AUS DEM LEHRSTUHL
FÜR INNERE MEDIZIN II
PROF. DR. MED. L. MAIER
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Association between human papillomavirus and primary lung cancer – a systematic review and pilot study

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Julia Karnosky

2020

„-Freiseite-„

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2. Abbreviations

- AC = adenocarcinoma
- ALK = anaplastic lymphoma kinase
- BRAF = v-Raf murine sarcoma viral oncogene homolog B1
- CDKN2A = TP53 and cyclin-dependent kinase inhibitor 2A
- CIN = cervical intraepithelial neoplasia
- COPD = chronic obstructive pulmonary disease
- DDR2 = discoidin domain receptor 2
- E = early region of HPV genome
- EBC = exhaled breath condensate
- EBUS = endobronchial ultrasound
- EGFR = epidermal growth factor receptor
- EML4-ALK = echinoderm microtubule associated protein-like protein 4 fused with Anaplastic Lymphoma Kinase
- FFPE = formalin-fixed paraffin-embedded
- FGR1 = fibroblast growth factor receptor 1
- HNSCC = head and neck squamous cell carcinoma
- HCC = hepatocellular carcinoma
- HPV = Human Papillomavirus
- ISH = In situ hybridization
- KRAS = Kirsten rat sarcoma viral oncogene homolog
- L = long region of HPV genome
- LC = lung cancer
- LCR = long control region of HPV genome
- MET = mesenchymal-epithelial transition factor
- MOOSE = Meta-analysis of Observational Studies in Epidemiology
- NSCLC = non-small cell lung cancer
- OS = overall survival
- PIK3CA = phosphoinositide-3-kinase catalytic subunit alpha isoform
- PTEN = phosphatase and tensin homolog
- PCR = Polymerase chain reaction
- PRISMA = Preferred Reporting Items for Systematic Reviews and Meta-Analysis

- PROSPERO = International prospective register of systematic reviews
- RET = rearranged during transfection
- ROS1 = c-ros oncogene 1
- SCLC = small cell lung cancer
- SCC = squamous cell carcinoma
- SOX2 = SRY related HMG box gene 2
- TP53 = tumor suppressor protein 53

3. Summary (Zusammenfassung)

3.1 Fragestellung

Im Jahr 1979 haben Syrjänen et al. erstmals über einen möglichen Zusammenhang zwischen einer HPV Infektion und der Entwicklung von Bronchialkarzinomen berichtet. Im weiteren Verlauf wurden zahlreiche Studien mit unterschiedlichsten Detektionsmethoden durchgeführt. In diesen zeigten sich sowohl geographische Unterschiede, als auch Unterschiede, welche auf die verwendete Analysemethode zurückgeführt wurden.

Ziel dieser Arbeit ist es, zum einen den aktuellen Stand in der Literatur bezüglich HPV Detektion mittels PCR in Lungenkrebsgewebe zu erfassen und zum anderen, dies mit den Ergebnissen unserer eigenen Kohorte zu vergleichen.

3.2 Methoden

Mittels einer strukturierten Literatursuche wurde zunächst versucht alle Publikationen zu erfassen, welche sich dem beschriebenen Thema widmen. Eine Einschränkung bezüglich des Publikationszeitraums oder der Sprache, in welcher die Publikation verfasst wurde, erfolgte nicht. Die erfassten Studien wurden anhand ihres Titels und, soweit verfügbar, Abstracts von zwei unabhängigen Personen begutachtet. Bei Uneinigkeit wurde die Entscheidung durch eine dritte Person getroffen. Anschließend wurde von allen eingeschlossenen Publikationen die Volltextversion beschafft und hinsichtlich der Ein- und Ausschlusskriterien geprüft.

Von allen eingeschlossenen Studien wurden die Fallzahlen sowie die Anzahl der HPV positiven und negativen Fälle und Kontrollen erfasst. Außerdem wurden allgemeine bibliographische Daten sowie Details bezüglich der analysierten Fälle dokumentiert und ausgewertet.

Aus der Gewebebank des Instituts für Pathologie der Universität Regensburg wurden 16 Fälle von Plattenepithelkarzinomen der Lunge, welche im Zeitraum von Mai 2017 bis März 2018 in der Klinik für Thoraxchirurgie operativ reseziert wurden, herausgesucht. Von 14 Patienten war ausreichend Gewebe vorhanden, sodass eine nested PCR nach der Methode von Sotlar et al. (1) durchgeführt werden konnte. Von zwei Patienten standen zusätzlich separate bioptische

Gewebeproben zur Verfügung, welche beide analysiert und in die Gesamtuntersuchung einbezogen wurden.

Die statistische Auswertung wurde mittels Microsoft Excel (2013) durchgeführt. Die Anzahl der HPV positiven und negativen Fälle und Kontrollen wurde aus den selektierten Arbeiten extrahiert und nachfolgend die HPV Prävalenzen anhand der einzelnen Patientendaten berechnet. Die Berechnung von Mittelwerten und Standardabweichungen erfolgte ebenfalls mittels Excel 2013. Die statistische Signifikanz der HPV Prävalenzunterschiede wurde mittels Chi-Quadrat-Test berechnet. Ein p-Wert < 0.05 wurde als statistisch signifikant eingestuft.

3.3 Ergebnisse

In der durchgeführten Literatursuche im Mai 2018 wurden 3884 Publikationen mit einem möglichen Themenbezug gefunden. Nach Deduplikation wurden 2624 Publikationen zum weiteren Screening mittels Titel und Abstract in die Literaturverwaltungssoftware Covidence eingeschlossen. Es wurden 340 Publikationen in die Volltextanalyse eingeschlossen. Nach Anwendung der Ein- und Ausschlusskriterien wurden 73 Veröffentlichungen in diesen Systematic Review eingeschlossen.

Die eingeschlossenen 73 Studien enthalten 8953 Lungenkrebspatienten und 754 Kontrollpatienten. Die HPV Prävalenz lag bei 13.6 % für alle HPV Arten kombiniert. Die HPV 16 Prävalenz war höher als die HPV 18 Prävalenz (6.0 % gegenüber 3.2 %, $p<0.01$). Studien aus Europa, Asien und Amerika erfüllten die Einschlusskriterien. Auf allen Kontinenten war die HPV 16 Prävalenz signifikant höher als die HPV 18 Prävalenz ($p<0.01$). Beim Vergleich der einzelnen Kontinente untereinander zeigten sich statisch signifikante Unterschiede ($p<0.01$) mit der geringsten HPV-Prävalenz in Europa (7.5%) gegenüber 16.6% in Asien und 11.6% in Amerika. Die HPV Prävalenz in Plattenepithelkarzinomen war höher als in Adenokarzinomen (18.6 % gegenüber 9.6 %). Dieser Unterschied war statistisch hochsignifikant ($p<0.01$). Die höchste HPV Prävalenz zeigte sich in Plattenepithelkarzinomen bei asiatischen Patienten (29.5 %).

Um eine zufällige HPV Detektion einschätzen zu können, wurden die eingeschlossenen Fall-Kontroll-Studien gesondert analysiert. In den 15 eingeschlossenen Fall-Kontroll-Studien wurden 1750 Lungenkrebspatienten und 754 gesunde Kontrollen analysiert. Es wurden nur Kontrollen in diese Subgruppenanalyse eingeschlossen, von welchen Lungenbiopsien analysiert wurden. Die HPV Prävalenz in den Lungenkrebsproben war statistisch signifikant höher als in der Proben der Kontrollgruppe (31.3 % gegenüber 5.4 %, $p<0.01$).

Um Unterschiede in der Prävalenz zu minimieren, die durch mögliche Einflussfaktoren zustande kommen können, wurden zum einen nur Veröffentlichungen eingeschlossen, welche PCR als Nachweismethode nutzen und zum anderen weitere Subgruppenanalysen durchgeführt.

Um einen möglichen Einfluss der Methode, mit welcher das Tumorgewebe behandelt wurde, zu untersuchen, wurden die Prävalenzen in den Studien, welche Formalin-fixierte und in Paraffin eingebettete Proben verwendeten, mit jenen verglichen, welche frische, gefrorene

Proben enthielten. Hierbei ergab sich kein statistisch signifikanter Unterschied der HPV Prävalenz (13.4 % gegenüber 13.5 %; p=0.9).

Da es in den letzten 30 Jahren zu großen Verbesserungen der PCR Methodik gekommen ist, wurden die Studien des 21. Jahrhunderts mit den in den 1990er Jahren durchgeführten Untersuchungen verglichen. Hier zeigte sich ein statistisch hoch signifikanter Unterschied zwischen den beiden Untersuchungszeiträumen ($p<0.01$) mit einer HPV-Prävalenz von 37.9% in den Studien aus den 90er Jahren gegenüber einer HPV-Prävalenz von 8.5 % in den Studien, welche im 21. Jahrhundert durchgeführt wurden

Das Durchschnittsalter der 14 Patienten, welche in Zusammenarbeit mit dem Institut für Pathologie der Universität Regensburg untersucht wurden, lag bei 69.6 Jahren. Acht Patienten waren männlich und alle Patienten waren aktuelle oder frühere Raucher. (Für einen Patienten war keine Raucheranamnese verfügbar.) Bei allen Patienten wurden primäre Plattenepithelkarzinome der Lunge diagnostiziert. Die mittels nested-PCR bestimmte HPV Prävalenz war 0 %.

3.4 Schlussfolgerung

Obwohl in den letzten Jahrzehnten große Fortschritte in der Lungenkrebstherapie gemacht wurden, ist Lungenkrebs immer noch mit einer sehr schlechten Prognose vergesellschaftet. Die Identifikation von vermeidbaren Risikofaktoren ist daher von besonderer Bedeutung, insbesondere bei Patienten ohne Raucheranamnese.

In vielen Studien konnte HPV DNA in Lungenkrebsgewebe nachgewiesen werden. Allerdings konnte bisher kein eindeutiger kausaler Zusammenhang belegt werden. Prävalenzunterschiede in Fall-Kontroll Studien deuten jedoch auf einen potentiellen Zusammenhang hin. Um dies kausal belegen zu können, sind jedoch weitere Studien notwendig, welche ergänzend die Expression der HPV Onkogene und eine mögliche Integration von HPV in das Hostgenom untersuchen.

Geographische Unterschiede der HPV Prävalenz wurden festgestellt mit einer höheren HPV Prävalenz in Asien gegenüber Europa und Nordamerika. Allerdings gibt es bisher noch keine Erklärungsansätze für diese Beobachtung.

Der Infektionsweg von HPV im Zusammenhang mit Lungenkrebs ist bisher ungeklärt. Nach aktuellem Stand der Forschung verursacht HPV keine Virämie, weshalb eine Blutstrominfektion unwahrscheinlich erscheint. Eine direkte Infektion wie beim Zervixkarzinom erscheint aber aufgrund der anatomischen Gegebenheiten ebenfalls unwahrscheinlich.

Zusammenfassend werden weitere Studien durchgeführt werden müssen, welche insbesondere versuchen, einen kausalen Zusammenhang zwischen HPV-Infektion und Lungenkrebs nachzuweisen und einige der obigen Fragen zu beantworten.

4. Introduction

4.1 Epidemiology

Lung cancer is estimated to be the leading cause of cancer related mortality worldwide with 2.1 million new lung cancer cases and 1.8 million predicted deaths worldwide. (1) This represents almost one in five cancer deaths worldwide in 2018 (18.4%).

This is the case not only in smokers. Even in never-smokers lung cancer is the most common cause of cancer death worldwide.

According to the GLOBOCAN 2018 report there will be an estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 worldwide. In this statistic all cancer subtypes except nonmelanoma skin cancer are included. In both sexes combined lung cancer is the cancer type most commonly diagnosed and the leading cause of cancer mortality. Lung cancer is the second most frequent type of cancer by incidence and the leading cause of cancer mortality in males. In females breast cancer is the most frequent type of cancer and the leading cause of mortality followed for incidence by colorectal and lung cancer. Concerning cancer mortality lung cancer is the second and colorectal cancer the third most common cause.

The most commonly diagnosed types of cancer underlie geographic variation depending on ethnicity, life style factors, virus infections and other still unknown factors.

Western Europe is one of the regions worldwide where cancer is the number one reason of premature death according to the WHO.

The incidence rate for lung cancer in Western Europe in 2018 is estimated to be 43.3 per 100.000 in males and 25.7 per 100.000 in females. The lung cancer incidence in males has been stable due to a lowering in smoking prevalence. But the lung cancer incidence in females is rising in most parts of the world. In some countries, like in the USA for example, the lung cancer incidence is now higher in young women then in young men. (2) This is mainly attributed to changes in smoking behavior.

In 2018 a group of Swiss and Canadian scientists estimated the lifetime and 10 year risk of developing lung cancer in a Swiss population between 1995 and 2003. (3) During that time the estimated lifetime risk decreased in men while it increased in women (7.1% to 6.9% and

2.5% to 4.1% respectively). The collected Data also showed that current and former smokers have an increased risk of developing lung cancer when compared to never smokers.

4.2 Lung Cancer

Lung cancer was historically divided into two major groups according to morphology: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC can be further divided into adeno-, squamous cell carcinoma, large cell carcinoma and more rare subtypes. Over time the distinction between in squamous cell carcinoma and adenocarcinoma has been shown to be important as they show differences in response to specific therapies. (4) In the last decade lung cancer is increasingly classified by its genetic alterations and oncogenic driver mutations.

Even though imaging techniques play an important role to determine the tumor stadium, in contrast to hepatocellular carcinoma (HCC) diagnosis of lung cancer to this day has to be verified by histology.

Approximately 30% of all non-small cell lung cancers are classified as squamous cell carcinomas (SCC). (5) SCC is defined as a malignant epithelial tumor expressing squamous differentiation in the form of keratinization or bridging. It can be further divided by the degree of keratinization into poorly, mildly or highly differentiated SCC. Smokers have a 10-20 fold higher risk of SCC of the lung than non-smokers.

The most commonly altered genes in SCC are: FGR1 (fibroblast growth factor receptor 1), PIK3CA (phosphoinositide-3-kinase catalytic subunit alpha isoform), DDR2 (discoidin domain receptor 2), MET, SOX2 (SRY related HMG box gene 2), PTEN (phosphatase and tensin homolog) and CDKN2A (TP53 and cyclin-dependent kinase inhibitor 2A). Some of these have therapeutic consequences already while others are still subject of ongoing research.

Adenocarcinomas (AC) represent approximately 50% of all NSCLC and approximately 38% of all lung cancers in the Western world. (6) Distribution of histological subtypes does underlie some geographic differences. Anatomically they are more often located in the periphery of the lung. Usually diagnosis is achieved by light microscopy where glandular morphology can be seen. This morphology can be further divided into acinar, leptic, papillary or solid with mucin patterns. Further evaluation of AC is done by immunohistochemistry

using adenocarcinoma marker. Apart from that adenocarcinomas are tested for oncogenic mutations which allow for targeted therapies.

The most commonly tested for altered genes in adenocarcinomas are:

- EGFR (epidermal growth factor receptor)
- EML4-ALK (echinoderm microtubule associated protein-like protein 4 fused with anaplastic lymphoma kinase)
- KRAS (Kirsten rat sarcoma viral oncogene homolog)
- MET (mesenchymal-epithelial transition factor)
- ROS1 (c-ros oncogene 1)
- RET (rearranged during transfection)
- BRAF (v-Raf murine sarcoma viral oncogene homolog B1)
- TP53 (tumor suppressor protein 53)

Some of these gene alterations are aims for targeted therapies (e.g. EGFR = Gefitinib, Erlotinib; ALK = crizotinib, alectinib) and are therefore routinely tested for in newly diagnosed lung cancers.

The symptoms of lung cancer are unspecific such as coughing or hemoptysis. In many cases B-symptomatic is the first sign of a malignant disease which usually only occurs in a more advanced tumor stage. This and the lack of a reliable screening method or tumor marker is the reason why lung cancer is most often diagnosed in an advanced tumor stage.

