thus excluded from analysis. The number was correct on the immunohistochemistry slide and diagnose confirmed microscopically so it was included.

The majority of patients was male (38/54, 70%) and ages ranged from 18 to 88 years with a mean age of 59 years. Nasal inverted papilloma was diagnosed in 37 of 54 patients whereas the diagnoses 'inverted papilloma of the sinus' and 'inverted urothelcellular papilloma' were registered 24 and 6 times, respectively. Thus, twelve patients had more than one benign diagnose.

Recurrence was defined as presence of two biopsies or more with a registration interval of at least 100 days since patients at our clinic commonly are operated within one month after receiving the diagnosis of inverted papilloma.

To determine if any of the patients' papilloma progressed to cancer, the pathology codes for 'squamous cell cancer of the nose', 'squamous cell cancer of the sinus', 'suspicious of squamous cell cancer of the nose', 'suspicious of squamous cell cancer of the sinus', 'recurrence of squamous cell cancer of the nose' and 'recurrence of squamous cell cancer of the sinus' where searched and matched with our patients with SIP.

3.2.2 Immunohistochemistry for p16

Slides were cut from paraffin embedded samples which a microtome. They were then pretreated with Tris- EDTA and staining was performed. The antibody against p16 was diluted 1:200 and visualized.

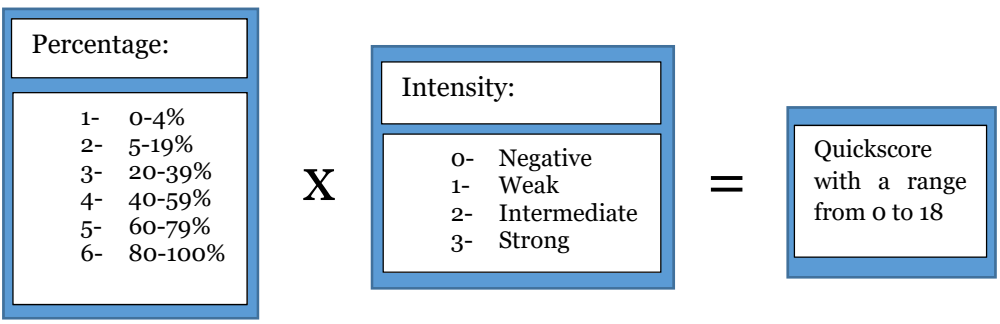
The immunohistochemical slides were evaluated independently by two researchers using a light microscope and online software. After evaluation, results were compared and consensus was reached.

3.2.3 P16 quickscore-system

P16 Intensity and percentage of cells expressing p16 was assessed using the quickscore system by Detre et al. [123]. It consists of a six-level score for

percentage of positive cells and a four-level score for intensity that are multiplied with one another and has been used previously by our group [124, 125] (Fig. 5.). The epithelium and the papilloma were scored separately.

Fig. 5. Quickscore system.



3.2.4 Polymerase Chain reaction

Tissue preparation
The tissue was first de-paraffinized and rehydrated, then dried and incubated with Proteinase K in ATL Buffer. The next day the biopsy samples were incubated again, thereafter a buffer ATL solution and 99,5% ethanol was added. The samples were centrifuged and transferred to the QlAmp MinElute column, washed and then centrifuged. DNA was removed using 100μl ATE buffer.

Spectrophotometric control
Prior to analysis with PapilloCheck®, specimens were assessed regarding quality and quantity of DNA. Quantity was controlled spectrophotometrically and quality based on DNA purity by spectrophotometric analysis of A260/280 ratio.

The degree of DNA fragmentation or integrity was determined by amplifiability of housekeeping β-globulin genes of three amplicon lengths (536bp, 268bp, 100bp). A size of about 350 bp is the minimum limit that can be successfully amplified. Amplification was achieved by general PCR thermal cycling.

PapilloCheck®
PapilloCheck® (Greiner BioOneGmbH, Frickenhausen, Germany) uses multiplex polymerase chain reaction (PCR) with fluorescent primers to amplify DNA fragments within the E1 open reading frame. Amplification of an internal HPV template present in the PCR master-mix leads to a signal on the PCR control spot on the chip. As a second control, used to access cellularity, an internal PCR control which targets a region within the human adenosine deaminase tRNA

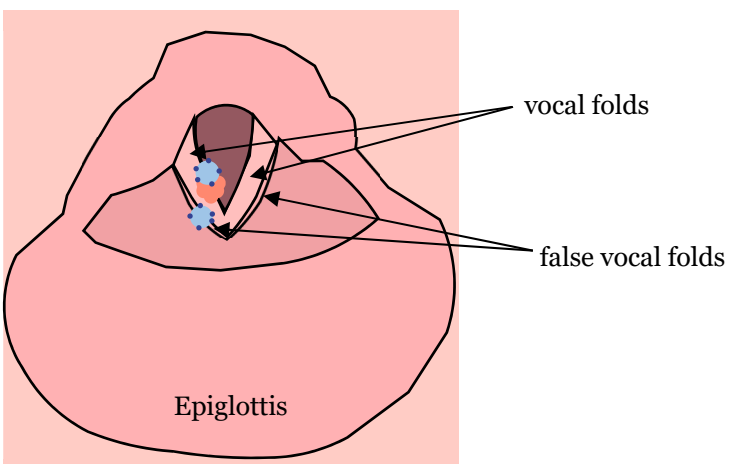
specific 1 gene, is used. PapilloCheck® identifies 18 high risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82) and six low risk types (HPV 6, 11, 40, 42, 43, and 44).

3.3 Study III

3.3.1 Study participants

In this study, all patients who underwent surgery for recurrent respiratory papilloma (RRP) at the University Hospital, Umeå between September 2015 and November 2016 were included after giving informed consent. Twenty-four patients underwent surgery for diagnostic/therapeutic reasons. The patients had a mean age of 40 years, 18 were males with a mean age of 43 years and 6 were females with a mean age of 29 years. Indications for surgery were hoarseness and airway obstruction and obtaining of biopsies for histopathological analysis in patients with macroscopically suspicious lesions. The surgical procedures were performed under general anesthesia. Two samples were obtained for this study: one 1-2mm biopsy from macroscopically unaffected false vocal folds (biopsy 1) and one 1-2 mm biopsy from the true vocal folds (biopsy 2) where apparent RRP lesions could be seen (Fig. 6.).

Fig. 6. Schematic drawing of biopsy sites. The biopsy sites are shown in blue with dotted lines. The RRP lesion is shown in reddish pink on the vocal fold. Illustration made by the author.



Three patients' VFs did not exhibit any macroscopical lesions, subsequently no biopsies were taken. After biopsy acquisition, papilloma were treated with laser

by Dr. Katarina Olofsson (phoniatrician). Twelve patients' samples were randomly assigned to be studied with HC staining for hyaluronan and IHC staining for CD44. The other 12 samples were studied with gas electrophoresis molecular mobility analyzer (GEMMA). The three patients whose VFs were not biopsied were assigned to the GEMMA group which implies that only nine vocal fold specimens but 12 false vocal fold specimens underwent evaluation with GEMMA. The biopsies were frozen at -80°C directly after acquisition.

3.3.2 Preparation for Immuno/histochemistry

Samples were fixed in buffered formaldehyde (4%) at room temperature prior to embedding in paraffin. The paraffin embedded samples were then cut in sections, mounted on slides and dried at 37°C overnight.

3.3.3 Histochemistry for hyaluronan

After deparaffination in xylene, slides were rehydrated, washed and incubated in a solution of 3%H_2O_2 in methanol. Slides were then washed in distilled water and in PBS. In order to block unspecific binding sites, the slides were incubated with bovine serum albumin (10mg/ml) for 30 minutes. Streptomyces hyaluronidase specifically degrades hyaluronan and was used for control slides to guarantee specificity.

Both control slides and regular slides were incubated with a HA binding probe thereafter with a Vectastain-Elite Avidin-Biotin complex reagent (Vector Laboratories, Burlingame, CA) and at last with 3,3'-diaminobenzidine (Vector Laboratories). Slides where then counterstained with Mayer's Hematoxylin and coverslipped.

A semiquantitive scoring scale by Opheim et al. [37] was modified slightly and used to evaluate intensity of staining of the slides according to the following grading: 0: no staining, 1: faint staining and 2: intense staining. Epithelium, stroma and papilloma were evaluated separately. Three researchers independently evaluated the stains, thereafter consensus was reached.

3.3.4 Immunohistochemistry for CD44

An automated Ventana Benchmark staining machine (Ventana Medical Systems, USA) was used for staining for CD44. Citrate buffer and EDTA buffer were used for antigen retrieval. Then, the primary antibody was applied. Secondary antibodies, which are part of the AEC Detection kit for Ventana BenchMark XT were used. As a positive control, human skin was used and as a negative control staining without primary antibody was performed.

For CD44, a two-graded scale was used for evaluation of staining in the epithelium, stroma and papilloma: 0: no stain, 1: visible staining. Additionally, it was evaluated if staining was congruent with HA (+ congruent with HA, - incongruent with HA).

3.3.5 GEMMA

GEMMA (gas-phase electrophoretic mobility molecular analysis) utilizes the electrophoretic mobility of molecules in air to estimate the molecular weight of the analyte. A nano-electrospray gas-phase electrophoretic molecular mobility analyzer (GEMMA) (TSI Corp., MN, USA) was used. HA has to be purified prior to analysis in order to avoid analysis of other molecules which would affect results greatly.

Preparation of samples for GEMMA

A modified protocol by Tolg et al. was used to isolate HA from the VF and FVF [126]. The tissue samples were dried using a vacuum rotary evaporator and homogenized manually by grinding. Thereafter, proteins were digested with proteinase K at 55° overnight.