In a British study using the data from the National Cancer Registration the results showed that with an increased tumor stage at diagnosis the one year survival decreased. (7) While in some tumor entities a major decrease in survival rates is associated with stage 4 cancer (e.g. breast, colorectal cancer) in lung cancer a decrease in survival rates can be seen in every tumor stage.

To this day lung cancer prognosis can best be determined according to the TNM stage at primary diagnosis. (6) According to the International Association for the Staging of Lung Cancer (IASLC) the current 5 year survival rate based on TNM classification decreased from 73% in stage IA cancer to 13% in stage IV.

Outcome in patients with SCLC is even worse with a 2 year survival rate of only 4.6%. (8)

4.3 Etiology

Tobacco smoking is still the main cause of lung cancer worldwide. (9) Usually the amount of smoking patients have done in their life is related to the amount of tobacco consumed over time ("pack-years"). (10) Even though there are lung cancer cases in never-smokers there is a strong correlation between smoking and lung cancer incidence, most prevalent in small cell lung cancer and squamous cell carcinoma. On the other hand adenocarcinoma is the most common lung cancer subtype in never-smokers. (4)

Apart from smoking different risk factors have been suggested especially in never-smokers such as environmental tobacco smoke, air pollution, cooking oil fumes. (11) Different pulmonary diseases have been identified that are associated with a higher risk for lung cancer e.g. former infection with tuberculosis, COPD, emphysema. (12) Up to this point the causal association between these diseases and the development of lung cancer has not been fully established. On the other hand there are risk factors specific to certain occupations (e.g. coal miners) or certain geographic regions (e.g. areas with domestic radon exposure).

A growing number of lung cancer incidence takes place in never-smokers. This group makes up between 15 and 25% of the lung cancer patients and accounts for up to 300.000 deaths every year. (13) If lung cancer in never smokers would be accounted for as an original entity among cancer types it would still be the seventh leading cause of mortality among solid tumors. Lung cancer in never-smokers is more frequent in women and there are distinct oncogenic mutational patterns. ALK and EGFR activating mutations are frequent and with consequences both for therapy and prognosis. Today's data on chemotherapy response in smokers as compared to never-smokers is not conclusive but an area of ongoing research. That is why some scientists suggest to treat lung cancer in never-smokers as an independent tumor entity.

In recent years cancer prevention has been a topic of increased interest in scientific research. It is undisputable that smoking cessation is the most important factor in prevention of lung cancer development. Smoking cessation reduces the risk for all subtypes of lung cancer, especially for SCC and SCLC.

To this day no reliable tumor marker for lung cancer screening has been established. In 2011 a large multi-center study on lung cancer screening in high risk patients was conducted by The National Lung Screening Trial Research Team in the United States. (14) From 2002

through 2004 approximately 53.000 patients in 33 US medical centers were included and randomly assigned into a group that was screened with low dose CT-scan or conventional chest X-ray. Results have shown that low dose CT scan was able to reduce lung cancer mortality in a high risk population. No lung cancer screening programs for high risk patients or for the general public have yet been established.

4.4 Human Papillomavirus

Since the first identification of human papillomavirus more than 200 different subtypes have been identified. They are classified into high-risk HPV types (16,18,31,33,39,45,51,52,58) and low-risk HPV types (6,11,42,43,44). (15) In some other publications a differentiation between high-, intermediate- and low-risk HPV types is made.

Human papillomaviruses are small non-enveloped viruses which belong to the Papillomaviridae family. They are between 50 and 60 nanometer in diameter and contain one circular, double-stranded DNA genome consisting of approximately 7000 to 8000 bp. The genome is divided into three regions: the long control region (LCR), the early region (E) and the late region (L). (16)

The long control region controls viral gene expression and replication while the late region encodes structural proteins. The early region encodes genes needed for viral gene expression, replication and survival. The names of the different regions refer to the phase in the viral life cycle in which they are expressed.

Those main regions can be further divided by the function of their gene products. According to today's research the early region encodes for six different proteins. Of those E5, E6 and E7 are the three oncogenes encoded by the virus. E5 is involved in disturbing growth-factor signaling pathways and avoiding the host immune system. E6 and E7 work synergistically to provide an environment that allows the virus to replicate. E6 of high-risk HPV-types also targets the tumor-suppressor protein p53 and inactivates it. E7 as the third encoded oncoprotein targets cell-cycle regulating proteins such as retinoblastoma protein (Rb).

As for the other early proteins: E1 possesses DNA helicase activity and is thereby the only enzyme encoded by a HPV virus, E2 controls the expression of the other proteins and it

recruits E1 to the viral origin. It thereby increases viral DNA replication. As a third function it plays a critical role in transferring the viral genome during division of the host cells. The function of E4 is still unknown. (17)

There are two late genes (L1 and L2) and one long control region (LCR) which regulate the expression of the early genes. L1 and L2 are the major and minor components of the viral capsid. The L1 region can form virus-like particles (VLPs) which are the basis for prophylactic vaccination.

Many different regions of the HPV genome can be detected by PCR: some, for example the L1 regions, can be used as consensus primers to detect many different types of HPV and some like the E6 and E7 as type specific primers (e.g. E6 HPV16 and E7 HPV18).

HPVs are epitheliotropic and their life cycle takes place in squamous epithelia. According to current knowledge HPV enters through microtrauma in the epithelia and reaches the basal cell which is believed to be the target cell for HPC infection. The infection method of HPV is not yet fully understood. After initial infection there seems to be an unproductive state in which only the early viral oncogenes are expressed. One of the specific features of HPV infection is its ability to persist over years. To achieve this the viral genome has to be preserved over multiple cell division. The method by which this is achieved is still unknown. When one of the daughter cells starts to differentiate the virus starts its productive stage. The virus redirects the host cells DNA replicative system and amplification and expression of late viral genes starts. Those are necessary to subsequently produce progeny virus and release them.

Almost all studies on HPV are done on cervical cancer cells. It has not yet been established whether all of the before mentioned stages and means of infection are also valid in other infection sites.

The transmission route of HPV into lung tissue has not been explained. One of the possible infection routes is oral sexual activity as a possible pathway to lung cancer cells. Another possible route that has been discussed is hematogenous. Neither of that could be proven by now. (18)

Some studies have tried to detect HPV DNA in blood cells. But even though HPV 16/18 prevalence has been shown to exist in some studies (19), these results are still very controversial because to this day it is believed that HPV infection does not cause viremia.

4.5 HPV and Cervical Cancer

In 1979 Kari J. Syrjänen first published a case report describing histological changes in bronchial epithelium resembling a squamous cell carcinoma of the lung similar to changes of condylomatous nature in the genital tract. (20) Even though an association of HPV with carcinogenesis was not established at that time it was proposed to observe such lesions closely until proven otherwise.

The Nobel Prize in Physiology or Medicine 2008 was awarded to Harald zur Hausen for establishing the connection between HPV infection and cervical cancer. He first drew attention to a possible association between HPV infection and carcinogenesis in 1976. (21)

In the 1960s there were first reports about double stranded DNA of the human papillomavirus which in the 1970s was further distinguished to be different types of HPV. (16) But it wasn't until 1974 when the human wart virus was isolated from plantar warts by zur Hausen et al. and used for hybridization of cutaneous, genital warts and cervical cancer. (HPV was still referred to as Human Wart Virus then).While high positive rates were found in cutaneous warts, no positive signal was seen in cervical cancer specimens. (22) The authors concluded that more research had to be done on the possible existence of different types of HPV virus. This hypothesis was later proven in different studies. (23)

The first experiments to establish a relationship between HPV and cervical cancer were initiated in 1972 by zur Hausen in Heidelberg. (24) In 1976 Meisels and Fortin first suggested that there might be an association between koilocytotic cells that were found in cervical cancer and HPV infection. (25)

In 1982 the first three publications on HPV DNA sequences in human tumors were published. They all referred to cervical cancer.

In the following years research about the association of HPV and cervical cancer increased proving for example the interaction of E6 with p53 and its resulting protein degradation. (26) Today the carcinogenetic potential of HPV is well established and it is known that more than 95% of cervical cancer biopsies contain high-risk HPV subtypes.

Three different types of HPV vaccination are currently available (e.g. Gardasil, Gardasil 9, Cervarix). (27) Since 2015 the WHO recommends a two dose program. And vaccination with Gardasil has been proven to be effective to reduce the incidence of HPV 16/18 infection. It

has not yet been fully established whether vaccination is also effective in cervical intraepithelial neoplasia (CIN) or adenocarcinoma in situ.

4.6 HPV and other Organ Sites

Since the oncogenic potential of HPV has been established there has been a lot of research on a possible association of HPV with cancers of other organ sites.

Shortly after HPV DNA was detected in cervical cancer some of the same as well as new HPV subtypes were detected in other anogenital cancers. But since many of the risk factors of cervical cancer are the same as for other anogenital cancers a causal relationship between HPV infection and those kinds of cancer is still subject to ongoing research. (28) Even though HPV DNA has been detected in vaginal, penil and anal cancer.

Recurrent respiratory papillomatosis is a rare disease where benign tumors (papilloma) form a long the aerodigestive tract. Although these papillomas usually cause a mechanical problem (e.g. obstruction of the airways), some cases of transformation of these papillomas into squamous cell carcinomas have been described. (29) Today it is well established that recurrent respiratory papillomatosis is associated with HPV infection.

So one of the organ sites that have been examined early are tumors of the upper aerodigestive tract and head and neck cancer. (30)

In recent years research was done on most other organ sites. There are still many questions concerning HPV infection: e.g. how long and where do transient infections take place, does HPV cause viremia, does HPV present an individual risk factor in other organ sites like it does in cervical cancer or does it present as an co-carcinogen and does every high-risk HPV infection impose a risk of developing cancer in every organ site.

HPV DNA has not only been detected in neoplastic tissue, but also in normal tissue in several organ sites. Its meaning has yet to be determined.

Since the first suggestion of a possible association between HPV and lung cancer numerous studies have been executed all over the world. Those include not only different types of detection but also different sampling methods. In this meta-analysis and the included analyzed tumor tissue we took a closer look at HPV detection by PCR in bronchoscopic biopsy specimens or resected lung tissue in primary squamous cell carcinoma of the lung.

5. Materials and Methods

5.1. Meta-analysis

The intention behind conduction of this systematic review was to get an idea of the prevalence of HPV infection in lung cancer both internationally and on the different continents as basis for further research and to detect the possibility of an association in our own patient collective.

Reporting of this meta-analysis is done according to the recommendation of Stroup et.al (31) for reporting observational studies published in 2000. They held a workshop 1997 in Atlanta with twenty-seven participants who were selected by a committee based on their clinical, statistical and other expertise. A systematic review of the published literature on reporting meta-analysis of observational studies was conducted. Based on the workshop results a checklist of recommendations on what to report was developed. These are referred to as "MOOSE" (Meta-analysis of Observational Studies in Epidemiology)-criteria.

5.1.1 Literature Research

Before starting the literature research we registered the concept for our meta-analysis on the international prospective register of systematic reviews (PROSPERO) in March 2018. At that point of time no other systematic reviews on the association of HPV infection and lung cancer were registered.

In cooperation with Dr. Helge Knüttel a librarian at the medical library of the University Clinic Regensburg we searched the digital databases EMBASE (via Ovid, 1974–present), MEDLINE (via Ovid, 1946–present), Cochrane Library (Cochrane Database of Systematic Reviews, Database of Abstracts of Reviews of Effect, Cochrane Central Register of Controlled Trials, Health Technology Assessment Database, NHS Economic Evaluation Database; from inception to present) and Science Citation Index Expanded (Web of Science, 1965–present) as well as the search engine Google Scholar (no date limit).

Using the default sort order by relevance only the first 200 records from Google Scholar were assessed. Records from Google Scholar were downloaded with Anne-Wil Harzing's "Publish or Perish" program (https://harzing.com/resources/publish-or-perish).

In addition to the bibliographic databases we searched the following registers of clinical trials for completed studies: WHO's International Clinical Trials Registry Platform, ClinicalTrials.gov, EU Clinical Trials Register and the German Clinical Trials Register.

We employed highly sensitive search strategies in order to identify all possibly relevant studies. We searched for the concepts "lung cancer" AND "HPV". Controlled terms from the databases' thesauri and a broad range of synonyms were used. Within each concept search terms were combined using the Boolean operator OR. No limits such as for study type, publication type, publication date or language were applied. We adjusted the search strategy according to the databases/search engines.

A draft search strategy for EMBASE (Ovid syntax) was published and is available from: http://doi.org/10.5283/epub.36830.

We screened the reference lists of included studies and of relevant systematic reviews for additional studies.

Records from searching were uploaded to a reference management software for deduplication.

Screening and eligibility assessment was done using the COVIDENCE online program for managing systematic reviews.

5.1.2 Acquisition of Publications

The full texts of the publications identified by the literature research were found via internet research or if the full text was not available online, by borrowing paper copies from our own library or by contacting other libraries through different services offered by our medical library ("Fernleihe", "Subito").

The full texts were obtained in the original language. If that was neither German nor English a translation was made using an online translation program and checked with a college or translator fluent in that particular language.

5.1.3 Data Selection

After deduplication the titles and abstracts of the remaining publications were analyzed by two independent reviewers (Dr. Franziska Koll, Julia Karnosky) for relevance and matching inclusion criteria. If there was no concordance between the two reviewers the final decision was made by an independent third reviewer (Prof. Christian Schulz). Those publications where no abstract was available were included into the full text review as well.

All studies that were recognized as possibly eligible for inclusion were analyzed using EndNote Citation Software (Version X9).

For all the included full texts the same analysis system with the same reviewers was used.

Analysis of the publications was done according to the following inclusion and exclusion criteria:

5.1.3.1 Inclusion Criteria

All studies reporting HPV prevalence in primary lung cancer cases in adults were included. Case reports were excluded. As detection method only PCR from fresh frozen and/or paraffin-embedded tissue were included. HPV detection in blood samples or any other means of detection was excluded. All types of tissue sampling method were included. HPV detection in archival tumor tissue was included as well.

No exclusion was done depending on whether HPV low-risk or high-risk were analyzed.

Only studies that provide data specific to HPV prevalence in lung cancer tissue were included.

No exclusions were made based on language. We included journal articles as well as abstracts and conference reports if they met the inclusion criteria. If an abstract and a journal article on the same patient population was available the journal article was included. Journal articles that reported about not only cases of HPV detection in primary lung cancer but e.g. in head and neck cancer as well, were included but only the data of the primary lung cancer group were extracted.

5.1.3.2 Exclusion Criteria

Exclusion Criteria were as following:

- Case Reports
- Reviews
- HPV detection in minors
- Any data on HPV prevalence that was done by a detection method other than PCR
- HPV detection in blood samples or any other biomaterial then tumor tissue
- HPV infection in non-primary lung cancer or metastatic disease of a different primary origin
- Publications for which neither abstract nor full text were available online or by borrowing a paper copy through a library

The excluded full text publications were documented stating the reason for exclusion.

5.1.4 Data Extraction

For all included studies title, first author, year of publication, country of research, journal in which the study was published and language of publication were collected. Additionally the study size, the detection method for HPV, the tissue sampling and processing method and the types of HPV detected were collected to be able to compare the overall prevalence of HPV infection in lung cancer to our own experiments. Not only the calculated HPV prevalence in the included studies but also the case numbers as well as the number of cases detected to be positive and negative were collected.

Data for a subgroup analysis for Squamous Cell Carcinoma and HPV types 16 and 18 were gathered as well.

In order to evaluate other possible factors influencing the results a subgroup analysis was done on basis of the used tissue processing method.

To exclude that any detected difference is influenced by improvement in PCR technology the studies were stratified by the time in which they were done and analyzed separately in another subgroup analysis.

5.2 Experimental Part

5.2.1 Biopsy Material

All the included materials were obtained from the tissue bank at the department of pathology at the University of Regensburg. Initially a list of 16 patients with primary SCC of the lung diagnosed in 2017 or 2018 was created. After obtaining the biopsy specimens it was determined that enough tissue for PCR was still available for 14 patients. In addition two separate biopsy specimens were available for two patients. Both were included in the final analysis. From all the included patients paraffin-embedded tissue from resected lung cancer was available. All of them were operated on by the Department of Thoracic Surgery at the University Clinic Regensburg between May 2017 and March 2018.