Chloroform was applied to extract HA by liquid-liquid extraction and the aqueous phases were dialyzed against 0.1M NaCl using Amicon Ultra 3K concentration units. Precipitation of HA was achieved in 99% ethanol overnight. Nucleic acids were removed by Benzonase® nuclease and incubation at 37°C for five hours. Thereafter, samples were again dialyzed against 0.1M NaCl using Amicon Ultra 3K concentration units and HA was precipitated in ethanol. Buffer chondroitinase ABC was used to digest chondroitin for 10 minutes in 37°C [127], followed by dialyzing against 0.1M NaCl using Amicon Ultra 3K concentration units and HA precipitation in ethanol. Anion exchange chromatography was used to remove sulfated glycosaminoglycans and other remaining contaminants. This was achieved by loading samples on prewashed anion exchange mini spin columns and centrifuging in order to wash out unwanted molecules based on NaCl-binding. Salt was removed by dialyzing the eluted HA samples against 20mM of ammonium acetate at pH 8.0 in prewashed Amicon Ultra 3K concentration units.

GEMMA analysis

HA samples were pressed through a capillary. At the tip of the capillary they were sprayed into an electric field, thereby electrically charged and turned into aerosols using a 3480 electro spray generator. Aerosols were then separated by a 3080 electrostatic classifier. A 3025A ultrafine condensation particle counter in high flow mode detected HA particles. Each HA sample underwent analysis thrice, thus the distribution spectrum is a sum of the three individual scans.

Molecular counts were calibrated as described by Malm et al. [128]. GEMMA analysis results in values resembling molecular diameter. These values were converted to HA molecular mass standard ranging from 30kDa to 2500kDa. Peak area in GEMMA spectrum in relation to HA concentration was calculated to estimate the relative concentration of different molecular masses of HA. This was calculated by normalizing peak areas under the curve from the GEMMA analysis to the dry weights of the samples. Low-molecular HA was estimated using the 30.6kDa HA standard. Of-note is the fact that the relative amount of HA with a mass less than 70kDa is not comparable to HA with a mass greater than 70 kDa due to the fact that HA with different masses behaves in different ways in the gas phase of the GEMMA analysis [128]. As a negative control, HA was degraded with hyaluronidase (from *Streptomyces hyalurolyticus*) and analyzed with GEMMA. HA from eight healthy skin biopsies was analyzed for comparison to HA from the RRP affected VFs. LMHA was defined as molecular mass ≤50kD and very high molecular HA (vHMHA) was defined as ≥10MDa. These definitions were based on the two observed hyaluronan peaks.

3.4 Paper IV

3.4.1 Study participants

In this study, tonsils from 40 patients, who had undergone surgery in Uppsala during 2014, were subject of investigation. Indications for surgery were tonsillar hypertrophy, chronic or recurrent infection. Thirty tonsils had been removed by tonsillectomy (total removal of tonsils) and ten patients were operated using tonsillotomy technique (partial removal of tonsils). Nineteen left and 21 right tonsils from 40 patients (19 females and 21 males, mean age 16.9 years, range 3-58 years) were included in this study.

Upon removal, the tissue was frozen. The specimens had been used in other studies [129-131] which implied that orientation of remaining material was difficult. Representative parts including both epithelium and lymphoid tissue/germinal centers were dissected for analysis with PapilloCheck® as well as p16 IHC and EBER in situ hybridization. PapilloCheck® and p16 IHC were performed as described in study II.

3.4.2 EBER-ISH

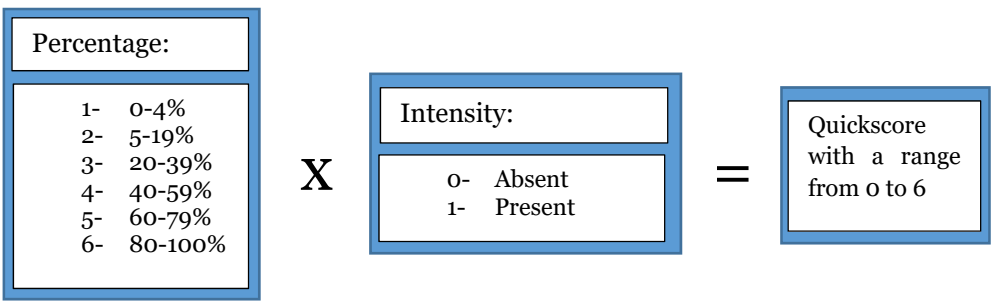
Epstein-Barr encoding region *in situ* hybridization (EBER-ISH) was performed using a commercially available kit.

Two sections were stained. The first one served as a control section to ensure presence of RNA and was for this purpose incubated with RNA positive control

probe. EBER 1 and 2 were traced by the Inform EBER Probe in the second section. The NBT-BCIP Detection System was used for visualization and staining was performed in a Bench Mark Ultra machine following manufacturer's guidelines. Two researchers manually counted EBER positive cells. Numbers were first counted independently and then compared to reach consensus.

There are several definitions regarding positivity of EBER expression. Cut-off levels described for EBV-positivity in diffuse large B-cell lymphoma vary from ≥20% to ≥50% to almost 100% of EBER positive cells [132]. Since no definition or scoring system for lower numbers of EBER expressing cells could be found, the quickscore system by Detre et al. [123] that is described in study II for scoring of p16, was modified and applied to the counted numbers of EBER expressing cells in the sections. The same values for percentage were used, but intensity was not quantified but assessed as present (1) or absent (0) in order to count only true positive, intensely stained, cells (Fig. 7.).

Fig. 7. Adapted quickscore for EBER.



3.5 Methodological considerations

Flow-cytometry
Immunoflow cytometry and fluorescence staining with monoclonal antibodies against phenotypic markers was used for characterizing the phenotypes of the immune cells since it is the method of choice for phenotyping lymphocytes in blood. Immunofluorescent staining is a very sensitive method and the use of monoclonal antibodies ensures the specificity of the staining. Immunohistochemistry could have been an alternative method for staining of lymphocyte markers if RRP tissue and tissue from healthy subjects had been available. Tissue from RRP patients could have been obtained intraoperatively, however, it was not considered ethically acceptable to biopsy tissue from healthy subjects as this could damage or scar VFs and thus impact voice production.

Real-time RT-qPCR
Cytokines can be assessed by mRNA profiles using real-time RT-qPCR or by protein assessment in the peripheral blood using ELISA or multiplex bead analysis by Luminex®. In this study, we chose to measure cytokine mRNA expression levels using real-time RT-qPCR. There are several reasons for that: 1. Chronic, low grade infections, such as HPV infections, rarely produce large enough quantities of cytokines to be present in the blood, to survive the exposure to serum metalloproteinases and be quantifiable by protein analysis; 2. The high specificity, sensitivity and stability of real-time RT-qPCR as a method and our own long term experience was another reason and 3. Another advantage of using quantitative real-time PCR is that not only presence of cytokines can be shown but also their cellular origin.

p16 immunohistochemistry
p16 immunohistochemistry (IHC) is used clinically to assess p16 positivity in e.g. tonsillar cancer but it is also commonly used in research [133]. A disadvantage of IHC can be that visual evaluation of the slides is subjective. In a study from 2017, a quantitative p16 mRNA assay was compared to digital reading of p16 IHC and visual interpretation of p16 IHC. Digital p16 IHC evaluation showed the weakest performance of all tests and visual IHC and p16-mRNA assay performed similarly [134]. P16 ELISA has been studied as an alternative screening method for cervical cancer in Kenya but it was considered to have too low specificity [135]. Considering this, the objective tests (ELISA or p16-mRNA) assessing p16 are either inferior or perform similarly to visual interpretation but the big advantage of IHC is, that it conveys more complex information regarding the location of the targeted molecule.

HPV PCR
Occurrence of HPV is most commonly studied with PCR (polymerase chain reaction). At the hospital in Umeå, Papillocheck® PCR is used due to its high specificity and sensitivity [136] as well as the fact that it is capable of detecting 24 different HPV types and this consideration also led to its usage in our projects.

GEMMA
Gas-phase electrophoretic mobility molecular analysis (GEMMA) is a very sensitive method that can be applied to detect hyaluronan [128]. It was used in our study since the biopsies taken from the VFs and FVFs were very small which implies that techniques such as gel-electrophoresis were considered inadequate.

Immunohistochemistry for CD44 & Histochemistry for HA
In order to visualize where CD44 and HA were situated in the laryngeal samples, immunohistochemistry and histochemistry were performed, respectively. Using

these methods, we were also able to evaluate if there was any co-staining that could indicate binding of CD44 and HA.

EBER-ISH
Epstein-Barr encoding region *in situ* hybridization (EBER-ISH) was applied to visualize EBV in our samples. EBV-PCR had been done previously on our samples and EBER-ISH was now chosen since we not only wanted to know if EBV was present but also how many cells were positive.

3.6 Statistics

Study I
For real-time RT-qPCR results the comparative Ct (ΔΔCt) method was applied for computing relative quantities (RQ) and average RQ (aRQ) were calculated for the four patients with $CD4^+/CD8^+$ ratio <1 and for the four patients with $CD4^+/CD8^+$ ratio >1. Individual fold difference values were calculated by dividing aRQs from the patient group with $CD4^+/CD8^+$ ratio <1 through aRQ values from the patient group with $CD4^+/CD8^+$ ratio >1. Thus, standard error and standard deviation were not applicable for calculation and presentation of the results. Student's T test was used to compare differences and p-values of ≤0.05 were considered significant.

Study II
Generally, descriptive statistics were used. For calculating the association between age and histopathological findings a Pearson's correlation analysis was performed and to describe incidence of lesions during the inclusion period a Poisson regression was used. Statistical analyses were performed using IBM SPSS Statistics software (version 23, NY, USA).

Study III
Differences between VFs and FVFs samples in GEMMA were calculated using Independent Samples Mann-Whitney U test. To estimate differences in variance between groups Levene's Test for Equality of Variances was used. For differences in the IHC groups calculations were performed using Wilcoxon's signed rank test. P-values ≤0.05 were considered significant. Statistical analyses were performed using IBM SPSS Statistics software (version 23, NY, USA).

Study IV
Descriptive statistics were performed. Wilcoxon's signed rank test was used for calculating differences between p16 score in epithelial and lymphatic tissue.

Statistical analyses were performed using IBM SPSS Statistics software (version 23, NY, USA).