5.2.2 HPV Detection

HPV detection was done according to the method reported by Sotlar et al. in 2004. (32) They evaluated a nested multiplex PCR assay. It incorporates consensus primers and type-specific primers. The viral E6/E7 oncogenes are used as primer targets. In the first round of amplification a broad spectrum of HPV subtypes is analyzed. 18 different HPV subtypes including all known high-risk HPV types are included. They were combined with type-specific primers used for nested PCR in following rounds of amplification. This novel PCR assay was initially tested on cervical scrapes. In their study the detected sensitivity of this new multiplex PCR was similar to the sensitivity of the nested PCRs done with consensus primers MY09/MY11 and GP5+/GP6+ combined. The sensitivity was higher than in a conventional PCR with either of the two. The advantage of this way of HPV detection is that it allows for exact HPV typing and detection of multiple HPV types at once.

All experimental analyses were carried out in cooperation with the Department of Pathology of the University Hospital Regensburg.The in this institute already established methodology of HPV-analytics was employed to the subject of this study. The evaluation of the multiplex-PCR-data was performed under the supervision and in cooperation with Prof. Dr. Wolfgang Dietmaier, chief of molecular pathological diagnostics in the Department of Pathology.

5.3 Statistical Analysis

All publications that matched the inclusion criteria were added to an Excel chart. Data was analyzed using Microsoft Excel (2013). The total number of cases as well as the number of positive and negative HPV detection were collected and the overall HPV prevalence was calculated. All tests for statistical significance were done using the Chi-squared-test. A p-value < 0.5 was determined to be statistically significant. Afterwards all the calculation were repeated using SPSS Version 22.

Other information e.g. on smoking behavior, gender etc. was collected from the individual publication. Mean value of age as well as standard deviation were calculated by means of Excel 2013.

If the information was not available in all of the included studies its information was collected for the studies that provide it and the number of studies which do so is acknowledged.

6. Results

6.1 Meta-analysis

6.1.1 Databank Research

An overview of all analyzed studies is documented in the PRISMA flowchart (Appendix Figure 3: Prisma flowchart).

6.1.2 General Literature Research

In the literature research done in May 2018 3884 publications with a possible connection to the research question were found in the following databases respectively:

- Medline (n=705),
- Embase (n=1376),
- Cochrane (n=12),
- Science Citation Index Expanded (n=1508),
- Google Scholar (n=200),
- WHO's International Clinical Trials Registry Platform (n=5),
- ClinicalTrials.gov (n=28)
- EU Clinical Trials Register (n=10).

After deduplication the remaining 2624 publications were imported into Covidence (management software for systematic reviews run by the Cochrane library).

They were evaluated by both reviewers on relevance for the research question. 2268 of the screened titles and abstracts did not relate to the current research and were excluded. For 16 publications no abstract was available, so it was not possible to evaluate whether the publication might be relevant. The decision was made to include these 16 publications into the full text review as well. In summary 340 publications were entered into the full text review.

The full text version of three of these publications was not available neither online nor by borrowing the paper version from a library (33-35).

The remaining 337 full text versions were screened by both investigators. After applying the inclusion and exclusion criteria 73 publications were included in this systematic review.

Reason for exclusion	**Number of publications**
No PCR	67
Overview articles without new data	28
Duplicates	22
Systematic Reviews or Meta-analysis	26
No data on HPV prevalence	28
No biopsies	25
Abstract of included full text	11
Case report	9
Comments on other articles	9
Same patient data in different publication	7
Biopsies from organs other than the lung	6
Studies on special patient groups	5
Correction of another article	5
Non primary lung cancers	6
Unfinished studies	2
No details on HPV detection method	2
Recurrent respiratory papillomatosis	2
No data on sampling method	2
English version included	2
Total number of excluded full text studies	**264**

Table 1: Excluded full text studies

The most common reason for exclusion was that no PCR was done to detect HPV. This was the case in 67 publications. In two studies the HPV detection method was not detailed. While manually screening the included title and abstracts 22 more duplicates were found and excluded. Nine publications were case reports. There were five corrections and nine comments on publications that were screened. Since none of the corrections changed the content of the publications they were excluded.

There were 26 systematic reviews and meta-analysis which were analyzed separately for additional studies but were not included. An additional 28 overview articles were excluded because they did not specifically provide data on HPV prevalence in lung cancer.

In 25 studies no lung biopsies were analyzed but HPV detection was done in other materials such as blood samples or cell lines. Six studies analyzed HPV prevalence in cancers other than lung cancer or on metastasis.

While 28 studies did give detailed information on different aspects of lung cancer no data on HPV prevalence was included.

We included study registers into our general literature research. After screening the titles and abstracts of the registered studies two were included into the full text review. The aspired date of conclusion for these two studies is set after this systematic review is finished.

The same patients were analyzed in two separate publications in seven cases. The newer or the one providing more detail was included if they fit the inclusion criteria. Four studies were published twice with the same information but in different languages. In these cases the English version was included.

In 11 cases an abstract was found published in a different journal than the full text. In these cases only the full text was included since the abstract did not provide additional information.

Five studies reported on HPV prevalence in lung cancer in special patient groups e.g. patients after lung transplantation, immunocompromised patients and butchers. Those were excluded because the spectrum of infections known to occur in such patient group differ from those in other patients. For the same reason two publications on HPV prevalence in patients with recurrent respiratory papillomatosis were excluded.

6.1.3 Overview of the Literature

After application of inclusion and exclusion criteria 73 publication were eligible for inclusion. A list of all included studies is recorded in Table 19: List of publications.

15 of the publications were case-control studies in which lung tissue was used as a control. In the case that studies did use other tumors as controls (e.g. cervical cancer tissue) or the control was not done on tissue but for example blood samples, only the data of the analyzed lung tumor tissue were included and the study was not counted as a case-control study.

The studies were stratified according to the geographical region in which the patients lived. No studies from Africa or Australia were found. There were 35 studies on patients from Asia, 23 studies on European patients and 15 studies carried out on the American continent.

The countries most represented were: Japan (n=11), China (n=10), USA (n=9) and Italy (n=5). Only three studies from Germany met the inclusion criteria. Six studies were done in multiple countries with the information summarized in one publication.

We did not exclude publications because of the language in which they were published. Most of the publications were written in English (n=68). The other publications were published in Chinese (n=3), French (n=1) and German (n=1). If a paper was published in more than one language but with the same content the more recent edition was included.

In order to get information on as many cases as possible not only journal articles but every type of available publication was included. Of the 73 included publications 62 were journal articles. Of the remaining publications 6 were abstracts, 3 were poster presentations and 2 were meeting abstracts.

The research included in this systematic review was published in more than 20 journals worldwide. The most common being: Lung Cancer (n=5), British Journal of Cancer (n=4), Oncology Reports (n=4) and Oncology Letters (n=4).

Of all the included studies 26 were performed in the 21st century. Of the remaining studies six were done in the 1990s. In 29 cases no data on the time of research was available. The time of research in the remaining 12 studies spanned both the 1990s and 21st century.

6.1.4 Patients Characteristics

A total number of 8953 lung cancer patients was included into this systematic review.

Of all the included studies 25 provided data on the patients age. The average age of the included patients was 62.2 years (SD: 4.9 years).

Information on patients' gender was available in 48 out of the 73 included studies. Those studies included 5957 patient. Of them 3756 were male and 2201 were female, 63.1 % and 36.9 % respectively.

Smoking behavior was detailed in 31 of the studies (42.5 %). There were 3409 current or former smokers, 1767 never smokers and in 3777 cases no information on smoking status was available. The rate of smokers was 38.1 %. If only the rate of smokers in the studies that

provide such information is calculated the rate of smokers is 62.7 %. This rate has to be seen very critical since both studies only including non-smokers as well as only smokers were included. It also has to be taken into consideration that there is no generally accepted rule to when a patient is considered to be a smoker. Definitions vary largely.

Only the information on primary SCC and primary AC of the lung was collected. There were 2629 cases of SCC and 2669 cases of AC. In the remaining cases it was neither one of them or the histological subtype was not detailed. In total 29.4 % of the included cases were squamous cell carcinomas and 29.8 % were adenocarcinomas.

6.1.5 Continents

6.1.5.1 Europe

	Reference	Country	No. of cases	Year	HPV prevalence in %	Specimen type used	Histologíc subtypes tested	HPV types detected
	Anantharaman et al. (36)	multiple European countries	290	2014	9.7	FFPE, fresh frozen	SCC/AC/others	11,16, 51,58
	Argyri et al. (37)	Greece	67	2017	3.0		SCC/AC/others	16,53
	Carpagnano et al. (38)	Italy	89	2011	16.4	FFPE	SCC/AC/others	16,30, 31,39
	Ciotti et al. (39)	Italy	38	2006	8.0	FFPE, fresh	SCC/AC/others	16,18
	Coissard et al. (40)	France	218	2005	1.8	fresh frozen	SCC/AC/others	16
	Eberlein-Gonska et al. (41)	Germany	55	1992	5.5	fresh	SCC/AC/others	16
	Galvan et al. (42)	Italy, United Kingdom	100	2012	0	fresh frozen	SCC/AC/others	none
	Gatta et al. (43)	Italy	50	2012	4.0	FFPE	SCC	
	Guliani et al. (44)	Italy	78	2007	12.8	fresh frozen	SCC/AC/others	16,18, 31,53
	Hennig et al. (45)	Norway	22	1999	13.6	FFPE	SCC/AC/others	6
	Miasko et al. (46)	Poland	94	2004	12.7		SCC/AC/others	
	Miasko et al. (47)	Poland	40	2001	10.0	FFPE	SCC/AC/others	
	Papadopoulou et al. (48)	Greece	52	1998	40.0	fresh frozen, FFPE	SCC	6,11, 16,18
	Podsiadlo et al. (49)	Poland	33	2012	3.0	fresh	NSCLC/SCLC	120
	Sagerup et al. (50)	Norway	334	2014	3.9	fresh frozen	SCC/AC/others	11,16, 33,66
	Sarchianaki et al. (51)	Greece	100	2014	19.0	FFPE	SCC/AC/others	6,11,16, 18,31, 33,59
	Shamanin et al. (52)	Germany	85	1994	0	fresh frozen	SCC/AC/others	none

	Spandidos et al. (53)	Greece	99	1996	15.0	FFPE	SCC/AC/others	11,16, 18,33
	Syrjanen et al. (54)	Finland	77	2012	5.2	FFPE, archival tissue	SCC/AC/others	6,16
	Van Boerdonk et al. (55)	Netherlands	211	2013	0	FFPE, archival tissue	SCC/AC/others	none
	Thomas et al (56)	France	31	1995	16.0	fresh frozen	SCC/AC/others	6, 11
	Welt et al (57)	Germany	38	1997	0	FFPE	SCC/SCLC	none
	Zafer et al (58)	Turkey	40	2004	5.0	fresh frozen	SCC/AC/others	18
Total			**2226**					

Table 2: European studies

From Europe 23 studies were included containing 2226 patients. The most common countries of origin were Italy (n=4) and Greece (n=4), followed by Germany (n=3) and Poland (n=3). HPV prevalence was tested in multiple countries and reported in one publication in two studies (36, 42).

21 publications were in English, one in French (56) and one in German (41).

Of all the European studies 19 publications were journal articles, two poster presentations, one meeting abstract and one was an abstract.

All of the included studies from Europe incorporated NSCLC tumor biopsies.

Two of the included studies only contained SCC samples.(43, 48) There were no studies only analyzing adenocarcinomas found in Europe.

The processing method of the analyzed tissue was not detailed in two of the included studies (37, 46).

Of all the included studies 13 used formalin-fixed paraffin-embedded tissue (56.5 %), nine studies used fresh frozen tissue, two studies used fresh tumor specimens (41, 49) and one study also included archival tissue(55). Four studies analyzed tissue that was processed by more than one of the methods.

Information on the used primers was available in seven studies (30.4 %). The primer used most often in the European subgroup was the consensus primer MY09/MY11 (n=5).

Four of the included studies were case-control studies (38, 41, 42, 51).

The total number of cases included in these studies was 351, the number of controls was 222. The number of HPV positive cases and controls was 37 and one case respectively. The overall HPV prevalence was calculated to be 10.5 % in the lung cancer cases compared to 0.45 % in the control cases from benign lung disease.

Only in nine studies information on the patients' age was provided. The average age was 64.7 years with a standard deviation of 2.3 years.

Information on the patients' gender was available in 13 of the included studies. 66.5 % of the patients were male and 33.5 % were female (935/472 cases).

Smoking behavior was detailed in 11 studies. 44.3 % were current or former smoker, 4.2 % were never smokers and in 51.5 % the smoking behavior was unknown.

All but five studies contained information on the tissue sampling method. It was resected lung tissue in nine studies and bronchoscopic biopsy in three. In six studies both surgically resected lung tissue and bronchoscopic biopsies were analyzed.

The total number of cases included from Europe was 2226. The maximum number of cases in one of the studies was 334, the minimal 22. On average there were 96.8 patients per study.

The most prevalent HPV subtype detected was HPV 16 (n=21), followed by HPV 18 (n=7). No data on the detected HPV subtype were available in three studies (43, 46, 47).

Of the included 2226 cases 168 were detected to be HPV positive by PCR. The HPV prevalence in the European subgroup was 7.5 %. The highest detected HPV prevalence in one of the European studies was 40 % (48). No HPV DNA was detected in four of the studies (42, 52, 55, 57).

Data on HPV 16 prevalence was included in 15 of the European studies. In total 1556 cases were tested of which 61 were found to be HPV 16 positive (3.9 %). Information on HPV 18 prevalence was available in 13 studies. HPV 18 prevalence was detected to be 0.8 %, meaning 11 out of the 1436 therefore analyzed cases.

	Positive	Negative	Total
HPV 16	61	1495	1556
HPV 18	11	1425	1436
	72	2920	2992

Table 3: HPV 16 and 18 prevalence in Europe compared

The difference between the detected HPV prevalence for HPV 16 and HPV 18 in the included European studies was statistically highly significant ($p<0.01$).

The number of included studies from Europe that contained information on HPV prevalence in squamous cell carcinoma of the lung was 19. Of the included 798 cases 43 were HPV positive (5.4 %). Four studies did not detect HPV DNA in SCC (42, 52, 55, 57).

Ten studies incorporate information on HPV 16 prevalence in SCC; it was calculated to be 2.8 %. Of the studies on SCC nine provided data on HPV 18 prevalence. Only three of the analyzed 408 lung cancer cases were detected to be HPV 18 positive (0.7 %).

Samples of adenocarcinomas of European patients were analyzed in 17 studies. In total 763 cases were examined. 52 cases were HPV positive (6.8 %). The prevalence for HPV 16 (n=9) and HPV 18 (n=8) in adenocarcinomas was 2.9 %. In eight studies AC samples were analyzed for HPV 18 DNA. None of the analyzed 365 cases were tested to be positive for HPV 18 DNA.

	HPV positive	HPV negative	Total
SCC	43	755	798
AC	52	711	763
	95	1466	1561

Table 4:HPV prevalence in SCC and AC in Europe compared

There was no statistically significant difference between the HPV prevalence in SCC and AC analyzed in the included studies from Europe ($p=0.24$).

6.1.5.2 Asia

	Reference	Country	No. of cases	Year	HPV prevalence in %	Specimen type used	Histologic subtypes tested	HPV types detected
	Aguayo et al. (59)	Pakistan, China	60	2010	13.0	FFPE	SCC/AC/others	16
	Baba et al. (60)	Japan	57	2010	19.3	FFPE	SCC/AC	6,16, 18,33
	Cheng et al. (61)	Taiwan	141	2004	38.3		SCC/AC	6,11
	Cheng et al. (62)	Taiwan	141	2001	54.6	FFPE, fresh frozen	SCC/AC	16,18
	Fan et al. (63)	China	262	2015	8.4	FFPE	SCC/AC	16,18, 31,58
	Goto et al. (64)	Japan/ Singapore/ China/ Korea	304	2011	7.9	FFPE	SCC/AC	6,11, 16,18
	Halimi et al. (65)	Iran	30	2011	10.0	FFPE	SCC	
	Hartley et al. (66)	Lebanon	20	2015	0	FFPE	SCLC	none
	Hirayasu et al. (67)	Japan	73	1996	60.3	FFPE	SCC	6,16,18
	Hiroshima et al. (68)	Japan	22	1999	4.5	FFPE	AC	16
	Ilahi et al. (69)	Pakistan	9	2016	11.1	FFPE	SCC/AC/others	16
	Isa et al. (70)	Japan	96	2015	1.0	FFPE	SCC/AC/others	6
	Ito et al. (71)	Japan	901	2014	0.9		SCC/AC/others	
	Iwakawa et al. (72)	Japan	297	2010	0	fresh frozen	AC	none
	Jafari et al. (73)	Iran	50	2013	18.0	FFPE	SCC/AC/others	6,18
	Jain et al. (74)	India	40	2005	5.0	fresh frozen	SCC/AC/others	18
	Kato et al. (75)	Japan	42	2012	16.7	FFPE	SCC/AC/others	16,58
	Kawaguchi et al. (76)	Japan	876	2016	0.3	FFPE	SCC/AC	16,62,66

	Kinoshita et al. (77)	Japan	36	1995	8.0	FFPE, fresh frozen	SCC/AC	18
	Lee et al. (78)	Korea	233	2016	0	FFPE	SCC/AC	none
	Li et al. (79)	China	50	1995	32.0	FFPE, fresh frozen	SCC/AC/others	16,18
	Lin et al. (80)	Taiwan	57	2005	50.9	FFPE	SCC/AC	16,18
	Lu et al. (81)	China	72	2016	45.8	FFPE	SCC/AC	16,18
	Miyagi et al. (82)	Japan	121	2001	33.9	FFPE	SCC/AC	6,16,18
	Nadji et al. (83)	Iran	129	2007	25.6	FFPE	SCC/AC/others	6,11,26, 31,16,18
	Ogura et al. (84)	Japan	29	1993	10.3	fresh frozen	SCC	16,18
	Park et al. (85)	Korea	112	2007	53.6		AC/NSCLC	16,18,33
	Wang et al. (86)	Taiwan	153	2006	45.1	fresh	SCC/AC	16,18
	Wang et al. (87)	China	313	2008	44.1	fresh frozen	SCC/AC	16,18
	Wang et al. (88)	China	45	2010	42.2	fresh frozen	SCC	16,18
	Xing et al. (89)	China	49	1993	14.2	FFPE	SCC	6,11,16
	Yang et al. (90)	China	50	1998	26.0	FFPE	SCC	16
	Yu et al. (91)	China	180	2015	55.6	FFPE	SCC/AC/SCLC	16,18
	Zhang et al (92)	China	68	2009	44.1	fresh frozen	SCC, AC	16,18
	Zhang et al (93)	China	104	2010	17.3	FFPE	SCC/AC/others	16
Total			**5222**					

Table 5: Asian studies

From Asia 35 studies were included containing 5222 patients. Two of those were done on patients in multiple Asian countries. The country most prevalent is Japan (n=11), followed by China (n=10) and Taiwan (n=4).

32 of these were published in English and three were published in Chinese. Of all the studies published in Asia, 34 were journal articles and one was an abstract.

NSCLC tumor specimens were analyzed in 30 of the included Asian studies. In one study only SCLC samples were analyzed. (66) Six of the studies only included SCC samples (65, 67, 84, 88-90). Two studies only included adenocarcinomas (68, 72).

The processing method of the analyzed tissue was not detailed in three of the included studies (61, 71, 85). Of all the included studies 25 used formalin-fixed paraffin-embedded tissue (71.4 %), of the remaining studies nine used fresh frozen tissue. One study analyzed fresh tumor specimens (86). In three studies tissue was analyzed in both processing forms (FFPE and fresh frozen) (62, 77, 79).

Information on the used primers was available in 14 studies (40 %). The consensus primers MY09/MY11 were used in five cases as the most commonly used PCR primer.

The number of case-control studies was ten out of the 35 included Asian studies. The total number of cases included in these studies was 1401, the number of controls was 538. The number of HPV positive cases and controls was 522 and 39 respectively. The overall HPV prevalence was calculated to be 37.3 % in the lung cancer cases compared to 7.2 % in the control cases from benign lung disease.

Only 12 of the studies provided data on the patients' age. The average age was 60.8 years with a standard deviation of 5.7 years.

Information on the patients' gender was available in 23 of the included studies. 2058 of the patients were male and 1265 were female (61.9 %/38.1 %)

Smoking behavior was detailed in 17 studies. 37.7 % were current or former smoker, 31.2 % were never smokers and in 31.1 % the information was not available.

26 studies contained information on the tissue sampling method. It was resected lung tissue in 24 studies and seven studies used bronchoscopic biopsies. Six of the studies analyzed both surgically resected lung tissue and bronchoscopic biopsies. In one study autopsy material was analyzed as well as surgically resected lung tissue (77). In eight of the studies no data on the tissue sampling method was available.

The total number of cases included from Asia was 5222. The maximum number of cases in one of the studies was 901 (71), the minimal amount was nine patients (69). On average there were 149.2 patients per study.

The most prevalent HPV subtype detected was HPV 16 (n=25), followed by HPV 18 (n=19). In two studies the HPV subtype was not determined.

Of all included cases 879 were HPV positive by PCR. The HPV prevalence in the Asian subgroup was calculated to be 16.8 %. In four studies no HPV DNA was detected (66, 72, 74, 78). The highest detected HPV prevalence in one of the studies was 60.3 % (67).

22 of the studies included data on HPV 16 prevalence. A total of 2946 cases from the included Asian studies were tested for HPV 16 DNA. 227 (7.7 %) were tested to be positive. Of all included studies 18 reported data on HPV 18 prevalence. Of the 2580 cases 127 were positive (4.9 %).

	Positive	**Negative**	**Total**
HPV 16	227	2719	2946
HPV 18	127	2453	2580
	354	5172	5526

Table 6: HPV 16 and 18 prevalence in Asia compared

In the comparison of HPV 16 and 18 prevalence in the studies included in this systematic review, there was a statistically significant difference ($p<0.01$).

21 of the included studies provided data on HPV prevalence of primary SCC of the lung. There were 1331 cases in Asia. 392 of the tumors were tested to be HPV positive (29.5 %). 12 studies provided information on HPV 16 prevalence in SCCs from Asia. Of the analyzed 561 cases 101 were positive for HPV 16 (18.1 %). Only 9 studies provided information on HPV 18 detection. 44 of the analyzed 428 cases were positive resulting in a prevalence of 10.3 %.

18 of the included studies from Asia analyzed primary adenocarcinomas, including 1226 cases. The HPV prevalence was 10.4 %. HPV 16 (n=12) and HPV 18 (n=10) prevalence in ACs was 8.7 % and 7.3 %, respectively.

	HPV positive	HPV negative	Total
SCC	392	939	1331
AC	128	1098	1226
	520	2037	2557

Table 7 HPV prevalence in SCC and AC in Asia compared

There was a statistically significant difference between the HPV prevalence in SCC and AC analyzed in the included studies from Asia ($p<0.01$).

6.1.5.3 The Americas

	Reference	Country	No. of Cases	Year	HPV prevalence in %	Specimen type used	Histologic subtypes tested	HPV types detected
	Aguayo et al. (94)	Chile	69	2007	29.0	FFPE	SCC/AC/others	6,16,18, 31,45
	Badillo-Almaraz et al. (95)	Mexico	39	2013	41.0		SCC/AC	16,18
	Bohlmeyer et al. (96)	USA	34	1998	5.9	FFPE	SCC	18
	Cardona et al. (97)	multiple South American countries	132	2013	39.4	FFPE	AC	16
	Carlson et al. (98)	USA	12	2007	0	FFPE	SCLC	none
	Castillo et al. (99)	Peru/ Colombia/ Mexico	36	2006	28.0	FFPE	SCC/AC/others	16,18, 33
	Garcia Falcone et al. (100)	Argentina	40	2017	25.0	FFPE	SCC	16,18
	Joh et al. (101)	USA	30	2010	16.7	FFPE	SCC/AC/others	11,16, other
	Koshiol et al. (102)	USA	399	2011	0	FFPE, ethanol fixed	SCC/AC	none
	Mehra et al. (103)	USA	36	2013	11.0		SCC/AC	16,18
	Pillai et al. (104)	USA	208	2013	14.9	FFPE	NSCLC	16,18
	Rezazadeh et al. (105)	USA	16	2008	25.0	FFPE	NSCLC	11,16
	Robinson et al. (106)	USA	70	2016	42.9	fresh frozen	SCC/AC	16,18,39, 44,51,52,68
	Suh et al. (107)	USA	48	2010	2.0	FFPE	SCC	no data
	Yanagawa et al. (108)	Canada	336	2013	1.5	FFPE	SCC/AC	16
Total			**1505**					

Table 8: American studies

There were 15 studies included that were done on patients from the American continent. Two of those studies contained patients from multiple countries. Of the remaining studies ten were carried out in North America, one in Middle America and two in South America.

These studies all were published in English. There were nine journal articles, four abstracts, one meeting abstract and one poster presentation.

All of the included studies but one analyzed NSCLC tumor specimens. One study only analyzed specimens of small cell lung cancer. Only one study was a case-control study. It only includes ten cases of benign lung disease as control. (106)

12 studies were performed with formalin-fixed paraffin-embedded tissue specimens, one with fresh frozen tissue and two studies did not provide information on the tissue processing method. Only five of the studies contained information on the PCR primer used.

Of the included studies four provided information on the patient age. The average age was 60.85 years (SD: 5.2).

Twelve of the included studies provided information on the patients' gender. 763 were male and 464 female, 62.2% and 37.8% respectively.

Three of the included studies provided information on smoking behavior (94, 96, 102). Of all included patients 30.0 % were current or former smokers, 2.8 % never-smokers and for 67.2 % of patients no information on smoking behavior was available.

The tissue sampling method was surgical resection in six cases, bronchoscopic biopsy in one case, archived tumor tissue in one case and in one case both resected lung tissue specimens and bronchoscopic biopsies. No data on the tissue sampling method was available in six cases.

The total number of cases in this subgroup was 1505. The largest study included 399 cases, the smallest included 12 cases. On average there were 100.3 cases per study.

The most commonly detected HPV subtype was HPV 16 (n=10), followed by HPV 18 (n=8). Low risk HPV subtypes were detected in three studies. (94, 101, 106)

Of all those cases 175 were detected to be HPV positive by PCR. The remaining 1330 cases showed no sign of HPV DNA. The HPV prevalence was detected to be 11.6 %. The highest detected HPV prevalence in any of the American studies was 42.9 % in a study by Robinson et al. from 2016 (106).

Of the included 15 studies 11 provided data on HPV 16 prevalence. 89 of the analyzed 1110 cases were HPV 16 positive (8.0 %). Nine of the included studies did PCR to detect HPV 18 DNA. Of the 642 cases analyzed under this aspect 12 were positive (1.9%). Four of the studies stated the HPV 18 prevalence to be zero. (98, 101, 102, 105) One of the studies did not provide information on the HPV subtype detected (107).

	Positive	**Negative**	
HPV 16	89	1021	1110
HPV 18	12	630	642
	101	1654	1752

Table 9: HPV 16 and 18 prevalence in the Americas compared

The difference detected between HPV 16 and HPV 18 prevalence detected in the American studies was statistically significant ($p<0.01$).

Ten of the included studies provided data on HPV prevalence of primary SCC of the lung. There were 500 cases in American studies. 54 of those cases were positive and 446 were negative. The HPV prevalence in SCC was determined to be 10.8 %. Eight of the included studies provided data on HPV 16 prevalence in SCC; it was calculated to be 6.4 %. Seven studies provided data on HPV 18 prevalence. 290 cases of SCC were analyzed. Of those 11 cases were positive for HPV 18 DNA (3.8%).

Only eight studies provided information on adenocarcinomas of the lung. Cumulative there were 680 cases of AC analyzed. 78 of them were determined to be HPV positive (11.5 %). Only three studies contained information of HPV 16 prevalence in AC. Two of them were negative. (102, 108) The highest HPV 16 prevalence was 39.4 %. (97) Only two studies contained data on HPV 18 prevalence. It was determined to be zero (102) and 15.4% (99) respectively.

	HPV positive	**HPV negative**	
SCC	54	446	500
AC	78	602	680
	132	1048	1180

Table 10: HPV prevalence in SCC and AC in the Americas compared

The difference between the HPV prevalence in SCC and AC was not statistically significant (p=0.78).

6.1.5.4 Continents compared

	Europe	**Asia**	**The Americas**	**Total**
Number of cases	2226	5222	1505	**8953**
Number of HPV positive cases	168	879	175	**1222**
HPV 16 positive cases	61	227	89	**337**
HPV 18 positive cases	11	127	12	**150**
HPV prevalence	7.5%	16.8%	11.6%	**13.6%**
HPV 16 prevalence	3.9%	7.7%	8.0%	**6.0%**
HPV 18 prevalence	0.8%	4.9%	1.9%	**3.2%**

Table 11: HPV prevalence on continents

A total number of 8953 lung cancer patients was included into this systematic review. Most of the cases included were analyzed in Asia. In total 58.3 % of cases were from Asia, 24.9 % of cases were from Europe and 16.8 % of cases from The Americas.

In the biopsies of all included patients with lung cancer HPV was detected to be positive in 1222 cases. The overall HPV prevalence was calculated to be 13.6 %. The highest HPV prevalence was detected in Asia with 16.8 %, followed by The Americas (11.6%) and Europe (7.5 %).

On all three continents the calculated prevalence of HPV 16 was higher than for HPV 18. The highest HPV 16 prevalence was detected in the Americas (8.0 %), followed by Asia (7.7 %), and Europe (3.9%). Overall the HPV 16 prevalence was calculated to be 6.0 %.

The calculated HPV 18 prevalence was calculated to be 3.2 %. The highest HPV 18 prevalence was found in Asia (4.9 %) followed by the Americas (1.9 %) and finally Europe

(0.8 %). The difference between the HPV 16 and HPV 18 prevalence detected worldwide was statistically highly significant ($p<0.01$).

The difference between the HPV prevalence for all HPV subtypes combined was statistically significant when the HPV prevalence in Europe was compared with the HPV prevalence in Asia or the Americas, as well as when the Asian and American HPV prevalence was compared ($p<0.01$ in each case).

6.1.6 Squamous Cell Carcinoma vs. Adenocarcinomas

	Europe	**Asia**	**America**	**Total**
Number SCC	798	1331	500	**2629**
Number SCC HPV positive	43	392	54	**489**
Number AC	763	1226	680	**2669**
Number AC HPV positive	52	127	78	**257**
HPV prevalence SCC	5.4%	29.5%	10.8%	**18.6%**
HPV prevalence AC	6.8%	10.4%	11.5%	**9.6%**

Table 12: HPV prevalence SCC vs. AC

Of all the included 8953 lung cancer cases 2629 were squamous cell carcinomas and 2669 were adenocarcinomas. For the remaining 3655 case no detailed information was on histology was available or they were classified as NSCLC without further specification. Most of these cases in both groups were from Asia.

The overall HPV prevalence in SCC (n=489) was calculated to be 18.6 %. The highest prevalence was calculated in Asia (29.5 %). The lowest in Europe (5.4 %).

In contrast the highest HPV prevalence in AC was calculated in The Americas (11.5 %), followed by Asia (10.4 %) and Europe (6.8 %). The overall HPV prevalence in adenocarcinomas (n=257) was calculated to be 9.6 %.

The HPV prevalence in SCC and AC was the lowest in both subgroups in Europe.