4. Ethical considerations

All four studies were conducted in accordance with the declaration of Helsinki. Samples of patients in study I and III were taken after receiving informed consent and with approval from the Regional Ethical Review board in Umeå (Approval numbers 2017-277-31M (2015-323-32M, 2012-379-32M, [2010-277-31M]); *2018-01-29*. Samples for study II were identified via the pathology register, accessed via the BioBank North and studied with approval from the Regional Ethical Review board in Umeå (Approval numbers: 2017-277-31M (2015-323-32M, 2012-379-32M, [2010-277-31M]); *2018-01-29*. Study IV was approved by the Uppsala Ethical Review board (Approval number: 2013/387/2) as well as the Regional Ethical Review board in Umeå (Approval numbers: 2017-277-31M (2015-323-32M, 2012-379-32M, [2010-277-31M]); *2018-01-29*. Studies had permission from the BioBank North 472-13-008, with latest update 2018-03-19.

5. Results

5.1 Study I

Phenotypic characterization of PBMC
Phenotypic characterization of the PBMC showed a higher proportion of $CD8^+$ cytotoxic T cells as well as a higher proportion of $CD56^+$ NK cells in the blood of RRP patients compared to controls. NKG2D receptor expression was elevated and there was a strong tendency for higher CD161 expression in RRP patients. MIC A/B were expressed in 40 % of RRP patients' lymphocytes. Proportions of $CD4^+$ helper T cells, B cells, monocytes and $\gamma\delta$ T cells were unchanged in RRP patients. Due to the fact that proportions of $CD8^+$ T cells were elevated and proportions of $CD4^+$ T cells were unchanged the ratio between $CD4^+/CD8^+$ cells was inverted to $CD4^+/CD8^+<1$. This was seen in 12 of 16 RRP patients. A ratio of >1 has been considered 'normal' in previous studies [137-139] whereas an inverted ratio is a marker for immune dysfunction [140].

Cytokine mRNA expression
Cytokine mRNA expression in four patients with the lowest inverted $CD4^+/CD8^+$ ratios were compared with cytokine mRNA expression in four patients with normal $CD4^+/CD8^+$ ratios. Cytokine expression levels in patients with normal ratio were used as reference =1. The cytotoxic response was downregulated in patients with inverted $CD4^+/CD8^+$ ratios. Th1 cytokines IFN-γ and IL-15, pro-inflammatory cytokines IL-1β, IL-6, TNFα and TNFβ/lymphotoxin were downregulated in the group with inverted $CD4^+/CD8^+$ ratios. These cytokines are important in clearing viral infections. IL-2 was reduced in all lymphocyte subpopulations except for NK-cells.

5.2 Study II

HPV in sinonasal inverted papilloma
Analysis with PapilloCheck® was performed in 53 samples, in 15 of these, DNA was fragmented and analysis failed. In the remaining 38 samples HPV 11 was found in 2 samples (2/38, 5%) and no other HPV type was found. The remaining 36 samples were negative for HPV.

p16 in sinonasal inverted papilloma
p16 IHC was performed in all 54 samples. P16 was expressed in the inverted papilloma parts of the sections in 52 cases (mean quickscore 7,17) and in the epithelium (mean quickscore 6,65) in all 54 samples. In 38 patients with successful HPV analysis p16 was present in the epithelium in all lesions and in

the inverted papilloma parts of the sections in 37 cases. Mean scores for p16 in the HPV negative samples were 7.25 and 6.65 in inverted papilloma lesions and epithelium, respectively. Mean scores in the two HPV positive samples were 3.00 and 6.00 for inverted papilloma lesions and epithelium, respectively.

p16 in regard to age
Patients ages were settled in 53 cases, in these the Pearson r for the two variables age and p16 in inverted papilloma was 0.13 (*p=0.367*) and p16 in the epithelium was -0.03 *(p=0.837)*.

Recurrence rates
19 of 54 patients experienced recurrence according to the definition decided for this study (at least 2 inverted papilloma diagnoses with at least 100 days apart).

Synchronous and metachronous malignancy
Synchronous sinonasal squamous cell carcinoma (SCC) was seen in one patient with inverted papilloma of the nose and sinus. One patient was synchronously to the diagnosis of inverted urothelcellular papilloma diagnosed with a histology 'suspicious of SCC' and carcinoma was later confirmed. One patient with inverted urothelcellular papilloma of the nose was synchronously diagnosed with nasal SCC. The biopsy of one patient with inverted papilloma of the nose and sinus and inverted urothelcellular papilloma was suspicious of SCC but no confirming diagnose was registered.

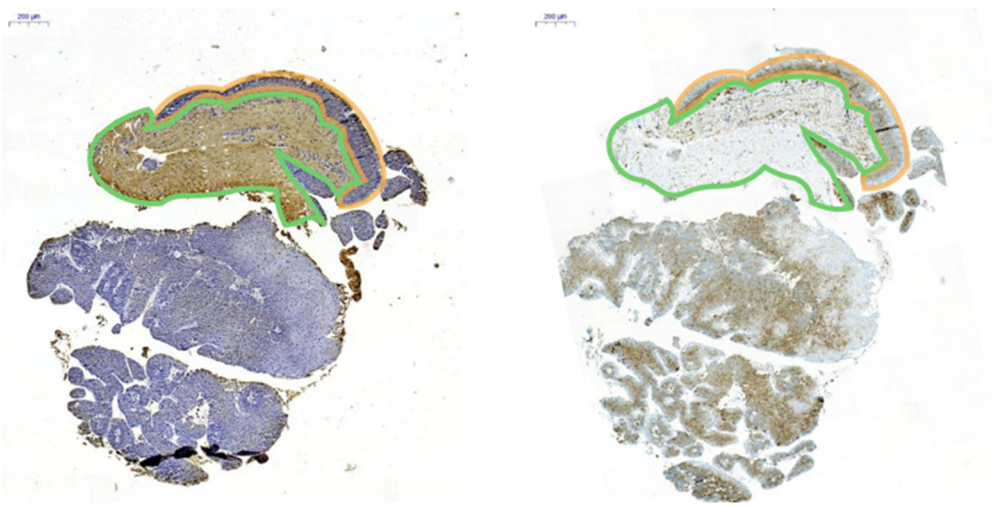
SIP incidence over time
Apart from the fact that six samples had been transferred from Norrbotten to Umeå for second opinion, information regarding patients' residence was not available since permission to assess patients' medical journals was not included in our ethical approval at that time. In order to be able to make an assumption regarding SIP incidence over time it was decided to assume that patients were admitted to the tertiary referral hospital, University Hospital in Umeå from all four northern counties (Västerbotten, Västernorrland, Norrbotten, Jämtland) and that the per capita rate in these counties are equal. Thereafter, Poisson regression was performed with time as covariate and using the population of Västerbotten as an offset. Based on this the incidence rate was estimated to increase with 7.6%/year (approximate 95% confidence interval: 4.7%-10.6%).

5.3 Study III

Hyaluronan and CD44 distribution in the tissue
Staining for HA was successful in the FVFs in 11 samples and in all VFs samples (n=12). IHC succeeded in ten VF and FVF samples. Faint HA staining was seen

in the epithelium of the FVFs in one of 11 stains and in the epithelium of the VFs in two of 12 stains. HA staining was more prevalent in the stroma: in ten of 11 FVF samples and in ten of 12 VF samples HA staining was seen (Fig. 8.), mostly with faint intensity and in occasional cases with intense staining. RRP lesions were seen in five of 11 macroscopically unaffected FVF samples. In one of these, faint HA staining was seen. In the VF samples, microscopic papilloma lesions were present in seven of 12 cases and HA staining could be shown in two of the seven. CD44 was seen in the epithelium, stroma and in papilloma lesions in all samples but it did not co-stain with HA (Fig. 8.).

Fig. 8. Histochemistry for Hyaluronan (left) and Immunohistochemistry for CD 44 (right) staining in the vocal fold of one sample. The epithelium is outlined in orange, the stroma in green and the remaining tissue resembles papilloma.



Molecular mass of Hyaluronan

Analysis with GEMMA succeeded in 8 of 9 VF samples and in 4 of 12 FVF samples. The remaining samples did not contain enough HA to be analyzed. A small amount of Low-Mass HA (LMHA) was detected and there was no difference between FVFs and VFs. Two large peaks were seen in the GEMMA curve in the high mass region that ranged from 50kDa to very High-Mass HA (vHMHA) (>10MDa). Levels of High-Mass HA (HMHA) were comparable in VFs and FVFs. Regarding vHMHA, the amount varied more among VF samples and was in some samples larger than in FVFs ($p=0.046$). The means for the groups did not differ ($p=0.808$).

5.4 Study IV

HPV in benign tonsils
Analysis with PapilloCheck® did neither detect presence of the analyzed 18 defined high-risk HPV nor the six low-risk types.

p16 in benign tonsils
p16 was found in the epithelium in all samples and in the lymphatic tissue in 92.5% of all samples. P16 quickscores ranged from 1 to 9 in the epithelium with a mean of 3.2, whereas p16 quickscore ranged from 0 to 4 in the lymphoid tissue with a mean of 1.1. Calculation with Wilcoxon signed ranks test revealed a significant difference between p16 quickscores in the epithelium and the lymphoid tissue ($p=<0.001$).

EBV in benign tonsils
Control slides showed presence of RNA in all samples. At least 1 EBER+ cell could be seen in 65% of samples. The largest amounts of positive cells were seen in a 4-year-old girl with tonsillar hypertrophy (76 cells) and a 22-year-old male with tonsillar hypertrophy (~850 cells). EBER quickscores were low and ranged from 0 to 2, with a mean of 0.7.

6. Discussion

The role of human papillomavirus in the development of both benign and malignant diseases encountered by the otorhinolaryngologist has been studied extensively and is still subject of investigation. This can be attributed to the fact that these diseases imply extensive morbidity but also mortality to the patients suffering from for example recurrent respiratory papilloma or tonsillar cancer.

The purpose of this thesis was to improve the understanding of HPV, EBV, p16 and hyaluronan in upper airway mucosa in benign (benign tonsils) and semimalignant conditions (SIP and RRP). The latter two, as well as tonsillar cancer, mainly affect men, who are not included in the general vaccination program in Sweden as of today. If SIP and benign tonsillar disease were associated with HPV, disease in the general population could be prevented with vaccinations in the long term. Additionally, staff involved in the surgical care-taking of patients with HPV infected airways could be vaccinated in order to minimize the risk of HPV infection.