To analyze the differences between detected HPV prevalence in SCC and AC the provided HPV prevalence detailed in the included studies was compared. The highest identified HPV prevalence in SCC in one of the studies was 60.3 % (67) in a study from Japan from 1996. The highest HPV prevalence in AC was 55.5 % (95) in a study from Mexico from 2013.

	HPV positive	**HPV negative**	
SCC	489	2140	2629
AC	257	2412	2669
	746	4552	5298

Table 13: HPV prevalence SCC vs. AC worldwide compared

When the provided HPV prevalence of SCC and AC are compared the difference is statistically highly significant ($p<0.01$).

6.1.7 Cases vs. Controls

	Case group	**Control group**
Number of cases	1750	754
Number of HPV detections	548	41
HPV prevalence	31.3%	5.4%

Table 14: HPV prevalence Case vs. Control Group

Of the included studies 15 are case-control studies (Appendix Table 11). One of them is from America, ten are from Asia and four from Europe.

In all of those studies 1750 lung cancer cases and 754 controls were analyzed. The number of HPV positive cases and controls was 548 vs. 41 respectively. The HPV prevalence was detected to be 31.3 % in the lung cancer group and 5.4 % in the control group.

The difference of the detected HPV prevalence between the case group and the control group was statistically highly significant ($p<0.01$).

	Europe	Asia	America	Total
Number cases	279	1401	70	**1750**
Number HPV positive cases	17	522	9	**548**
Number controls	206	583	10	**754**
Number HPV positive controls	1	39	1	**41**
HPV prevalence cases	6.0%	37.3%	12.9%	**31.3%**
HPV prevalence controls	0.5%	6.7%	10%	**5.4%**

Table 15: Case-Control studies on the different continents

When the difference between cases and controls is compared separately in each of the continents, it is statistically significant as well in Europe as in Asia. Only one case-control study from The Americas was included.

6.1.8 FFPE vs. fresh frozen

	FFPE	Fresh frozen
Number of studies	40	15
Number of cases	4476	1763
Number of HPV detections	599	238
HPV prevalence	13.4%	13.5%

Table 16: HPV prevalence in FFPE and fresh frozen tissue

40 of all included studies analyzed solitarily formalin-fixed paraffin-embedded tissue. (Appendix Table 12)

The total number of included cases was 4476. In the FFPE cases the number of HPV detection was 599 which leads to a HPV prevalence of 13.4 %.

15 of the included studies analyzed fresh frozen tumor tissue. (Appendix Table 13)

The number of fresh frozen tumor tissue cases analyzed in these studies was 1763 of which 238 were found to be positive. The HPV prevalence was calculated to be 13.5 %.

The difference between the HPV prevalence detected in the studies was not statistically significant between the FFPE and fresh frozen tissue (p=0.9).

6.1.9 1990s vs 21st century

	1990s	**21st century**
Number of studies	6	26
Total number of cases	425	4005
Number of HPV positive cases	161	339
HPV prevalence	37.9%	8.5%

Table 17: HPV prevalence in studies from the 1990s and 21st century

Six of the included studies were conducted in the 1990s. (Appendix Table 14) Of the 425 cases 161 were positive by PCR. The calculated HPV prevalence was 37.9 %.

Of all the included 73 studies 26 were done in the 21st century. (Appendix Table 15) A total of 4005 cases were analyzed. The number of positive cases was 339 which sums up to a HPV prevalence of 8.5 %.

The difference between the HPV prevalence stated in both subgroups was statistically highly significant (p<0.01).

6.2 Experimental Part

6.2.1 Patient Characteristics

A total of 16 tissue samples of 14 patients were included. The average age of the included patients was 69.6 years (SD: 9.6). 57 % are male (8/14) and 43 % female. Information on smoking behavior was available for all but one patient. There were five current smokers and eight former smokers. The average number of pack years was 65 for the current smokers at time of diagnosis and 39 for the former smokers, respectively. 43 % were previously diagnosed with COPD. All of them were diagnosed with primary lung cancer between 2017 and 2018 and have been operated on by the Department of Thoracic Surgery at the University Clinic Regensburg.

Table 18: Patient characteristics

		Total number of patients	**Range**	**Percentage**
Total number of patients		14		
Mean age (range) years		69.6	60.0 - 79.2	
Sex				
	male	8		57.0
	female	6		43.0
Tumor histology				
	squamous cell carcinoma	14		100
Smoking behavior				
	current smokers	5		35.7
	former smokers	8		57.2
	no information	1		7.1

Histology was determined to be squamous cell carcinoma in all 14 patients. As an example for the light microscopic picture two photographs of an included tumor sample of primary SCC of the lung are documented here. They are taken with a microscopic enlargement of 40 and 100 fold respectively. Both pictures were taken by Dr. Kirsten Utpatel, Department of Pathology University Clinic Regensburg using a Nikon Eclipse Ci microscope.

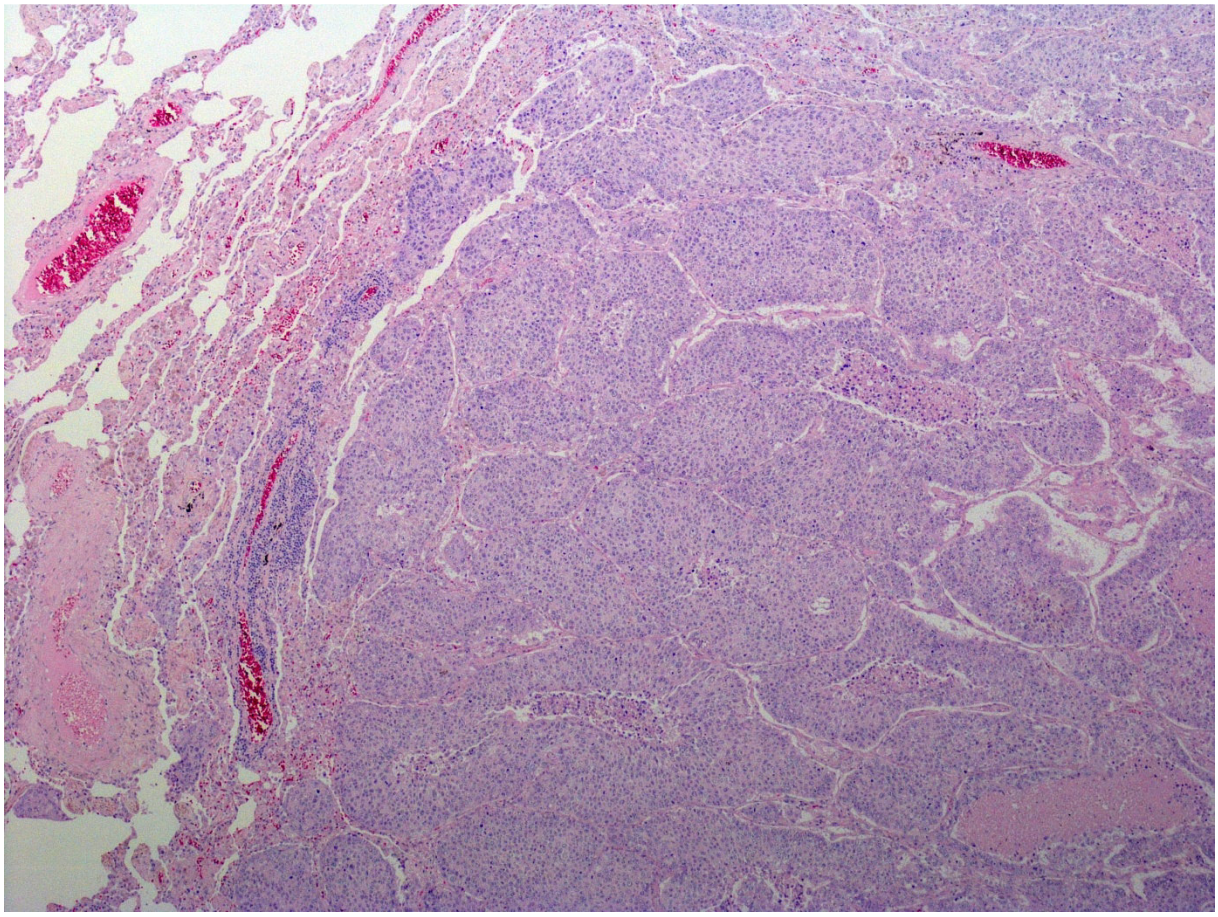

Figure 1: Histology SCC 40 fold

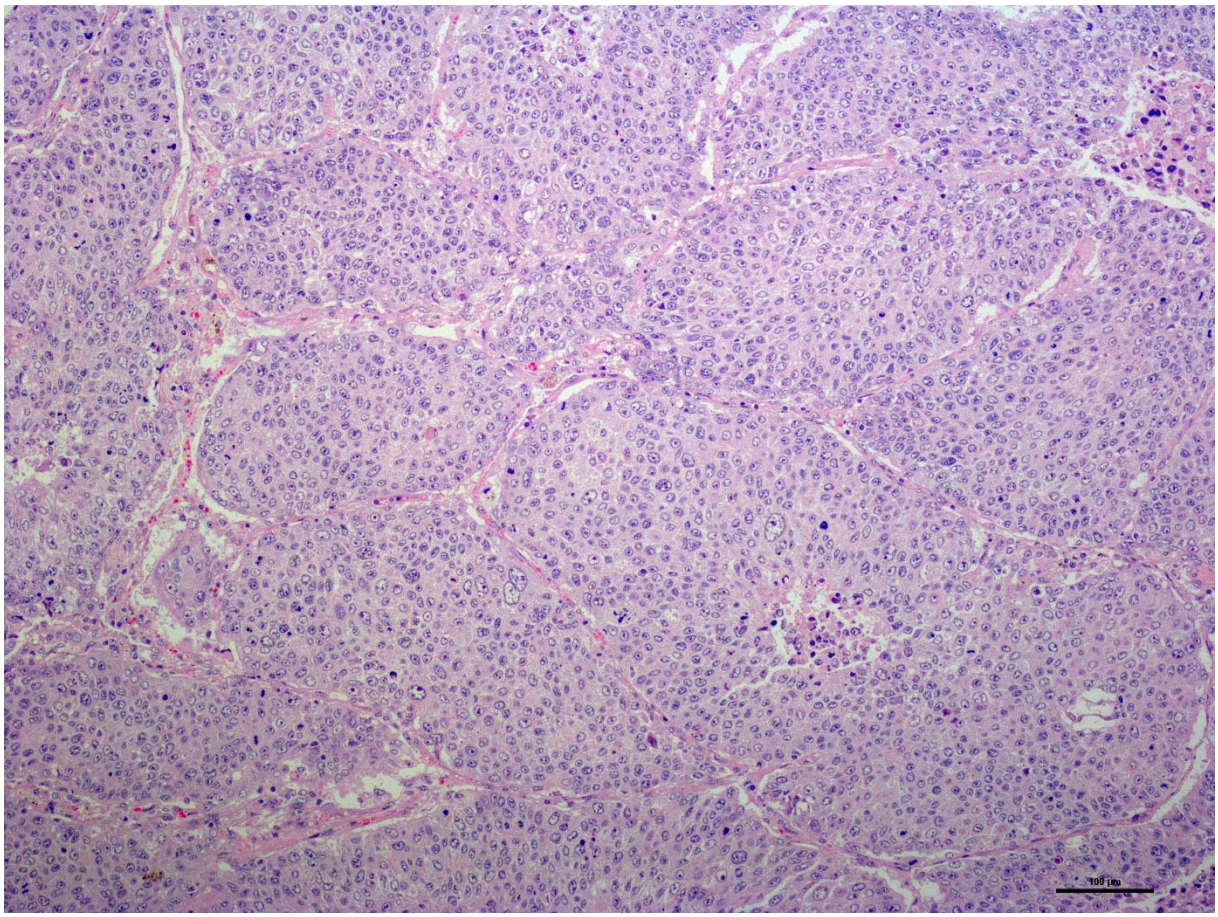

Figure 2: Histology SCC 100 fold

6.2.2 HPV Detection

All of the analyzed tumor biopsies were negative for HPV by nested PCR (0/14). The prevalence of HPV infection in SCC detected by nested PCR in our sample was zero percent.

7. Discussion

Case-Control studies

In the literature the question of a possible causality between HPV infection and lung cancer is discussed very critically. To further evaluate this topic case-control studies on HPV prevalence in both lung cancer samples and benign lung disease samples have been done in different countries.

In a case-control study conducted by Zhang et al. (92) in 2009 HPV was detected in 44.1 % of lung cancer cases and 8.3 % of benign lung disease respectively which shows a significant difference. They concluded that HPV is an important risk factor for lung cancer development. While studies like these have been showing significant difference of HPV infection levels in Asia, the studies done in the other parts of the world have not been as distinct.

In another case-control study from Iran (83) the prevalence of HPV DNA was significantly higher in the case group compared to the control group, 25.6 % vs. 9 % respectively, as well. The control group consisted of patients with lung diseases other than cancer. Even though the HPV prevalence in the lung cancer group is significantly lower than in the Chinese study, the prevalence in the control group is on a similar level.

Both studies used samples from patients with benign lung disease as controls. One of the problems with these samples could be that the risk factors for those diseases are the same as for the development of lung cancer, e.g. smoking. Some publications have discussed an association between smoking and HPV infection due to the higher vulnerability of the bronchial epithelium. In this case smoking might be the initiating factor for the development of lung cancer and HPV infection might only follow as a co-factor.

In a European case-control study carried out in Italy and the United Kingdom 100 tissue samples of primary SCCs of the lung and adherent non neoplastic lung tissue (n=85) were tested for the presence of HPV DNA. (42) There was no HPV DNA detected, neither in the tumor nor in the control samples. Since the positive control for DNA quality was successfully amplified, all tested specimens were declared free of HPV infection.

This might indicate that in fact HPV in lung tissue, whether malignant or not, is not very common in Europe. Our study result on primary SCC samples is in concordance with these results.

In this systematic review the difference between HPV positive cases and controls was statistically significant, 31.3 % versus 5.4 % ($p<0.01$). When the difference between cases and controls is compared separately in each of the continents, it is statistically significant as well in Europe as in Asia. Only one case-control study from The Americas was included (106). The difference in HPV prevalence was statistically significant.

But there have also been studies that did not find a statistical difference between the HPV prevalence detected in lung cancer cases and control samples. An Italian study on 50 cases of primary SCC of the lung and 23 control cases of wedge resections for non-neoplastic pneumothorax the HPV prevalence was detected to be 4 % and 4.3 % respectively. (43) Even though the number of cases was relatively low it shows presence of HPV DNA in squamous cell carcinoma of the lung, but not statistically significant when compared to non-neoplastic lung tissue.

There is a strong indication that HPV prevalence in lung cancer is higher than in benign lung tissue even though there have been some studies which established contradictory results. Whether HPV prevalence does play a causal role in lung cancer development is not yet decided either.

Studies from Germany and other European Countries

In a study conducted in France by Thomas et al. HPV prevalence was detected to be 16 % (109), which is a lot lower than the average detection rates in Asia.

Miasko et al. found a similar HPV prevalence conducted in Poland in two separate studies of 10 % and 12.7 % respectively. (46, 47) In these studies low risk as well as high risk HPV subtypes were detected.

To this day only one study conducted in Germany showed HPV DNA in primary lung tumor tissue. (41) Eberlein-Gonska et al. tested primary lung cancer tissue of 50 patients and 15 biopsies of normal bronchial mucosa in 1992. HPV DNA was detected two cases of SCC and one case of AC by PCR. None of the control biopsies were positive. Two other studies

conducted in Germany met the inclusion criteria (52, 57). Neither of them detected HPV DNA in the tested lung cancer tissue.

The only Dutch study that met the inclusion criteria (55) detected no HPV DNA in 211 cases of primary lung cancer by PCR with consensus primers GP5+/GP6+ in 2013.

In another French study carried out in 2005 HPV prevalence was found to be only 1.8 % in NSCLC. (40) Of all the 218 cases only four were positive for HPV 16. A Polish study from 2012 showed HPV DNA only in one of 33 cases and the one detected was HPV 120. (49)

A Finish study from 2012 did detect HPV DNA in primary lung cancer cases even though the prevalence was low 5.2 % (54).

Another Greek study analyzed 67 tumor samples of NSCLC for presence of HPV DNA and E6/7 mRNA. (37) Two of the tumor samples were detected to contain HPV DNA but the corresponding test for mRNA turned out to be negative. Only few studies have tested tumor samples for active transcription of mRNA. The authors concluded that most likely, at least in this population, HPV DNA does not play a role in lung cancer development. More studies should be done including detection of active protein transcription of HPV. Thereby a better understanding of the role of HPV in the tested tissue could be gathered and a better understanding of its possible role in carcinogenesis could be gained.

The highest detection rate for HPV was found in a study conducted in Greece as well (48). In this study 52 cases of SCC were tested for the presence of HPV by PCR using consensus primers followed by Southern blot hybridization. 21 cases showed positivity by PCR (40 %). Another Greek study by Sarchianaki et al. found HPV prevalence to be 19 % (51) which is the second highest HPV prevalence found in Europe. This might indicate that there is a geographic difference in the distribution of HPV infection within Europe similar to the geographic difference in HPV prevalence in Asia compared to Europe.

It has to be noticed that in the included study from neighboring Turkey the detected HPV prevalence was only 5.0 % (58). Another Turkish study conducted by Zafer et al. (58) detected HPV in 2 out of 40 non-small cell lung cancers. Both were squamous cell carcinomas and all the included patients were smokers.

In general there seems to be a lower rate of HPV detection rates in Europe with some difference in geographic distribution. Germany to this day is one of the countries with the lowest HPV detection rates in Europe and worldwide.

Tissue sampling and processing method

An American study on a cohort of 399 primary SCC and AC of the lung did not detect any HPV DNA either. (102) Testing was done on formalin-fixed and ethanol-fixed tissue specimens. A difference in detected HPV prevalence depending on the tissue processing has been previously discussed. The most common tissue processing method is formalin-fixed paraffin-embedded tissue because it is the method used most often to store tissue samples for long periods of time. Some scientists have annotated that certain parts of HPV DNA might be lost during this processing which might explain the low HPV prevalence in certain studies depending on the tissue processing methods used.

In another study done on archival tumor tissue in the United States HPV DNA prevalence was detected to be 16.7 %. (101) All of the tumor specimens employed in their study were obtained from the Tumor Tissue Bank at the University of Louisville. They were stored not as paraffin-embedded samples but in their frozen form. This might be a possible explanation for the higher detection rate of HPV DNA.

Some of the included studies analyzed tissue samples processed in both ways (36, 48, 62, 77, 79). Unfortunately none of these studies directly compared the detected HPV prevalence in tissue processed either way. The results were reported as one.

In our systematic review the detected HPV prevalence in FFPE and fresh frozen lung cancer samples was almost identical 13.4 % and 13.5 % respectively. The difference was not statistically significant.

Another aspect of the analyzed tumor tissue specimens that has to be taken into account is the tissue sampling method. Most of the included studies analyzed HPV in resected lung tissue. The second most common tissue sampling method is bronchoscopic biopsy. The histological samples obtained by operation provide a lot more tissue which allows for several rounds of PCR detection if needed. It also allows for samples to be stored for later analysis.

Another factor that might bias the results is that operative lung resection is only done in early stage or locally advanced lung cancers. Even though only few of the included studies provided information on the lung cancer stage of the included patients, it has to be assumed that those were not stage IV cancer patients since they have no indication for lung resection. There might be a possible association between HPV infection and tumor stage which might not be resembled in this systematic review.

Our own analysis was done on resected lung tissue that was formalin-fixed and paraffin-embedded which is the most common tissue specimen analyzed in the included studies. Still we did not detect HPV DNA by nested PCR.

Histological subtypes

Some authors have discussed whether HPV infection might be more strongly related with a specific histological subtype of lung cancer.

A study conducted on 313 fresh frozen lung cancer samples from central China in 2008 analyzed 215 SCCs and 98 ACs. The HPV prevalence in SCC was significantly higher than that in AC, 52.1 % vs. 26.5 % respectively.

In contrast a study from Norway (50) showed a significantly higher HPV prevalence in lung adenocarcinoma compared to squamous cell carcinoma, e.g. 5,6 % and 1,2 % respectively. Even though the detected HPV prevalence in Europe is lower than in many Asian studies. There are several studies from Asia showing a higher HPV prevalence in lung adenocarcinomas than in squamous cell carcinomas. (60, 62)

In contrast a study conducted by Iwakawa et al. on 297 primary adenocarcinomas of the lung in Japan in 2010 did not detect any HPV DNA in any of the samples. (72) To exclude possible wrong negative results because of the implemented detection method they tested the tumor samples with both nested PCR and a one-step multiplex PCR with type specific primers. Both detection methods showed negative results, so that the authors came to the conclusion that the results were not based on low sensitivity of any of the methods as it had been previously described by other scientists.

Only very few studies included primary SCLC of the lung into their analysis. A small study from the USA conducted in 2007 analyzed 12 tumor biopsies of primary SCLC of the lung. No HPV DNA was detected in any of them. (98) Since there is a strong association between SCLC and smoking behavior the HPV prevalence in SCLC would be expected to be higher if HPV infection was in fact connected to smoking behavior. The data available in the literature on this topic is scarce so that no real trend can be derived from it.

An association specifically between SCC and HPV infection has been discussed since an association between squamous cell carcinomas and HPV in other organs sites has been shown. To this day the data is not conclusive on this question.

In our own experiments we did not detect HPV DNA in primary SCC of the lung by nested PCR. In this systematic review the difference between HPV prevalence in SCC and AC was statistically highly significant ($p<0.01$). The higher HPV prevalence was detected in SCC. The same is true if all continents are analyzed individually even though the difference between the individual HPV prevalence in the subgroups was only statistically significant in Asia ($p<0.01$).

HPV Subtypes

Regional differences in the distribution of different HPV subtypes have been discussed previously. Park et al. found a significantly higher prevalence of HPV 33 compared to HPV 16 and 18 in a study conducted on NSCLC samples from Korea. (85)

Another case-control study from China detected HPV 18 to be the most dominant type of HPV in NSCLC. HPV detection rate was significantly higher in the lung cancer group compared to the control group and was associated with the degree of histological differentiation and lymph node metastasis and thereby tumor stadium. (81)

In a study conducted in Iran in 2013 no HPV 16 DNA was detected but the prevalence of HPV 18 was 16 %. (73) In this study only Squamous Cell Carcinomas were included which are known to be closely related to smoking behavior. Until now no correlation between smoking and special subtypes of HPV has been found.

In contrast a study conducted in China and Pakistan only HPV 16 was detected in 60 cases of primary lung carcinoma. (59) All of these were cases were Squamous Cell Carcinoma.

In our systematic review the HPV prevalence of HPV 16 was higher than the HPV 18 prevalence in all continents, 6.0% and 3.2% respectively. The difference was statistically highly significant ($p<0.01$). The same is true if all continents are analyzed separately.

Detection method

There is huge variety of HPV prevalence in the lung detected in the included studies and in other literature reflecting on the topic. The detection methods mostly used for HPV detection are PCR and ISH.

A Chinese study analyzed 141 NSCLC tumor specimens with both nested PCR and in situ hybridization (ISH). (62) The concordance between HPV 16 and HPV 18 detection by PCR and ISH were 73% and 85,5% respectively. In both cases the HPV detection rates acquired by nested PCR were higher. In this study not only two different detection methods were used but the tissue was previously processed in two different ways: formalin-fixed paraffin-embedded and fresh frozen. Unfortunately no formalin-fixed probes of the non-lung cancer tissue were available. No HPV DNA was detected in the control group.

In a study from The Americas the concordance rate between ISH and PCR was 100 %. (108) The overall HPV prevalence in this study was only 1.5 %.

There has been some discussion about low specificity in PCR, which might lead to false negative results and as a final result to a higher HPV prevalence detected. While PCR is in fact a very sensitive method it does not allow to analyze which kind of cells etc. are infected by HPV but only provides the information that HPV DNA is included in the analyzed sample.

A study conducted in 2012 tested both lung tumor tissue and adjacent non-tumor tissue from the same patients by PCR using primers detecting the L1 region of HPV. They did not detect HPV in neither cases nor adjacent tissue.

Not only different detection methods but also differences between the executions of the methods could play a role in discrepancies of HPV prevalence detected.

A study from Bulgaria detected HPV prevalence in lung cancer samples by three parallel PCRs. They used the consensus primer GP5+/6+ and type-specific primers for HPV 16 and 18. The HPV prevalence detected by type-specific primers was higher than by consensus primers, 25 % vs. 3.8 % respectively. (110)

In our systematic review not enough studies provided information on the used PCR primers to calculate the differences in the resulting HPV prevalence detected. Only 26 of the included studies contained information on the PCR primers used. There was a huge variety of primers

used with MY09/MY11 and GP5+/6+ being ones most frequently implemented. Unfortunately the number of different primers used resulted in subgroups that were too small to provide statistically reliable results.

Many discrepant results have been published on the subject of HPV prevalence in lung cancer over the years. To create a more uniform group of studies for our systematic review we only included studies using PCR as detection method. Still there was a huge variability of HPV prevalence in the studies meeting the inclusion criteria.

In the last 30 years PCR technology has evolved. To understand whether this development has had an effect on the detected HPV prevalence the included studies conducted in the 1990s and those conducted in the 21st century were analyzed separately and the results were compared. The HPV prevalance detected in the studies conducted in the 1990s was higher than in the studies conducted in the 21st century, 37.9 % and 8.5 % respectively. The difference was statistically highly significant ($p<0.01$). PCR technique has developed and new methods like e.g. nested PCR are more frequently applied which might lead to a higher sensitivity. In our own analyzes we used nested PCR but did not detect HPV DNA in any of the samples.

It also has to be taken into consideration that the methods applied to detect HPV DNA in the included studies have been evaluated by analyzing cervical cancer specimens. Due to the biological difference between cervical and lung tissue there might be some difference in sensitivity and specificity of the applied methods.

In our study the tissue processing method did not make a statistical difference on the HPV prevalence. Some studies have discussed that certain parts of the HPV genome might be lost if the tissue is paraffin-embedded. If the primers engaged are using this regions as an aim the results would be false negative. There are no studies proving such a theory to our knowledge but it has to be taken into consideration when comparing study results.

Previous history of malignancy

One of the theories that has been discussed is that HPV prevalence might not be a marker for HPV infection but for a possible metastatic disease. This could be the case for example in women suffering from cervical intraepithelial lesions or cervical squamous cell cancers who

have gone undiagnosed. Many of the included studies did not provide information on previous malignancies of the included patients.

A Norwegian study from 1999 compared HPV prevalence in women with both CIN III (cervical intraepithelial neoplasia) lesion and second primary cancers of the lung compared to women with primary lung cancers without previous history of CIN. (45) There results indicate that HPV might be a factor in both CIN III lesions and second primary lung cancers after CIN III lesions. Even though the HPV prevalence in the control group was lower than in the group of the secondary bronchopulmonary carcinomas (14 % and 32 % respectively), the difference was not significant (p=0.13). In this study only low risk HPV subtype 6 was detected in the control group which has to be taken into consideration.

In Canada a study on NSCLC cases was conducted in 2013. (108) Yanagawa et al. tested HPV prevalence in 336 cases of surgically resected primary NSCLC. All samples were formalin-fixed paraffin-embedded and histology was determined to be SCC in 132 and AC in 203. This study showed a low HPV prevalence in NSCLC in North American patients. Even though all included tissue samples were classified as primary lung cancers, all of the patients who were positive for HPV DNA had a previous history of cervical cancer or head and neck cancer. HPV prevalence was detected 1.5 % with all five positive cases being SCC. In this study all patients that were found to have a HPV positive tumor had a previous history of either head and neck cancer (n=2) or cervical squamous cell carcinoma (n=3).

In our own study two of the patients did have a previous history of malignant disease. One patient previously had a head and neck tumor and the other suffered from thyroid cancer.

The lungs are one of the most common places of metastatic disease. Differentiation between primary lung cancer and metastatic disease is especially difficult if both tumors show the same histology in light microscopy.

A Dutch study conducted in 2013 tried to differentiate tumors presenting in the lung by HPV infection. They analyzed both primary lung cancer (from patients without or unrelated malignancies in their past) and lung tumors whose origin was not determined at the time of diagnosis. None of the primary lung cancers were found to be HPV positive but three of the equivocal carcinomas were. All of these patients had a history HPV associated malignancies (one tonsillar carcinoma and two cervical carcinomas).

Since a possible association between primary lung cancer and HPV infection has not been excluded a differentiation between lung metastasis and primary lung cancer cannot be made.

However if the radiological findings in the lung seem to be those of a metastatic lung disease and histological biopsies are obtained, HPV infection could be used as a hint to the possible primary since some tumors have been proven to have a strong association to HPV infection (e.g. cervical cancer, head and neck cancer, tonsillar cancer). (30)

Detection in different organ sites

Since the association between cervical cancer and HPV infection has been proven a lot of research on a possible association between HPV infection and malignancies has been done not only concerning lung cancer.

A study from Pakistan compared the HPV prevalence in lung and breast cancer. (69) The HPV detection rate was 11 % and 17 % respectively. HPV has not yet been identified to play a significant role in breast cancer but it has been in other organ sites, especially in squamous cell carcinomas.

In an Iranian HPV prevalence study 30 cases of SCC of the lung and oral cavity were compared. (65) While three samples in the lung cancer group were detected to be HPV positive, six SCCs of the oral cavity were positive. But the difference was not statistically significantly higher.

In a systematic review on head and neck cancer conducted by Kreimer et al. in 2005 the overall HPV prevalence was calculated to be 25.9 %. It was significantly higher in oropharyngeal SCCs than in oral SCCs and laryngeal SCCs. (111) Almost all detected HPV subtypes in this systematic review were HPV 16 or 18. Because of the high HPV prevalence detected in head and neck cancers the authors concluded that there might in fact be a causal relationship even though it has not been proven yet. Since the risk factors for lung and head and neck cancer are similar another possibility might be that HPV infection only plays a role as a co-carcinogen in combination with risk factors like smoking for example.

Some researchers have tried to differentiate between primary lung cancer and metastases from previous head and neck SCC. (112) They concluded that HPV DNA could be an additional factor that could be useful to differentiate but it cannot be used as a single deciding factor.

The association between cervical cancer and HPV infection is well established and the association with anogenital cancers is commonly accepted. On the other hand a possible

association between head and neck cancer and other cancers e.g. breast cancer, bladder cancer, esophageal cancer is still subject to ongoing research and no real trends can be seen in the literature.

Possible microscopic correlations of HPV infection

The theory that there might be an association between lung cancer and HPV infection was first published by Syrjänen in 1980. (113) Histological findings similar to condyloma of the cervix were described and further investigated.

Since those findings different types of microscopic and electron microscopic investigations in lung cancer were described that were discussed to be associated with a possible HPV infection.

One of the observations made for lung cancer in some studies was koilocytosis. Koilocytosis is a feature of HPV infection in squamous epithelial cells. (114) Some of the cellular changes are: nuclear enlargement, hyperchromasia and a perinuclear halo.

In a study from China analyzing NSCLC specimens koilocytosis was found in several cases that were tested to be HPV positive. (87) In contrast an American study on 34 cases of primary SCC of the lung discovered koilocytosis in 11 cases but none of these cases were HPV positive by PCR. (96)

Some publications have reported Langerhans cell infiltration in lung cancer. (115) A study on HPV-infected squamous cell carcinoma and adenocarcinoma of the lung detected a high Langerhans cell infiltration. (82) The authors discuss that the high Langerhans cell infiltrations might be caused by HPV infection. High Langerhans cell infiltration was associated with HPV infection in both SCC and AC. In the HPV negative lung cancers the Langerhans cell infiltration was lower. In their study SCCs with high Langerhans cell infiltration was associated with a better prognosis. Studies on uterine cervical intraepithelial neoplasia have focused on Langerhans cell infiltration and even though there are some conflicting results, Langerhans cells seem to be HPV antigen-presenting cells. According to current knowledge they take part in initiating the immune response. (116) An association between smoking behavior and Langerhans cell infiltration has been discussed as well. Since smoking is the main risk factor for lung cancer development most of the patients analyzed in the studies were smokers.