The thesis addresses four interrelated aims: Firstly, to increase the understanding of changes in the immune system in patients with RRP. Secondly, to assess the prevalence of HPV and its surrogate marker p16 in SIP. Thirdly, we aimed to investigate if changes in hyaluronan are present in RRP and where hyaluronan and its receptor CD44 are located. Finally, we aimed to assess prevalence of HPV, EBV and p16 in benign tonsillar disease. These objectives are discussed under coming headings.

6.1 Recurrent respiratory papilloma

RRP is commonly caused by HPV 6 and 11 and most often affects the VF itself [50]. Factors that could influence development and/or persistence of RRP lesions were reported in study I and III.

An inverted $CD4^+/CD8^+$ ratio was seen in 12 of 16 patients. Generally, an inverted $CD4^+/CD8^+$ ratio can occur when cell death of $CD4^+$ T cells occurs, when $CD8^+$ T cells are overexpressed or due to a combination of the two conditions [139]. Inverted $CD4^+/CD8^+$ ratios can otherwise be seen in HIV patients [141] as well as in immunosenescence [142]. In our patients, the proportion of $CD4^+$ cells was close to normal, whereas the proportion of $CD8^+$ cells was elevated which could indicate an insufficient T-helper cell response. Previous studies by Bonagura et al. did not find inverted $CD4^+/CD8^+$ ratios in their patients with RRP [32, 143] and 4 of 12 patients studied in our study had $CD4^+/CD8^+$ ratios >1. It can be theorized that $CD4^+/CD8^+$ ratios change during the disease course, however, to

our knowledge, there are no studies available that assessed $CD4^+/CD8^+$ ratios over time.

NK cell numbers were elevated and a significantly increased expression of activating NK cell receptors NKG2D and CD161 was seen. These receptors are expressed on both NK cells and T cells [25, 144-146]. MIC A and B, ligands of NKG2D receptor [147], make cells potential targets for NK cells as they tag cells with damaged DNA. MIC A and B were seen in a large proportion of PBMC which labels these cells for recognition by NK cells. It can be speculated that MIC A/B expressing $CD4^+$ were subjected to NK cell attack which then led to the inverted $CD4^+/CD8^+$ ratio. Abnormally high expression of MIC A/B leading to dysregulation of NK cell response has previously been shown in infections [148, 149], autoimmune disease [150-152] and cancer [153, 154]. Elevated MICA expression has previously been reported in HPV infected cell lines [155], thus HPV infection could play a role in the elevated MIC A/B expression.

Cytokine mRNA profiles for monocytes, $CD4^+$ cells, $CD8^+$ cells and NK cells were depressed when comparing patients with $CD4^+/CD8^+ <1$ to patients with normal $CD4^+/CD8^+$ ratio (>1). NK cells further showed downregulated IFNγ mRNA expression but upregulated expression of immunosuppressive cytokines. This suggests a depressed cytotoxic function and T-helper cell function as well as inhibition of the NK-cell cytokines responsible for promoting Th1 response rather than Th2 response.

Both a peak resembling regular High-Mass HA (HMHA) and a peak resembling very HMHA (vHMHA) was found in VFs and FVFs in patients with RRP. The amount of vHMHA showed larger variability in VFs compared to FVFs. vHMHA is normally not present in humans but it has previously been shown in the naked mole rat, a rodent that is famous for its cancer resistance [156]. The origin of this vHMHA in our patients is not clear, however, the fact that a clearly distinguished vHMHA peak can be seen, indicates that a specific mechanism lies behind this phenomenon. If the distribution of vHMHA had been more even it could have been regarded as random but the clear peak seen makes cross-linking with another specific molecule, with HA itself or HA entanglement more likely. One molecule that could be involved in cross-linking is inter-α-inhibitor, a plasma protein that has been shown to form complexes with HA in inflamed tissues, and it is possible that inter-α-inhibitor has anti-inflammatory properties [157]. Complex-formation is mediated by tumor necrosis factor stimulated gene 6 (TSG 6) [158] whose expression can be induced by TNFα. This suggests that complex formation could be influenced by anti-TNFα drugs (such as infliximab or adalimumab). It can also be speculated that vHMHA in patients with RRP prevents cancer formation as it does in the naked mole rat.

The facts that amounts of Low-Mass HA (LMHA) were low in our RRP patients and that histochemistry showed that HA did not co-stain with its main cellular receptor CD44 could be part of the explanation for the non-inflammatory response seen in HPV infections such as RRP [5]. However, it has to be kept in mind that few patients were studied and analysis did not succeed in all samples proposing a cautious interpretation until larger studies have been performed.

6.2 Sinonasal inverted papilloma

Presence of HPV was found in 5% of 38 successfully studied SIP. This number is low, considering a meta-analysis by Syrjänen et al. showed presence of HPV in 37,8% of SIP [67], though numbers of patients studied in the individual reports ranged from 1 to 101 and detection rates ranged from 0 to 100%. Varying geographical locations for the studies as well as different detection methods could be reasons for the differences. P16 scores found in study II, however, were relatively high. This could be explained by a theory proposed by Lawson et al. [159] in which it is speculated that HPV induces the lesions but cannot prevail within them. However, p16 can also be elevated due to factors independent of HPV such as loss of pRB in tumors [100]. It has to be considered that p16 is a tumor suppressor protein and elevated expression in a condition that has the potential to malignify could be protective. In fact, Lin et al. [160] showed p16 positivity in benign SIP in 64% but only 14% in SIP with carcinomatous degeneration which implies that p16 is working as designed in the non-malignant lesions, protecting the host from malignant conversion. It can be concluded that p16 cannot be used as a marker for HPV in SIP. Larger prospective studies on fresh frozen samples as well as analysis of p16 in normal mucosa are needed to gain more insight into these questions.

6.3 Non-malignant tonsillar disease

Analysis with Papillocheck® did not reveal presence of HPV in benign tonsils; this finding is in line with a large previous study [161] and could indicate that there is no precursor for malignant tonsillar disease in contrast to cervical cancer development. Other groups found presence of hr-HPV and lr-HPV in benign tonsils in 12,5% and 15%, respectively [162]. HPV presence in mouth washes (10,3%) has been compared to HPV presence in tonsillar specimens (0%) [163] in the same patients. The differences found could be explained by transient HPV infection as it is possible that the immunologic activity present in the tonsils clears most infections. Moreover, most studies only analyze one tonsil, and Rusan et al. found presence of HPV in one tonsil but not the other in the one patient in which HPV was found [164]. Compared to tonsillar cancer [165], low p16 quickscore values were seen in the lymphoid portions of the specimens in 92,5% of cases and in the epithelium of all specimens. These values could resemble

normal activity of p16 in the tonsillar tissue that is incessantly exposed to cellular stress induced by viruses and bacteria. Klingenberg et al. showed presence of p16 in 28% of cases whereas only 1 was positive for HPV [166] and Quabius et al. showed 73% weak and 18% moderate epithelial p16 staining with no HPV positive case [167]. As scoring systems vary in literature it is difficult to compare p16 values among studies. At least one EBER positive cell could be seen in 65% of cases which is in line with previous studies that found EBV DNA via PCR in tonsils in 43-80% of cases [168-170]. EBER was used as a method in order to be able to quantify the number of EBV infected cells in the slides. EBER gene transcription is reduced when EBER switches from latent infection to lytic replication [171]. Two patients showed larger numbers of EBER positive cells (76 and ~850 cells) which could indicate switching into latent infection.

Larger studies are needed to address the question when EBV and HPV infections occur. Most patients in Sweden undergo tonsillectomy or tonsillotomy at a younger age (mean age: 13,3 [172] which implies that samples across all relevant ages can be difficult to obtain.

6.4 Limitations of the studies

Several limitations need to be considered when interpreting the results of the studies.

RRP and SIP are rare diseases and thus only a limited number of patients was studied. This implies low statistical power, which is defined as a reduced likelihood that the obtained significance is a true effect. As a consequence, the results can be difficult to be reproduced.

Another limitation is that three of four studies lack controls: In study I, blood samples were studied. Thus, it was possible to obtain matched controls. However, for study II-IV controls could not be obtained. It is difficult and unethical to acquire tissue from healthy vocal folds since this traumatizes the tissue and could ultimately lead to scarring of the vocal folds and negative consequences for voice function. It would be possible to obtain tissue from deceased and this option was considered, however, it would still be difficult to match controls and to be certain that control tissue was not altered due to the fact that it was non-viable. Ethical permission to obtain healthy nasal mucosa had not been received at the start of study II. However, we are currently planning a larger, prospective, multi-center study assessing SIP that includes controls in the form of healthy nasal mucosa. Completely healthy tonsillar tissue, that has not been exposed to viral or bacterial antigens unlikely exists and if it did, it also would be difficult to obtain.

6.5 Future perspectives

Since RRP and SIP are rare diseases, larger, multi-center studies are needed to increase the number of patients studied and thus increase statistical power.

We are currently planning a multi-center study including the University hospital of Uppsala and Umeå as well as the county hospital in Östersund. The aim of the study is to increase knowledge regarding the origin of SIP through study of adenovirus, HPV and EBV as well as p16 in SIP tissue embedded in paraffin as well as fresh-frozen tissue.

In light of HPV vaccine, it would be interesting to study vaccine effects on the incidence of RRP in Sweden, especially considering that incidence of JoRRP in Australia significantly decreased since the introduction of the vaccine [173]. Also, it would be interesting to further study the use of vaccines as adjuvant therapy in the treatment of RRP as a study by Young et al. has suggested that Gardasil vaccine can modulate the severity of RRP and induce remission in some patients [174].

A large multicenter study including all tonsils removed in Sweden, could possibly answer the question when HPV infection in tonsils occurs. This would be particularly interesting, since there is no known precursor lesion for HPV positive tonsillar cancer as there is for cervical cancer. If we knew when HPV infects tonsils options for screening for tonsillar cancer could emerge.

7. Conclusions

The findings demonstrate an immune dysregulation with inverted CD4+/CD8+ ratios compared to healthy controls. Aberrant cytokine mRNA production as well as downregulated T-helper cell response was seen in patients with the lowest CD4+/CD8+ ratio as compared with patients with normal CD4+/CD8+ ratio. The speculative clinical value might be to use these parameters in order to identify RRP patients who are at greater risk for widespread disease and thus in need of a more aggressive local and systemic treatment intervention.

We conclude, that p16 cannot be used as a surrogate marker for high-risk HPV-infection in SIP and that HPV infection rates were low (5%). P16 could, however, be involved in protecting the host from malignant conversion.

We report laryngeal HA distribution in RRP patients mainly in the stroma. CD44 did not bind to HA. The speculative clinical relevance is that the absence of co-expression could explain the non-inflammatory response described in RRP patients. We also present possibly crosslinked vHMHA in both VFs and FVFs, with more variable amounts in VF samples. In clinical practice, this finding could lead to a medical option that counteracts HA crosslinking as a possible treatment option in RRP. It is possible, that the vHMHA enables better voice function despite the RRP lesions as viscoelastic properties are improved.

We report absence of HPV in non-malignant tonsils, whereas EBV EBER DNA was present in 65% of cases and p16 was expressed in low numbers. The clinical value of the study outcome is the reproducibility of previous studies reporting no precursor lesion for tonsillar cancer caused by HPV [161]. The low amount of p16 expression could resemble normal p16 activity in the tonsillar tissue that is continuously exposed to viral and bacterial antigens.

8. Acknowledgements

In a way research is just like one of my favorite past-time activities – SCUBA diving. At first someone needs to catch your interest and make it sound like something you would like to try. In my case that is my grandfather **Stig** Holm who worked here in Umeå as a professor and published hundreds of papers. Unfortunately, Stig did not get the chance to read this little book but I am pretty sure nobody would have ever read one written by me if it hadn't been for him. He also told me, while I was struggling during my medical school research project, that the most important thing when it comes to starting a research career is your mentor, the second one is your mentor and the third, you guessed right… is you mentor! This in a way is also like SCUBA diving. You need to find a dive master you trust with your life as you have no idea what you are doing and what you really shouldn't be doing under water. During my research, my dive masters or maybe we should call them research masters to add to the confusion were truly amazing. Without them I would never have gotten to the point where I am now, I simply wouldn't have dared to dive into this new field or I would have drowned in the sometimes messy world of research.

My main mentor **Katarina** was there from the very beginning to make sure I wouldn't drown, that I would learn all the important techniques to survive. Later on, during the project, when I felt more comfortable with the basics, she was there to show me the beauty of research. This too, is just like SCUBA diving. In the beginning you are focused on your equipment but once you've mastered that, you start seeing all the beautiful corals and miraculous animals. Thank you, Katarina, for always having my back, teaching me all necessary skills but most of all for showing me the beauty and the fun of it. Much unlike diving, my research master, also paid for everything, including way too many lunches in which we discussed everything from research to clinical questions or private problems. Words cannot describe how thankful I am but on a scale from one to ten it would be a 12.

I am lucky to have two more wonderful research masters helping me on the way, **Karin** and **Göran**.

Thank you, Karin, for giving me confidence when I did not really believe in myself and especially when I did not believe I was ready for the next step. I am not sure you know just how important that was during the way. Without you, I would not have dared to dive deeper and swim just a little faster. You, too, showed me a new, beautiful world when we were sitting in your office going through histology slides.

Thank you, Göran, for always making sure that my diving mask was as clean as possible so I would be able to see and to stay focused on the exact research

questions so the answers would be just as clear. You, too, are a true master of your profession. Thank you for making sure that I would not be harmed by predators that can be lurking in the murky waters of the researching world.

My three wonderful research masters have complemented each other in a way I didn't think was possible. In my mentoring world there were no problems with ego, trying to get the most benefit out of my work or not responding to e-mails. Thank you!

When diving at other sites you always learn new fascinating new things, you see new interesting animals and corals.

In the four different projects we co-operated with other research masters in their fields and I would like to thank **Lucia, Ivan and Olga** for introducing me to the fascinating world of immunology, **Urban and Claude** for showing me that a little glycosaminoglycan named hyaluronan is of great importance in almost every tissue and **Annika and Irene** for always enthusiastically helping us with virus analysis and for making everything sound so very simple. Thank you, **Kalle and Tanel**, for giving us some of your carefully collected tonsillar tissue to study!

A big thank you to all patients who agreed to take part in our studies!

Thank you, **Anders L.**, for your invaluable statistical support. Statistics are a bit lite diving time tables: Incredibly difficult to interprete and calculate alone and much easier with a master and a diving computer (or in this case SPSS) on your side.

Thank you, **Michael**, for always enthusiastically and quickly helping us with language correction!

What makes diving especially fun is diving with others to share the experience with. **Alexandra**, you are a bit newer to this field of research than I am but you are teaching me so much! You are caring, interested and incredibly bright! You question things I never thought of which helps me understand problems on a deeper level. I really enjoy research diving together with you and I know you are on the way to becoming an amazing research master!

Mohammad, in this diving analogy you are a bit of the head surfer. You haven't started with your own research just yet but - to stay with water sports - you are a head surfer and as such my surf master (to everyone confused by my odd analogies, Mohammad is my clinical mentor at the hospital). Thank you for helping me make sure that my surfing (clinical) education wouldn't be too

affected by all the research diving! You really are more that just my mentor and I really appreciate your friendship and all good advice I got during the years!

Christos, thank you so much for swimming a bit of the way together with me. You showed be how beautiful the corals are and also that becoming a dive master really can be done! Also, you are great at surfing and it's just so good to see that this combination is possible!

Mattias and Clas, you are two great role models and you were always there to encourage me when I thought combining surfing and diving wasn't possible. I will never forget the lunch-meeting you, Mattias, organized together with Clas with the sole purpose to cheer me up and cheer me on. Thank you! On a side note, Mattias, we really need to go to Australia so we can go SCUBA diving together (maybe we can combine it with some research diving).

Thank you, **Anders and Ellen**, my two favorite surf (work) roomies for your support and encouragement. Thank you, Ellen, for your endless pep-talks and believing in my skills more than I do! Special thanks to you, Anders, for taking the picture of my larynx seen in the background information of the thesis, and for checking my surfing administration while I was gone research diving. You saved me from worrying about missing important X-ray or pathological results while I was gone, which really is invaluable!

A big thank you to all collegues at work for cheering me on during these years and for covering for me when I was away due to my research! Thank you, **Lena and Barbro**, for making room in my crowded surfing schedule for my research diving!

Thank you, **Susanne**, for lending your throat to be photographed for the background info in this thesis, but most of all, thank you for an endless number of evenings spent at your place with wonderful food, great conversations and silly tickling games with your wonderful son **Bertil**.

Thank you **Hannerl and Lizzie,** for your endless support during so many years. Even though there are a couple thousand kilometers between us most of the time, I know I can always count on your loving advice. You both question me and my decisions in a very constructive and sometimes painfully honest way. Thank you so much for that!

Thank you, **Marina and Rasmus**, for lovely evenings spent together, squash battles at IKSU and great coffee breaks at Kahls where most issues in life can be solved. Good luck, Rasmus, with your thesis!

Thank you, my wonderful family for all your support during the years and most of all for helping me focus on other things than reseach when we were spending time together.

When diving you also need a dive buddy, someone who is always with you in case something doesn't work out and you need to be bailed out. I had quite a few dive buddies on my way:

Pappa, you were one of my first buddies along the way but of course also an inspiration like Stig. You have helped me on the way and encouraged me when it felt tough. You have taught me that it cannot always be easy and that some things are difficult for a reason. I really enjoyed facetiming you Sunday evenings to proof-read articles to make sure that the language was understandable and clear. During these talks we also always talked about the surrounding aspects of research and life and again about how important your mentors are. I'm not sure Katarina, Karin and Göran know how big of a fan of theirs you are ☺

Mama, you don't really care about all this research stuff I'm doing. Don't get me wrong, I know you're proud of me but you and I exist in bubble outside of research which feels incredibly good. We never really talk about my research, we talk about other things, so talking to you feels like taking a vacation. When things feel tough you always have my back, regardless if I'm wrong or right. Thank you for your unbeatable support!

Niklas, you too don't really care all too much about what I'm writing about and we rarely talk about the content of my research. However, I'd like to thank you for supporting me when it comes to discussing sometimes odd or even rude behaviours common in the research world that you would describe as 'insane'. Thank you for having my back and getting angry at things and people instead of me or together with me when necessary.

Niclas, you are my buddy in life and as such also my real SCUBA dive buddy. You have bailed me out of pretty dangerous situations SCUBA diving and research diving. Thank you for making sure that research wouldn't swallow me as a whole and introducing a more balanced way of approaching research. You never knew who I was before research and I am afraid you will never know who I will be after because I don't think I can stop anymore, it's just too much fun. Please continue telling me when it is too much and when you think my behavior (like working too many evenings/weekends during our holidays and taking my computer everywhere I go) is going out of bounds. I promise I will listen and try to make sure we both find the right buoyancy. Thank you for putting up with all this (and me) during the years!