HPV detection in material other than tumor biopsies

To secure lung cancer diagnosis a histological sample is needed. Usually such samples are gathered by bronchoscopy either via transbronchial fine needle puncture or by endobronchial ultrasound puncture of lymph node metastases. These sampling methods do not provide large tumor samples and due to recent advances in research more tests on tumor tissue are made e.g. immunohistochemistry, molecular pathology. Since the most established method for HPV detection is PCR this means additional tests of the bioptic material can be problematic. For this reason some studies have tried to detect HPV infection in less invasive material.

One possibility is to test cells found in pleural effusions. (117) In the majority of cases malignant pleural effusion is caused by lung cancer. (118) Still not all patients with lung cancer develop pleural effusion which is associated with a higher tumor stadium and pleural effusion very often only provides few tumor cells which can be difficult to process even to secure diagnosis.

In an Italian study by Carpagnano et al. (38) HPV detection by PCR was not only performed on tumor tissue but also in exhaled breath condensate (EBC). A total of 12 (16.4 %) of the included 89 tumor tissue specimens were detected to positive by PCR. All of them were matched with their EBC of which 11 (15.1 %) turned out positive. Not only did these two different tested materials provide similar prevalence rates but the detected HPV subtypes were the same as well. (e.g. 16, 30, 31, 39) All of the controls turned out negative both in EBC as well as in the paired lung tissue.

Several studies have tested blood samples for presence of HPV DNA. Buyru et al. tested blood samples from lung cancer patients and healthy control subjects for the presence of HPV DNA by PCR using consensus primers MY09/MY11. (119) They detected HPV DNA only in one of 65 patients (HPV prevalence 1.54 %).

A Chinese study tested serum samples of 183 lung cancer cases and 217 controls for antigen specific antibodies. No correlation between HPV 16 L1 or HPV18 L1 seropositivity and lung cancer risk was found. (120) These results were consistent with the results found in a study analyzing serum samples from the USA conducted by the same researchers. (121)

One meeting abstract from 2011 reports an increased lung cancer risk in the presence of HPV antibodies in European patients. (122) Unfortunately the abstract provides only very few details and a follow-up study could not be found. It reports that in a case-control study the serological antibodies for high-risk HPV was statistically significantly higher in lung cancer patients than in the control group. The author states that these findings are only descriptive and further research has to be done to establish a causal association between serum antibodies and lung cancerogenesis.

No marker for HPV infection in blood samples has been identified yet. According to current research HPV does not create viremia so most research is not directed toward detecting HPV DNA in blood samples but to find a marker for seropositivity and activity of a possible infection. (19) Interpretation of HPV antibodies is difficult since research on CIN has shown that HPV infection can be persistent for years in cervical epithelium before causing malignant transformation.

Another approach was tested by Mehra et al. (103) who conducted a pilot study to determine whether p16 could be used as a surrogate marker for HPV infection as it has been proposed in HNSCC. They detected HPV 16/18 prevalence to be 11 % but found no association with p16 expression.

Possible association between HPV infection and oncogenic mutations

A Japanese study done in 2016 analyzed the mutational spectrum in newly diagnosed NSCLC as well as the prevalence of HPV DNA in 876 lung cancer specimens. (76) The HPV prevalence was detected to be only 0.3 % and no association with any of the detected driver mutations was found. They did detect the mutational spectrum to be directly related to the environmental factors BMI and smoking. Mutations TP53, KRAS, EGFR and SMAD4 were proportionally linked to the smoking dose. A possible correlation between smoking dose and HPV prevalence as has been discussed by the authors but could not be detected. This might be in part because of the extremely low HPV prevalence.

Another Japanese study from 2012 found a significant correlation between EGFR mutation and presence of HPV DNA. (75) HPV prevalence was detected to be 16.7 % in 42 NSCLC probes and EGFR mutation was found in 31 %.

There have been more studies researching a possible association between EGFR mutations and HPV infection and some also focused on the gefitinib-response in that context. Gefitinib is a medication used in targeted cancer therapy and belongs to the class of EGFR inhibitors. It´s a selective inhibitor of the epidermal growth factor receptor's (EGFR) tyrosine kinase domain.

A Japanese study analyzed gefitinib-response in lung adenocarcinomas with high-risk HPV prevalence. (60) High-risk HPV DNA was found in six out of eight gefitinib-responsive adenocarcinomas (75 %). None of the 12 remaining adenocarcinomas (without gefitinib-response) were detected to be HPV positive. Unfortunately EGFR mutations were not tested in the screened adenocarcinomas. EGFR is known to be more prevalent in females, never-smokers and patients of Asian ethnicity.

In a Chinese study lung cancer tissue samples were analyzed for both HPV infection and EGFR mutations. (123) HPV DNA was significantly associated with EGFR mutations. HPV prevalence was 28.4 % and 46.3 % had EGFR mutations. These results are in concordance with a study conducted by Marquez-Medina et al.. (124)

EGFR mutations are more common in nonsmoking women from Asia and are strongly associated with adenocarcinoma. (125) The reasons behind these findings are not yet understood.

Some authors have discussed that a similar reason might be involved in the higher HPV prevalence in nonsmoking women in Asia and the high rate of EGFR mutations. What that might be is still undecided and subject to ongoing research. A possible explanation could e.g. be an association with ethnicity and hormonal factor.

In 1996 a Greek researcher group tested for HPV DNA and KRAS mutation in 99 surgically resected lung cancers. (53) They detected HPV DNA and KRAS mutation in the same case in 50 %. The HPV prevalence was 15 %, while KRAS mutation was detected in 18 % of the examined specimens. KRAS is the most frequent oncogene driver mutation in NSCLC in the western countries. It is associated with smoking and includes different genotypes that are still subject to research. (126) KRAS-mutation is associated with poor chemotherapy response so that extensive research has been done to develop treatment for these patients. The large biological heterogeneity of this mutations makes development of targeted therapies extremely difficult.

A possible explanation for these findings could be that HPV infection by itself is not sufficient for the development of lung cancer but might be involved as a co-factor in carcinogenesis.

Detection of active transcription of HPV

Most of the studies done on HPV prevalence in lung cancer use PCR on HPV DNA. It is known from studies done on cervical and oropharyngeal cancer specimens that presence of HPV DNA does not allow to draw conclusions on a causal relationship. Only few studies have tested for active transcription either by the detection of mRNA or the expression of one of the viral proteins. (39, 77, 127, 128)

A study conducted by Yang et al. tested bronchial brushing of patients with lung cancer and of benign lung disease for mRNA expression levels via real-time PCR. (129) They detected the levels of mRNA to be increased in the lung cancer group and the difference to the control-group was statistically significant.

Ciotti et al. analyzed lung tumor tissue as well as neighboring healthy tissue from the same patients from Italy for HPV E6 and E7 oncogenes and their transcripts. (39) Of the included 38 cases eight were positive for HPV DNA (21 %). Detection of HPV oncogene transcripts was done only on fresh tissue of HPV 16 positive cases. The E7 transcript was found in all but one case while in two rounds of amplification for E6 transcript only four patients were detected to be positive. All but one of the tumor specimens tested for transcripts contained either E6 or E7 transcripts. In one patient no viral transcripts was detected which could mean that in this case the detection of HPV DNA was accidental.

In future studies PCR for HPV transcripts to further evaluate whether the detected HPV prevalence is accidental or if in fact viral oncogenes are expressed.

Comparison with other systematic reviews and meta-analysis

Several systematic reviews and meta-analysis have been done concerning HPV prevalence in lung cancer.

A systematic review done in 2009 by Srinivasan on 44 studies evaluated the HPV prevalence of HPV type 16 and 18 in primary lung cancer including studies using PCR. (130) The detected prevalence for HPV 16 and HPV 18 was 7.1 % and 5.6 % respectively. The detected HPV prevalence ranged from 0 % to 78.3 %. In our own systematic review the prevalence for HPV 16 DNA was higher than for HPV 18 as well. But the prevalence was lower for both HPV 16 and HPV 18 (6.0 % vs. 3.2 %). They also analyzed whether the used PCR primers had an influence on the detected HPV prevalence. The studies using type-specific HPV primers resulted in higher HPV detection rates. In this study only HPV DNA was tested which is not sufficient to establish a causal relationship.

Another meta-analysis tested HPV prevalence in lung cancer cases compared to a control group. (131) Nine studies met the inclusion criteria resulting in 1094 analyzed cases and 484 controls. The detected HPV prevalence in the lung cancer cases was higher than in the control group, 37.57 % and 10.54 % respectively. The association between LC and HPV was statistically significant. In our analysis the HPV detection rates in both subgroups were lower, 31.3 % and 5.4 %. Nonetheless the detected difference in the HPV prevalence was statistically significant as well.

A study focusing on the HPV incidence in lung cancer analyzed 53 studies with a total of 4508 cases total. (132) The overall HPV prevalence was higher than in our analysis (24.5 %), similar to our own analysis the HPV prevalence in Asia was the highest of the compared continents. The most prevalent high-risk HPV subtypes were 16, 18, 31 and 33. This is in concordance with the HPV subtypes most frequently detected in our own systematic review. In this study the detected heterogeneity between studies was high as well. They discussed that different detection methods or tissue processing methods might be the reason. Our subgroup analysis did not find a statistic significant difference between FFPE vs. fresh-frozen tissue and only studies using PCR were included.

A systematic review and meta-analysis from 2017 researched HPV infection as a potential prognostic marker for lung adenocarcinomas. (133) The pooled hazard ratio did not show a significant correlation between HPV positivity and overall survival (OS).

Another specific topic analyzed is a possible association between HPV in NSCLC and never smokers. (134) The included studies in this systematic review used PCR as HPV detection method. The HPV prevalence with NSCLC in never-smokers was 31.4 %. They also compared the HPV prevalence between smokers and never smokers on the different

continents. HPV prevalence was significantly higher in never smokers in Europe, while the HPV prevalence in smokers and never smokers was similar in Europe.

Since HPV infection could be an important risk factor in women and never smokers most of all in East Asia Bae et al. analyzed the available literature focusing on HPV prevalence in female never smokers. (135) Four case-control studies were included and the results showed a significant effect on lung cancer in female never smokers.

Overall the detected HPV prevalence in the other systematic reviews was similar to our own findings. (128, 136)

Publication and methodical bias

To avoid possible publication bias not only journal articles but also meeting abstracts, poster presentations and information from study registrations were included. Still it has to be assumed that there is unpublished information which could not be found and reviewed for inclusion. Negative results are less likely to be published which can distort the overall results. One of the disadvantages of including shorter publications such as poster presentations is that they usually do not provide a lot of details which does influence the information that can later be analyzed. For example the included patient samples might have been discussed in another study but this information might not be included in the provided abstract. As a last consequence this might lead to information counted multiple times.

By including all languages and time periods a broad range of studies was detected. Even though the initial data bank research was done using English search vocabulary which might lead to publications not being detected if neither title nor abstract was included in an English version. Internet search engines are usually only check for Arabic letters which improves the chances of studies written in languages using Arabic letters compared to study e.g. using Chinese letters.

Heterogeneity between the studies was high in our systematic review. The subgroup analysis did not provide an explanation. We tried to minimize methodical bias by only including studies that used PCR for HPV DNA as primary detection method. The decision to include studies using PCR was made because of its high sensitivity and specificity. (137)

8. Conclusion

In this systematic review conducted on 73 studies that provided information on HPV prevalence in primary lung cancer tissue examined by PCR the overall HPV prevalence detected was 13.6 %. This rate was lower than in most systematic reviews made.

A possible explanation might be that only studies using PCR were included while studies using ISH are discussed to detect higher prevalence.

The HPV prevalence was analyzed for Europe, Asia and The Americas since no studies from Australia or Africa met the inclusion criteria. The highest HPV prevalence was detected in Asia followed by The Americas and Europe. This is in concordance with the information available in the literature even though no explanation has been found to this day. A geographic distribution as well as an ethnic component to HPV infection in lung cancer have been discussed.

In a subgroup analysis researching HPV 16 and 18 prevalence in the three continents of which studies were included the difference was statistically significant in all of them. HPV 16 prevalence was higher on all three continents. Overall HPV prevalence 16 and 18 was 6.0 % and 3.2 % respectively which was statistically highly significant as well ($p<0.01$).

In our review HPV detection was more strongly associated with SCC than with AC. The difference was statistically significant. (18.6 % vs. 9.6 %, $p<0.01$)

Even though the tissue processing method (FFPE vs. fresh frozen) did not result in a statistical difference in the detected HPV prevalence the time-period in which the analysis was made (1990s vs. 21st century) resulted in a statistically highly significant difference in the HPV prevalence that was detected ($p=0.9$ vs. $p<0.01$ respectively).

The difference between HPV prevalence in the cancer and the control group in our analysis was statistically highly significant (31.3 % vs. 5.4 %, $p<0.01$) This result has been found in other systematic reviews as well which has led many authors to discuss a possible association between primary lung cancer and HPV infection.

In the last 20 years lung cancer therapy has constantly developed. Targeted therapies and immunotherapy have not only improved the overall survival rates but have also changed the reality of cancer therapy for patients with more treatment being done by oral medication or in

an ambulant setting. Also the side effects that have to be expected during these therapies are different and often more manageable.

Still lung cancer has a very high mortality rate and is often diagnosed in a very late tumor stage. HPV infection as a modifiable risk factor has been discussed for a while now and since a vaccination is available some authors discuss a possible indication not only for young girls but also for young boys. A causal connection has not yet been made between lung cancer and HPV infection but since it has been between HPV infection and anogenital and other cancers Australia has established the National HPV Vaccination Program**.** It provides free vaccination in schools for both boys and girls aged 12-13 years. Since 2018 HPV vaccination for boys is recommended in Germany as well.

Still the most important modifiable risk factor for lung cancer is smoking. Therefore smoking cessation has to remain one of the most important goals in lung cancer prevention.

Mechanisms behind lung cancer development are still not fully understood. Neither are there established screening methods that allow earlier diagnosis.

The information in our systematic review and in other publications suggests that HPV infection could play a role in lung cancer development but many questions are still unanswered.

Most of the studies published used PCR on HPV DNA to determine the HPV prevalence in primary lung cancer specimens. Proof of HPV DNA in tumor samples is not enough evidence to prove a causal relationship between HPV and lung cancer development. One way to collect further information is to test for oncoprotein expression or mRNA. Another way is to confirm that HPV DNA is in fact integrated into the tumor genome. Only few studies using these methods have been done and more data will have to be gathered before a definitive decision can be made.

Another point that can not yet be answered is why there seems to be a geographic difference in HPV prevalence in lung cancer. All of the published systematic reviews and meta-analysis published found the highest HPV prevalence in Asia which is in concordance with our own results. No explanation for this has been found.

Some studies have discussed whether HPV is not an independent risk factor for lung cancer but a co-factor. In many studies HPV is more strongly related with SCC which is known to have a strong correlation with smoking. The very low detection of HPV in SCLC which has a very strong correlation with smoking behavior as well can be considered as a

counterargument. In contrast other studies have found a very high HPV prevalence in nonsmoking females with adenocarcinoma.

Many different possible infection routes have been discussed. Based on todays knowledge HPV infection does not cause viremia even though that seems to be the most likely way of infection. Because of the given anatomy a direct infection of the bronchial epithelium seems unlikely. The way in which HPV enters host cells has not yet been fully understood either.

Further research should be done on the large heterogeneity of HPV prevalence worldwide. Some of the possible influencing factors might be e.g. different PCR protocols, different PCR primers used or differences in the host factors.

In conclusion further research has to be done most of all to examine the causality of HPV prevalence in lung cancer.