9. References

1. nobelprize.org. Nobel Media AB 2014. *Harald zur Hausen - Biographical*. 2008; Available from: http://www.nobelprize.org/nobel_prizes/medicine/laureates/2008/hausen-bio.htm.
2. de Villiers, E.M., *Cross-roads in the classification of papillomaviruses*. Virology, 2013. **445**(1-2): p. 2-10.
3. Satterwhite, C.L., et al., *Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008*. Sex Transm Dis, 2013. **40**(3): p. 187-93.
4. Ryndock, E.J. and C. Meyers, *A risk for non-sexual transmission of human papillomavirus?* Expert Rev Anti Infect Ther, 2014. **12**(10): p. 1165-70.
5. Stanley, M.A., *Epithelial cell responses to infection with human papillomavirus*. Clin Microbiol Rev, 2012. **25**(2): p. 215-22.
6. Doorbar, J., *The papillomavirus life cycle*. J Clin Virol, 2005. **32 Suppl 1**: p. S7-15.
7. Raff, A.B., et al., *The evolving field of human papillomavirus receptor research: a review of binding and entry*. J Virol, 2013. **87**(11): p. 6062-72.
8. Bergvall, M., T. Melendy, and J. Archambault, *The E1 proteins*. Virology, 2013. **445**(1-2): p. 35-56.
9. McBride, A.A., *The papillomavirus E2 proteins*. Virology, 2013. **445**(1-2): p. 57-79.
10. Koromilas, A.E., S. Li, and G. Matlashewski, *Control of interferon signaling in human papillomavirus infection*. Cytokine Growth Factor Rev, 2001. **12**(2-3): p. 157-70.
11. Fu, L., et al., *Degradation of p53 by human Alphapapillomavirus E6 proteins shows a stronger correlation with phylogeny than oncogenicity*. PLoS One, 2010. **5**(9).
12. Tomaic, V., *Functional Roles of E6 and E7 Oncoproteins in HPV-Induced Malignancies at Diverse Anatomical Sites*. Cancers (Basel), 2016. **8**(10).
13. Doorbar, J., *The E4 protein; structure, function and patterns of expression*. Virology, 2013. **445**(1-2): p. 80-98.
14. DiMaio, D. and L.M. Petti, *The E5 proteins*. Virology, 2013. **445**(1-2): p. 99-114.
15. Venuti, A., et al., *Papillomavirus E5: the smallest oncoprotein with many functions*. Mol Cancer, 2011. **10**: p. 140.
16. Burd, E.M., *Human Papillomavirus Laboratory Testing: the Changing Paradigm*. Clin Microbiol Rev, 2016. **29**(2): p. 291-319.
17. Wang, J.W. and R.B. Roden, *L2, the minor capsid protein of papillomavirus*. Virology, 2013. **445**(1-2): p. 175-86.
18. Pett, M. and N. Coleman, *Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis?* J Pathol, 2007. **212**(4): p. 356-67.

19. McBride, A.A. and A. Warburton, *The role of integration in oncogenic progression of HPV-associated cancers*. PLoS Pathog, 2017. **13**(4): p. e1006211.
20. Nulton, T.J., et al., *Patients with integrated HPV16 in head and neck cancer show poor survival*. Oral Oncol, 2018. **80**: p. 52-55.
21. Sasagawa, T., H. Takagi, and S. Makinoda, *Immune responses against human papillomavirus (HPV) infection and evasion of host defense in cervical cancer*. J Infect Chemother, 2012. **18**(6): p. 807-15.
22. Doorbar, J., *Molecular biology of human papillomavirus infection and cervical cancer*. Clin Sci (Lond), 2006. **110**(5): p. 525-41.
23. Stanley, M., *HPV - immune response to infection and vaccination*. Infect Agent Cancer, 2010. **5**: p. 19.
24. Woodworth, C.D., *HPV innate immunity*. Front Biosci, 2002. **7**: p. d2058-71.
25. Bauer, S., et al., *Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA*. Science, 1999. **285**(5428): p. 727-9.
26. Arreygue-Garcia, N.A., et al., *Augmented serum level of major histocompatibility complex class I-related chain A (MICA) protein and reduced NKG2D expression on NK and T cells in patients with cervical cancer and precursor lesions*. BMC Cancer, 2008. **8**: p. 16.
27. Stanley, M., *Immune responses to human papillomavirus*. Vaccine, 2006. **24 Suppl 1**: p. S16-22.
28. Dong, C. and R.A. Flavell, *Th1 and Th2 cells*. Curr Opin Hematol, 2001. **8**(1): p. 47-51.
29. Doorbar, J., et al., *Human papillomavirus molecular biology and disease association*. Rev Med Virol, 2015. **25 Suppl 1**: p. 2-23.
30. Bais, A.G., et al., *A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions*. J Clin Pathol, 2005. **58**(10): p. 1096-100.
31. Albers, A.E., et al., *Meta analysis: HPV and p16 pattern determines survival in patients with HNSCC and identifies potential new biologic subtype*. Sci Rep, 2017. **7**(1): p. 16715.
32. Bonagura, V.R., et al., *Recurrent respiratory papillomatosis: altered CD8(+) T-cell subsets and T(H)1/T(H)2 cytokine imbalance*. Clin Immunol, 1999. **93**(3): p. 302-11.
33. Andersen, A.S., et al., *The interplay between HPV and host immunity in head and neck squamous cell carcinoma*. Int J Cancer, 2014. **134**(12): p. 2755-63.
34. Piersma, S.J., et al., *High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer*. Cancer Res, 2007. **67**(1): p. 354-61.
35. Heusinkveld, M., et al., *Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer*. Int J Cancer, 2012. **131**(2): p. E74-85.
36. Laurent, T.C., U.B. Laurent, and J.R. Fraser, *The structure and function of hyaluronan: An overview*. Immunol Cell Biol, 1996. **74**(2): p. A1-7.

37. Opheim, L.R., et al., *Hyaluronan in Human Vocal Folds in Smokers and Nonsmokers-A Histochemical Study*. J Voice, 2016. **30**(3): p. 255-62.
38. Cowman, M.K., et al., *The Content and Size of Hyaluronan in Biological Fluids and Tissues*. Front Immunol, 2015. **6**: p. 261.
39. Cowman, M.K., *Hyaluronan and Hyaluronan Fragments*. Adv Carbohydr Chem Biochem, 2017. **74**: p. 1-59.
40. Monslow, J., P. Govindaraju, and E. Pure, *Hyaluronan - a functional and structural sweet spot in the tissue microenvironment*. Front Immunol, 2015. **6**: p. 231.
41. Jiang, D., J. Liang, and P.W. Noble, *Hyaluronan as an immune regulator in human diseases*. Physiol Rev, 2011. **91**(1): p. 221-64.
42. Pardue, E.L., S. Ibrahim, and A. Ramamurthi, *Role of hyaluronan in angiogenesis and its utility to angiogenic tissue engineering*. Organogenesis, 2008. **4**(4): p. 203-14.
43. Delmage, J.M., et al., *The selective suppression of immunogenicity by hyaluronic acid*. Ann Clin Lab Sci, 1986. **16**(4): p. 303-10.
44. Bohaumilitzky, L., et al., *A Trickster in Disguise: Hyaluronan's Ambivalent Roles in the Matrix*. Front Oncol, 2017. **7**: p. 242.
45. Jiang, D., J. Liang, and P.W. Noble, *Hyaluronan in tissue injury and repair*. Annu Rev Cell Dev Biol, 2007. **23**: p. 435-61.
46. Mackay, C.R., et al., *Expression and modulation of CD44 variant isoforms in humans*. J Cell Biol, 1994. **124**(1-2): p. 71-82.
47. Naor, D., et al., *Involvement of CD44, a molecule with a thousand faces, in cancer dissemination*. Semin Cancer Biol, 2008. **18**(4): p. 260-7.
48. Lesley, J., et al., *Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation*. J Exp Med, 1995. **182**(2): p. 431-7.
49. Misra, S., et al., *Interactions between Hyaluronan and Its Receptors (CD44, RHAMM) Regulate the Activities of Inflammation and Cancer*. Front Immunol, 2015. **6**: p. 201.
50. Derkay, C.S. and B. Wiatrak, *Recurrent respiratory papillomatosis: a review*. Laryngoscope, 2008. **118**(7): p. 1236-47.
51. Kashima, H., et al., *Sites of predilection in recurrent respiratory papillomatosis*. Ann Otol Rhinol Laryngol, 1993. **102**(8 Pt 1): p. 580-3.
52. Loizou, C., et al., *Recurrent respiratory papillomatosis in northern Sweden: clinical characteristics and practical guidance*. Acta Otolaryngol, 2015. **135**(10): p. 1058-64.
53. Dedo, H.H. and K.C. Yu, *CO(2) laser treatment in 244 patients with respiratory papillomas*. Laryngoscope, 2001. **111**(9): p. 1639-44.
54. Derkay, C.S., *Task force on recurrent respiratory papillomas. A preliminary report*. Arch Otolaryngol Head Neck Surg, 1995. **121**(12): p. 1386-91.
55. Omland, T., et al., *Epidemiological aspects of recurrent respiratory papillomatosis: a population-based study*. Laryngoscope, 2012. **122**(7): p. 1595-9.
56. Larson, D.A. and C.S. Derkay, *Epidemiology of recurrent respiratory papillomatosis*. APMIS, 2010. **118**(6-7): p. 450-4.

57. Kashima, H.K., et al., *A comparison of risk factors in juvenile-onset and adult-onset recurrent respiratory papillomatosis.* Laryngoscope, 1992. **102**(1): p. 9-13.
58. Silverberg, M.J., et al., *Condyloma in pregnancy is strongly predictive of juvenile-onset recurrent respiratory papillomatosis.* Obstet Gynecol, 2003. **101**(4): p. 645-52.
59. Wiatrak, B.J., et al., *Recurrent respiratory papillomatosis: a longitudinal study comparing severity associated with human papilloma viral types 6 and 11 and other risk factors in a large pediatric population.* Laryngoscope, 2004. **114**(11 Pt 2 Suppl 104): p. 1-23.
60. Omland, T., et al., *Risk factors for aggressive recurrent respiratory papillomatosis in adults and juveniles.* PLoS One, 2014. **9**(11): p. e113584.
61. Karatayli-Ozgursoy, S., et al., *Risk Factors for Dysplasia in Recurrent Respiratory Papillomatosis in an Adult and Pediatric Population.* Ann Otol Rhinol Laryngol, 2016. **125**(3): p. 235-41.
62. Avelino, M.A., T.C. Zaiden, and R.O. Gomes, *Surgical treatment and adjuvant therapies of recurrent respiratory papillomatosis.* Braz J Otorhinolaryngol, 2013. **79**(5): p. 636-42.
63. Chadha, N.K. and A. James, *Adjuvant antiviral therapy for recurrent respiratory papillomatosis.* Cochrane Database Syst Rev, 2012. **12**: p. CD005053.
64. Szeps, M., et al., *Human papillomavirus, viral load and proliferation rate in recurrent respiratory papillomatosis in response to alpha interferon treatment.* J Gen Virol, 2005. **86**(Pt 6): p. 1695-702.
65. Gerein, V., et al., *Use of interferon-alpha in recurrent respiratory papillomatosis: 20-year follow-up.* Ann Otol Rhinol Laryngol, 2005. **114**(6): p. 463-71.
66. Best, S.R., M. Mohr, and K.B. Zur, *Systemic bevacizumab for recurrent respiratory papillomatosis: A national survey.* Laryngoscope, 2017. **127**(10): p. 2225-2229.
67. Syrjanen, K. and S. Syrjanen, *Detection of human papillomavirus in sinonasal papillomas: systematic review and meta-analysis.* Laryngoscope, 2013. **123**(1): p. 181-92.
68. Bielamowicz, S., T.C. Calcaterra, and D. Watson, *Inverting papilloma of the head and neck: the UCLA update.* Otolaryngol Head Neck Surg, 1993. **109**(1): p. 71-6.
69. Bhandary, S., et al., *Sinonasal inverted papilloma in eastern part of Nepal.* Kathmandu Univ Med J (KUMJ), 2006. **4**(4): p. 431-5.
70. Lawson, W. and Z.M. Patel, *The evolution of management for inverted papilloma: an analysis of 200 cases.* Otolaryngol Head Neck Surg, 2009. **140**(3): p. 330-5.
71. Lund, V.J., et al., *European position paper on endoscopic management of tumours of the nose, paranasal sinuses and skull base.* Rhinol Suppl, 2010. **22**: p. 1-143.
72. Bewes, T., et al., *Incidence of neoplasia in patients with unilateral epiphora.* J Laryngol Otol, 2015. **129 Suppl 3**: p. S53-7.

73. Lisan, Q., O. Laccourreye, and P. Bonfils, *Sinonasal inverted papilloma: From diagnosis to treatment*. Eur Ann Otorhinolaryngol Head Neck Dis, 2016. **133**(5): p. 337-341.
74. Yamashita, K., et al., *Studies of Tonsils in Basic and Clinical Perspectives: From the Past to the Future*. Adv Otorhinolaryngol, 2016. **77**: p. 119-24.
75. Ingram, D.G. and N.R. Friedman, *Toward Adenotonsillectomy in Children: A Review for the General Pediatrician*. JAMA Pediatr, 2015. **169**(12): p. 1155-61.
76. Marcus, C.L., et al., *Diagnosis and management of childhood obstructive sleep apnea syndrome*. Pediatrics, 2012. **130**(3): p. 576-84.
77. Busaba Nicholas, D.S., *Tonsillectomy in adults: Indications*. uptodate.com.
78. Hoddeson, E.K. and C.G. Gourin, *Adult tonsillectomy: current indications and outcomes*. Otolaryngol Head Neck Surg, 2009. **140**(1): p. 19-22.
79. Paradise, J.L., et al., *Efficacy of tonsillectomy for recurrent throat infection in severely affected children. Results of parallel randomized and nonrandomized clinical trials*. N Engl J Med, 1984. **310**(11): p. 674-83.
80. Baugh, R.F., et al., *Clinical practice guideline: tonsillectomy in children*. Otolaryngol Head Neck Surg, 2011. **144**(1 Suppl): p. S1-30.
81. Nasman, A., et al., *Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma?* Int J Cancer, 2009. **125**(2): p. 362-6.
82. WHO, I.A.f.R.A.C., *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. 2007.
83. Syrjanen, S., *HPV infections and tonsillar carcinoma*. J Clin Pathol, 2004. **57**(5): p. 449-55.
84. Ang, K.K., et al., *Human papillomavirus and survival of patients with oropharyngeal cancer*. N Engl J Med, 2010. **363**(1): p. 24-35.
85. Markowitz, L.E., et al., *Human papillomavirus vaccine introduction--the first five years*. Vaccine, 2012. **30 Suppl 5**: p. F139-48.
86. Kirby, T., *FDA approves new upgraded Gardasil 9*. Lancet Oncol, 2015. **16**(2): p. e56.
87. Shi, L., et al., *GARDASIL: prophylactic human papillomavirus vaccine development--from bench top to bed-side*. Clin Pharmacol Ther, 2007. **81**(2): p. 259-64.
88. Inglis, S., A. Shaw, and S. Koenig, *Chapter 11: HPV vaccines: commercial research & development*. Vaccine, 2006. **24 Suppl 3**: p. S3/99-105.
89. Schiller, J.T., X. Castellsague, and S.M. Garland, *A review of clinical trials of human papillomavirus prophylactic vaccines*. Vaccine, 2012. **30 Suppl 5**: p. F123-38.
90. Stanley, M., L.A. Pinto, and C. Trimble, *Human papillomavirus vaccines--immune responses*. Vaccine, 2012. **30 Suppl 5**: p. F83-7.
91. Arnheim-Dahlstrom, L., et al., *Autoimmune, neurological, and venous thromboembolic adverse events after immunisation of adolescent girls*

with quadrivalent human papillomavirus vaccine in Denmark and Sweden: cohort study. BMJ, 2013. **347**: p. f5906.
92. McCormack, P.L., *Quadrivalent human papillomavirus (types 6, 11, 16, 18) recombinant vaccine (gardasil((R))): a review of its use in the prevention of premalignant anogenital lesions, cervical and anal cancers, and genital warts*. Drugs, 2014. **74**(11): p. 1253-83.
93. Garland, S.M., et al., *Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases*. N Engl J Med, 2007. **356**(19): p. 1928-43.
94. Serrano, M., G.J. Hannon, and D. Beach, *A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4*. Nature, 1993. **366**(6456): p. 704-7.
95. Russo, A.A., et al., *Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a*. Nature, 1998. **395**(6699): p. 237-43.
96. LaPak, K.M. and C.E. Burd, *The molecular balancing act of p16(INK4a) in cancer and aging*. Mol Cancer Res, 2014. **12**(2): p. 167-83.
97. Sharpless, N.E., *INK4a/ARF: a multifunctional tumor suppressor locus*. Mutat Res, 2005. **576**(1-2): p. 22-38.
98. Gruis, N.A., et al., *Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds*. Nat Genet, 1995. **10**(3): p. 351-3.
99. Shapiro, G.I., et al., *Reciprocal Rb inactivation and p16INK4 expression in primary lung cancers and cell lines*. Cancer Res, 1995. **55**(3): p. 505-9.
100. Romagosa, C., et al., *p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors*. Oncogene, 2011. **30**(18): p. 2087-97.
101. Lewis, J.S., Jr., et al., *Human Papillomavirus Testing in Head and Neck Carcinomas: Guideline From the College of American Pathologists*. Arch Pathol Lab Med, 2018. **142**(5): p. 559-597.
102. Crawford, D.H., *Biology and disease associations of Epstein-Barr virus*. Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1408): p. 461-73.
103. Shannon-Lowe, C.D., et al., *Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells*. Proc Natl Acad Sci U S A, 2006. **103**(18): p. 7065-70.
104. Straus, S.E., et al., *NIH conference. Epstein-Barr virus infections: biology, pathogenesis, and management*. Ann Intern Med, 1993. **118**(1): p. 45-58.
105. Dunmire, S.K., K.A. Hogquist, and H.H. Balfour, *Infectious Mononucleosis*. Curr Top Microbiol Immunol, 2015. **390**(Pt 1): p. 211-40.
106. Cohen, J.I., *Epstein-Barr virus infection*. N Engl J Med, 2000. **343**(7): p. 481-92.
107. Niedobitek, G., et al., *Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells*. J Pathol, 1997. **182**(2): p. 151-9.
108. Babcock, G.J., et al., *EBV persistence in memory B cells in vivo*. Immunity, 1998. **9**(3): p. 395-404.

109. Balfour, H.H., Jr., et al., *Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students*. J Infect Dis, 2013. **207**(1): p. 80-8.
110. Straus, S.E., *The chronic mononucleosis syndrome*. J Infect Dis, 1988. **157**(3): p. 405-12.
111. Molyneux, E.M., et al., *Burkitt's lymphoma*. Lancet, 2012. **379**(9822): p. 1234-44.
112. Burkitt, D., *A "tumour safari" in East and Central Africa*. Br J Cancer, 1962. **16**: p. 379-86.
113. Niedobitek, G., et al., *Heterogeneous expression of Epstein-Barr virus latent proteins in endemic Burkitt's lymphoma*. Blood, 1995. **86**(2): p. 659-65.
114. Geser, A., et al., *Final case reporting from the Ugandan prospective study of the relationship between EBV and Burkitt's lymphoma*. Int J Cancer, 1982. **29**(4): p. 397-400.
115. Hesseling, P., et al., *Endemic Burkitt lymphoma: a 28-day treatment schedule with cyclophosphamide and intrathecal methotrexate*. Ann Trop Paediatr, 2009. **29**(1): p. 29-34.
116. Mbulaiteye, S.M., et al., *Sporadic childhood Burkitt lymphoma incidence in the United States during 1992-2005*. Pediatr Blood Cancer, 2009. **53**(3): p. 366-70.
117. Young, L.S. and C.W. Dawson, *Epstein-Barr virus and nasopharyngeal carcinoma*. Chin J Cancer, 2014. **33**(12): p. 581-90.
118. Pathmanathan, R., et al., *Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia*. Am J Pathol, 1995. **146**(6): p. 1355-67.
119. Tyan, Y.S., et al., *Detection of Epstein-Barr virus and human papillomavirus in head and neck tumors*. J Clin Microbiol, 1993. **31**(1): p. 53-6.
120. Atula, S., et al., *Human papillomavirus and Epstein-Barr virus in epithelial carcinomas of the head and neck region*. Anticancer Res, 1997. **17**(6D): p. 4427-33.
121. Jiang, R., et al., *Association between human papilloma virus/Epstein-Barr virus coinfection and oral carcinogenesis*. J Oral Pathol Med, 2015. **44**(1): p. 28-36.
122. Al Moustafa, A.E., et al., *Association between human papillomavirus and Epstein-Barr virus infections in human oral carcinogenesis*. Med Hypotheses, 2009. **73**(2): p. 184-6.
123. Detre, S., G. Saclani Jotti, and M. Dowsett, *A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas*. J Clin Pathol, 1995. **48**(9): p. 876-8.
124. Loizou, C., et al., *Incidence of tonsillar cancer in northern Sweden: Impact of human papilloma virus*. Oncol Lett, 2015. **10**(6): p. 3565-3572.
125. Sgaramella, N., et al., *Expression of p16 in squamous cell carcinoma of the mobile tongue is independent of HPV infection despite presence of the HPV-receptor syndecan-1*. Br J Cancer, 2015. **113**(2): p. 321-6.

126. Tolg, C., et al., *A RHAMM mimetic peptide blocks hyaluronan signaling and reduces inflammation and fibrogenesis in excisional skin wounds*. Am J Pathol, 2012. **181**(4): p. 1250-70.
127. Grondahl, F., et al., *Easy HPLC-based separation and quantitation of chondroitin sulphate and hyaluronan disaccharides after chondroitinase ABC treatment*. Carbohydr Res, 2011. **346**(1): p. 50-7.
128. Malm, L., U. Hellman, and G. Larsson, *Size determination of hyaluronan using a gas-phase electrophoretic mobility molecular analysis*. Glycobiology, 2012. **22**(1): p. 7-11.
129. Assadian, F., et al., *Distribution and Molecular Characterization of Human Adenovirus and Epstein-Barr Virus Infections in Tonsillar Lymphocytes Isolated from Patients Diagnosed with Tonsillar Diseases*. PLoS One, 2016. **11**(5): p. e0154814.
130. Assadian, F., et al., *Expression profile of Epstein-Barr virus and human adenovirus small RNAs in tonsillar B and T lymphocytes*. PLoS One, 2017. **12**(5): p. e0177275.
131. Assadian, F., et al., *Efficient Isolation Protocol for B and T Lymphocytes from Human Palatine Tonsils*. J Vis Exp, 2015(105).
132. Ok, C.Y., et al., *EBV-positive diffuse large B-cell lymphoma of the elderly*. Blood, 2013. **122**(3): p. 328-40.
133. Dreyer, J.H., et al., *pRb and CyclinD1 Complement p16 as Immunohistochemical Surrogate Markers of HPV Infection in Head and Neck Cancer*. Appl Immunohistochem Mol Morphol, 2017. **25**(5): p. 366-373.
134. Vasiljevic, N., et al., *Role of quantitative p16(INK4A) mRNA assay and digital reading of p16(INK4A) immunostained sections in diagnosis of cervical intraepithelial neoplasia*. Int J Cancer, 2017. **141**(4): p. 829-836.
135. Wu, T.J., et al., *Performance of p16INK4a ELISA as a primary cervical cancer screening test among a large cohort of HIV-infected women in western Kenya: a 2-year cross-sectional study*. BMJ Open, 2016. **6**(9): p. e012547.
136. Crosbie, E.J., et al., *The PapilloCheck Assay for Detection of High-Grade Cervical Intraepithelial Neoplasia*. J Clin Microbiol, 2015. **53**(11): p. 3553-9.
137. Wikby, A., et al., *The immune risk profile is associated with age and gender: findings from three Swedish population studies of individuals 20-100 years of age*. Biogerontology, 2008. **9**(5): p. 299-308.
138. Luz Correa, B., et al., *The inverted CD4:CD8 ratio is associated with cytomegalovirus, poor cognitive and functional states in older adults*. Neuroimmunomodulation, 2014. **21**(4): p. 206-12.
139. McBride, J.A. and R. Striker, *Imbalance in the game of T cells: What can the CD4/CD8 T-cell ratio tell us about HIV and health?* PLoS Pathog, 2017. **13**(11): p. e1006624.
140. Lu, W., et al., *CD4:CD8 ratio as a frontier marker for clinical outcome, immune dysfunction and viral reservoir size in virologically suppressed HIV-positive patients*. J Int AIDS Soc, 2015. **18**: p. 20052.

141. Serrano-Villar, S., et al., *The CD4:CD8 ratio is associated with markers of age-associated disease in virally suppressed HIV-infected patients with immunological recovery*. HIV Medicine, 2014. **15**(1): p. 40-49.
142. Olsson, J., et al., *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study*. Mech Ageing Dev, 2000. **121**(1-3): p. 187-201.
143. Bonagura, V.R., et al., *Enriched HLA-DQ3 phenotype and decreased class I major histocompatibility complex antigen expression in recurrent respiratory papillomatosis*. Clin Diagn Lab Immunol, 1994. **1**(3): p. 357-60.
144. Fergusson, J.R., et al., *CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages*. Cell Rep, 2014. **9**(3): p. 1075-88.
145. Jamieson, A.M., et al., *The role of the NKG2D immunoreceptor in immune cell activation and natural killing*. Immunity, 2002. **17**(1): p. 19-29.
146. Lanier, L.L., *NKG2D Receptor and Its Ligands in Host Defense*. Cancer Immunol Res, 2015. **3**(6): p. 575-82.
147. Raulet, D.H., et al., *Regulation of ligands for the NKG2D activating receptor*. Annu Rev Immunol, 2013. **31**: p. 413-41.
148. Groh, V., et al., *Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells*. Nat Immunol, 2001. **2**(3): p. 255-60.
149. Das, H., et al., *MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function*. Immunity, 2001. **15**(1): p. 83-93.
150. Allez, M., et al., *CD4+NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions*. Gastroenterology, 2007. **132**(7): p. 2346-58.
151. Hue, S., et al., *A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease*. Immunity, 2004. **21**(3): p. 367-77.
152. Fernandez-Morera, J.L., et al., *Soluble MHC class I chain-related protein B serum levels correlate with disease activity in relapsing-remitting multiple sclerosis*. Hum Immunol, 2008. **69**(4-5): p. 235-40.
153. Chen, D. and U. Gyllensten, *MICA polymorphism: biology and importance in cancer*. Carcinogenesis, 2014. **35**(12): p. 2633-42.
154. Groh, V., et al., *Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation*. Nature, 2002. **419**(6908): p. 734-8.
155. Del Toro-Arreola, S., et al., *MHC class I-related chain A and B ligands are differentially expressed in human cervical cancer cell lines*. Cancer Cell Int, 2011. **11**: p. 15.
156. Tian, X., et al., *High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat*. Nature, 2013. **499**(7458): p. 346-9.
157. Fries, E. and A. Kaczmarczyk, *Inter-alpha-inhibitor, hyaluronan and inflammation*. Acta Biochim Pol, 2003. **50**(3): p. 735-42.
158. Milner, C.M. and A.J. Day, *TSG-6: a multifunctional protein associated with inflammation*. J Cell Sci, 2003. **116**(Pt 10): p. 1863-73.

159. Lawson, W., N.F. Schlecht, and M. Brandwein-Gensler, *The role of the human papillomavirus in the pathogenesis of Schneiderian inverted papillomas: an analytic overview of the evidence*. Head Neck Pathol, 2008. **2**(2): p. 49-59.
160. Lin, G.C., et al., *Epidermal growth factor receptor, p16, cyclin D1, and p53 staining patterns for inverted papilloma*. Int Forum Allergy Rhinol, 2013. **3**(11): p. 885-9.
161. Palmer, E., et al., *Human papillomavirus infection is rare in nonmalignant tonsil tissue in the UK: implications for tonsil cancer precursor lesions*. Int J Cancer, 2014. **135**(10): p. 2437-43.
162. Duray, A., et al., *High prevalence of high-risk human papillomavirus in palatine tonsils from healthy children and adults*. Otolaryngol Head Neck Surg, 2011. **145**(2): p. 230-5.
163. Grun, N., et al., *Human papillomavirus prevalence in mouthwashes of patients undergoing tonsillectomy shows dominance of HPV69, without the corresponding finding in the tonsils*. Infect Dis (Lond), 2017. **49**(8): p. 588-593.
164. Rusan, M., et al., *Prevalence of tonsillar human papillomavirus infections in Denmark*. Eur Arch Otorhinolaryngol, 2015. **272**(9): p. 2505-12.
165. Gronhoj Larsen, C., et al., *Correlation between human papillomavirus and p16 overexpression in oropharyngeal tumours: a systematic review*. Br J Cancer, 2014. **110**(6): p. 1587-94.
166. Klingenberg, B., et al., *p16 INK4A overexpression is frequently detected in tumour-free tonsil tissue without association with HPV*. Histopathology, 2010. **56**(7): p. 957-67.
167. Quabius, E.S., et al., *SLPI and AnxA2 expression in neoplasm-free palatine tonsils is associated with smoking habit of individuals*. Mol Clin Oncol, 2017. **7**(3): p. 427-434.
168. Berger, C., et al., *Distribution patterns of beta- and gamma-herpesviruses within Waldeyer's ring organs*. J Med Virol, 2007. **79**(8): p. 1147-52.
169. Seishima, N., et al., *EBV infection is prevalent in the adenoid and palatine tonsils in adults*. J Med Virol, 2017. **89**(6): p. 1088-1095.
170. Gunel, C., et al., *Detection of the Epstein-Barr virus, Human Bocavirus and novel KI and KU polyomaviruses in adenotonsillar tissues*. Int J Pediatr Otorhinolaryngol, 2015. **79**(3): p. 423-7.
171. Greifenegger, N., et al., *Epstein-Barr virus small RNA (EBER) genes: differential regulation during lytic viral replication*. J Virol, 1998. **72**(11): p. 9323-8.
172. Hallenstal, N., et al., *Tonsil surgery in Sweden 2013-2015. Indications, surgical methods and patient-reported outcomes from the National Tonsil Surgery Register*. Acta Otolaryngol, 2017. **137**(10): p. 1096-1103.
173. Novakovic, D., et al., *A Prospective Study of the Incidence of Juvenile-Onset Recurrent Respiratory Papillomatosis After Implementation of a National HPV Vaccination Program*. J Infect Dis, 2018. **217**(2): p. 208-212.
174. Young, D.L., M.M. Moore, and L.A. Halstead, *The use of the quadrivalent human papillomavirus vaccine (gardasil) as adjuvant*

therapy in the treatment of recurrent respiratory papilloma. J Voice, 2015. **29**(2): p. 223-9.