9. Table of illustrations

9.1. Pictures

9.2 Tables

10. Appendix

Figure 3: Prisma flowchart

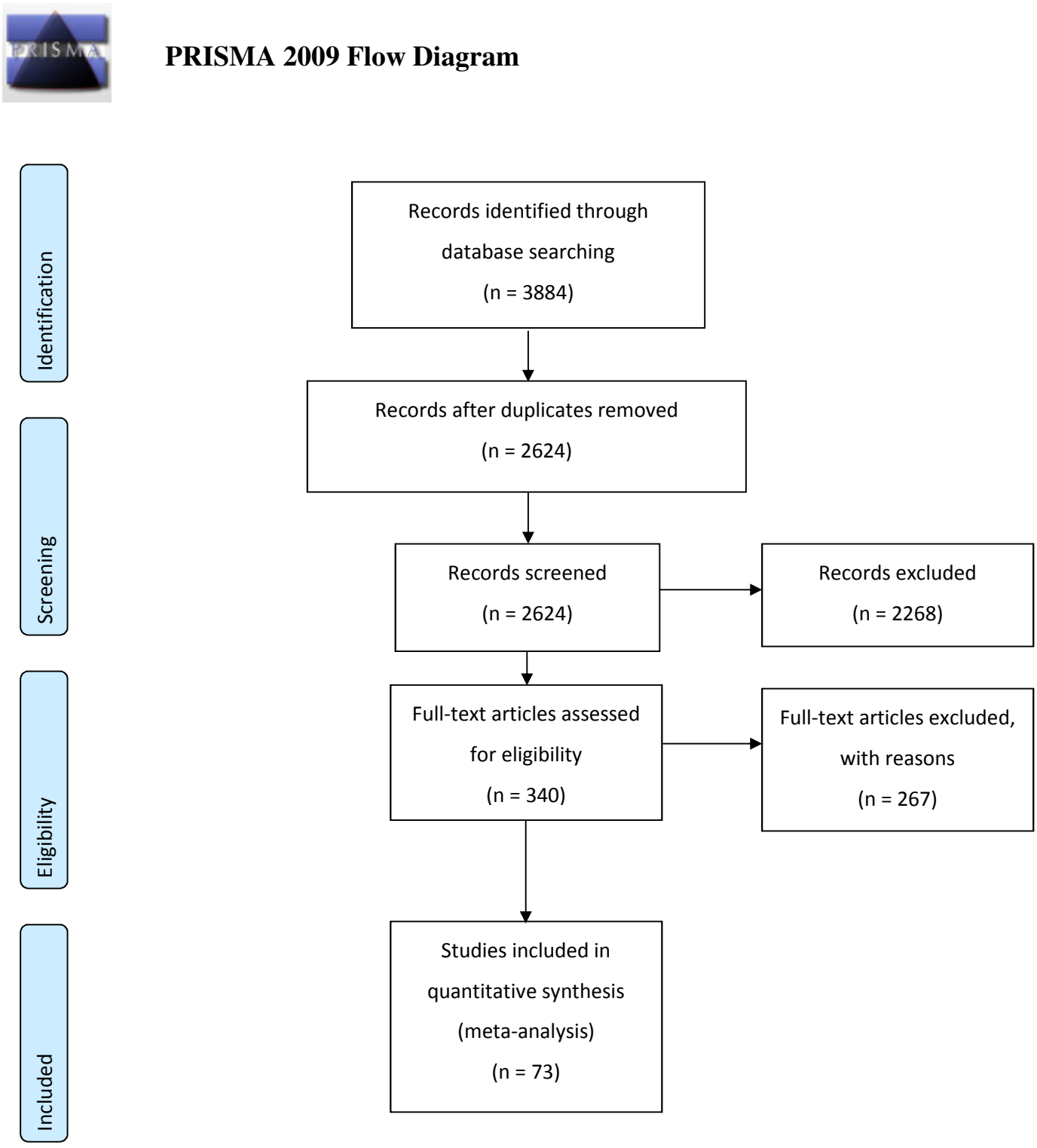

Table 19: List of publications

Number	Titel	First author	year	Reference number
1	Human papillomavirus-16 presence and physical status in lung carcinomas from Asia	Aguayo, F.	2010	(59)
2	Human papillomavirus-16 is integrated in lung carcinomas in Chile	Aguayo, F.	2007	(94)
3	No causal association identified for human papillomavirus infections in lung cancer	Anantharaman, D.	2014	(36)
4	Investigating the role of human papillomavirus in lung cancer	Argyri, E.	2017	(37)
5	Human papillomavirus is frequently detected in gefitinib-responsive lung adenocarcinomas	Baba, M.	2010	(60)
6	Human papillomavirus 16/18 infections in lung cancer patients in Mexico	Badillo-Almaraz, I.	2013	(95)
7	Detection of human papillomavirus in squamous cell carcinomas of the lung by polymerase chain reaction	Bohlmeyer, T.	1998	(96)
8	EGFR and KRAS mutations in patients having lung adenocarcinoma associated with human papilloma virus infection	Cardona, A.F.	2013	(97)
9	Biomarker-assisted diagnosis of ovarian, cervical and pulmonary small cell carcinomas: The role of TTF-1, WT-1 and HPV analysis	Carlson, J. W.	2007	(98)
10	HPV in exhaled breath condensate of lung cancer patients	Carpagnano, G.E.	2011	(38)
11	Human papillomavirus in lung carcinomas among three Latin American countries	Castillo, A.	2006	(99)
12	Gender difference in human papillomavirus infection for non-small cell lung cancer in Taiwan	Cheng, Y. W.	2004	(61)
13	The association of human	Cheng, Y. W.	2001	(62)

	papillomavirus 16/18 infection with lung cancer among nonsmoking Taiwanese women			
14	Detection and expression of human papillomavirus oncogenes in non-small cell lung cancer	Ciotti, M.	2006	(39)
15	Prevalence of human papillomavirus in lung carcinomas: A study of 218 cases	Coissard, C.J.	2005	(40)
16	Polymerase chain reaction demonstration of human papillomavirus type 16 in a lung adenocarcinoma and two squamous cell carcinoma.	Eberlein-Gonska, M.	1992	(41)
17	Correlation between squamous cell carcinoma of the lung and human papillomavirus infection and the relationship to expression of p53 and p16	Fan, X.	2015	(63)
18	Testing of human papillomavirus in lung cancer and non-tumor lung tissue	Galvan, A.	2012	(42)
19	Human papillomavirus infection in lung squamous cell carcinoma and correlation to p16 INK4A expression from an Argentine population	Garcia Falcone, M.M.	2017	(100)
20	Human papillomavirus DNA and p16 gene in squamous cell lung carcinoma	Gatta, L.B.	2012	(43)
21	Detection of oncogenic viruses (SV40, BKV, JCV, HCMV, HPV) and p53 codon 72 polymorphism in lung carcinoma	Giuliani, L.	2007	(44)
22	Human papillomavirus infection in lung and esophageal cancers: Analysis of 485 Asian cases	Goto, A.	2011	(64)
23	Human papillomavirus infection in lung vs. Oral squamous cell carcinomas: A polymerase chain reaction study	Halimi, M.	2011	(65)
24	Small cell neuroendocrine carcinomas of the lung do not harbor high-risk human	Hartley, C.P.	2015	(66)

	papillomavirus			
25	HPV positive bronchopulmonary carcinomas in women with high-grade cervical intraepithelial neoplasia (CIN III)	Hennig, E.M.	1999	(45)
26	Human papillomavirus DNA in squamous cell carcinoma of the lung	Hirayasu, T.	1996	(67)
27	Ultrastructural study if intranuclear inclusion bodies of pulmonary adenocarcinomas	Hiroshima, K.	1999	(68)
28	Detection of human papillomavirus-16 Dann in archived clinical samples of breast and lung cancer patients from North Pakistan	Ilahi; N. E.	2016	(69)
29	Molecular analysis of human papillomavirus in never-smokers with non-small cell lung cancer	Isa, S.I.	2015	(70)
30	Driver mutations associated with smoking and other environmental factors:	Ito, N.	2014	(71)
31	Prevalence of human papillomavirus 16/18/33 infection and p53 mutation in lung adenocarcinoma	Iwakawa, R.	2010	(72)
32	Genotyping of human papillomavirus and TP53 mutations at exons 5 to 7 in lung cancer patients from Iran	Jafari, H.	2013	(73)
33	Infection of human papillomavirus type 18 and p53 codon 72 polymorphism in lung cancer patients from India	Jain, N.	2005	(74)
34	Human papillomavirus (HPV) and Merkel cell polyomavirus in non small cell lung cancer	Joh, J.	2010	(101)
35	EGFR mutations and human papillomavirus in lung cancer	Kato, T.	2012	(75)
36	Prospective analysis of oncogenic driver mutations and environmental factors: Japan molecular epidemiology for lung cancer study	Kawaguchi, T.	2016	(76)

37	Human papillomavirus type 18 DNA and E6-E7 mRNA are detected in squamous cell carcinoma and adenocarcinoma of the lung	Kinoshita, I.	1995	(77)
38	Assessment of human papillomavirus in lung tumor tissue	Koshiol, J.	2011	(102)
39	No Detection of Episomal or Integrated High-Risk Human Papillomavirus in Nonsmall Cell Lung Carcinomas among Korean Population	Lee, J. E.	2016	(78)
40	Detection of human papillomavirus types 16, 18 DNA related sequences in bronchogenic carcinoma by polymerase chain reaction	Li, Q.	1995	(79)
41	An association of DNMT3b protein expression with P16INK4a promoter hypermethylation in non-smoking female lung cancer with human papillomavirus infection	Lin, T. S.	2005	(138)
42	Expression of HIF-1alpha and P-gp in non-small cell lung cancer and the relationship with HPV infection	Lu, Y.	2016	(81)
43	A pilot study of the association and prevalence of the human papillomavirus (HPV) in non-small cell lung cancer (NSCLC)	Mehra, R.	2013	(103)
44	Presence of human papillomavirus DNA (HPV DNA) in non-small-cell lung cancer	Miasko, A.	2004	(46)
45	Detection of human papillomavirus in non-small cell lung carcinoma by polymerase chain reaction	Miasko, A.	2001	(47)
46	Extremely high Langerhans cell infiltration contributes to the favorable prognosis of HPV-infected squamous cell carcinoma and adenocarcinoma of the lung	J. Miyagi	2001	(82)
47	Relationship between lung cancer and human papillomavirus in north of Iran, Mazandaran province	Nadji, S. A.	2007	(83)
48	Human papillomavirus DNA	Ogura, H.	1993	(84)

	in squamous cell carcinomas of the respiratory and upper digestive tracts			
49	Detection of human papillomaviruses in squamous cell carcinomas of the lung	Papadopoulou, K.	1998	(48)
50	The prevalence of human papillomavirus infection in Korean non-small cell lung cancer patients	Park, M. S.	2007	(85)
51	Human papillomavirus (HPV)-associated early stage non-small cell lung cancer (NSCLC)	Pillai, R. N.	2013	(104)
52	Detection, genotyping and phylogenesis of human papillomavirus (HPV) and Epstein-Barr virus (EBV) in patients with lung cancer	Podsiadlo, L.	2012	(49)
53	Detection of HPV in different subtypes of non-small cell lung cancer (NSCLC)	Rezazadeh, A.	2008	(105)
54	Molecular evidence of viral DNA in non-small cell lung cancer and non-neoplastic lung	Robinson, L. A.	2016	(106)
55	Human papilloma virus detection and typing in 334 lung cancer patients	Sagerup, C. M. T.	2014	(50)
56	Detection and genotype analysis of human papillomavirus in non-small cell lung cancer patients	Sarchianaki, E.	2014	(51)
57	Development of a broad spectrum PCR assay for papillomaviruses and its application in screening lung cancer biopsies	Shamanin, V.	1994	(52)
58	Detection of human papilloma virus (HPV) and K-ras mutations in human lung carcinomas	Spandidos, D. A.	1996	(53)
59	A strong inverse correlation between p16INK4a and pRb expression is observed at the level of individual tumor cells in HPV-negative primary squamous cell lung cancer	Suh, J. H.	2010	(107)
60	Detection of human papillomavirus genotypes in bronchial cancer using	Syrjanen, K.	2012	(54)

	sensitive multimetrix assay			
61	Detection of human papillomavirus DNA in primary lung carcinoma by nested polymerase chain reaction	Thomas, P.	1995	(109)
62	High-risk human papillomavirus-positive lung cancer: Molecular evidence for a pattern of pulmonary metastasis	Van Boerdonk, R. A. A.	2013	(55)
63	Frequent FHIT gene loss of heterozygosity in human papillomavirus-infected non-smoking female lung cancer in Taiwan	Wang, J.	2006	(86)
64	Human papillomavirus type 16 and 18 infection is associated with lung cancer patients from the central part of China	Wang, Y.	2008	(87)
65	The relationship among human papilloma virus infection, survivin, and p53 gene in lung squamous carcinoma tissue	Wang, Y. H.	2010	(88)
66	Human papillomavirus infection is not associated with bronchial carcinoma: Evaluation by in situ hybridization and the polymerase chain reaction	Welt, A.	1997	(57)
67	Detection of human papillomavirus DNA in squamous cell carcinomas of the lung by multiple polymerase chain reaction.	Xing, L. Q.	1993	(89)
68	Human papilloma virus genome is rare in North American non-small cell lung carcinoma patients	Yanagawa, N.	2013	(108)
69	A study on the relationship between HPV infection and the oncogenesis of primary squamous carcinoma of the lung	Yang, Y	1998	(90)
70	Effect of FHIT loss and p53 mutation on HPV-infected lung carcinoma development	Yu, Y.	2015	(91)
71	Detection and Typing of Human papillomavirus in Non-Small Cell Lung Cancer	Zafer, E.	2004	(58)
72	Variation of human papillomavirus 16 in cervical and lung cancers in Sichuan, China	Zhang, J.	2010	(93)

73	The relationship between HPV infection and the expression of insulin-like growth factor II in lung cancer and its clinical significance	Zhang, M.	2009	(92)

Table 20: Included Case-Control studies

	Author	Year	No. Cases	No. pos. Cases	HPV prevalence Cases	No. controls	No. controls positiv	HPV prevalence controls
	Carpagnano, G.E. (38)	2011	89	12	16.4%	68	0	0%
	Cheng, Y.W. (61)	2004	141	54	38.3%	60	1	1.%
	Cheng, Y. W. (62)	2001	141	77	54.6%	60	16	26.7%
	Eberlein-Gonska, M. (41)	1992	55	3	5.5%	15	0	0%
	Fan, X. (63)	2015	262	22	8.4%	19	0	0%
	Galvan, A. (42)	2012	85	0	0%	100	0	0%
	Gatta, L.B. (43)	2012	50	2	4.0%	23	2	4.3%
	Li, Q. (79)	1995	50	16	32.0%	22	0	0%
	Lu, Y. (81)	2016	72	33	45.8%	54	2	3.7%
	Nadji, S. A. (83)	2007	129	33	25.6%	89	8	9.0%
	Robinson, L. A. (106)	2016	70	9	30.0%	10	1	10.0%
	Wang, Y. (87)	2008	313	138	44.1%	96	4	4.2%
	Wang, Y. H. (88)	2010	45	19	42.2%	16	0	16.0%
	Yu, Y. (91)	2015	180	100	55.6%	110	7	7.3%
	Zhang, M. (92)	2009	68	30	44.1%	12	1	8.3%
Total			**1750**	**548**	**31.3%**	**754**	**41**	**5.4%**

Table 21: Included studies using FFPE processed tissue

	Author	Year	No. Cases	No. pos. Cases	HPV prevalence
	Aguayo, F. (59)	2010	60	8	13.0%
	Aguayo, F. (94)	2007	69	20	29.0%
	Baba, M. (60)	2010	57	11	19.3%
	Bohlmeyer, T. (96)	1998	34	2	5.9%
	Cardona, A.F. (97)	2013	132	52	39.4%
	Carlson, J. W. (98)	2007	12	0	0%
	Carpagnano, G.E. (38)	2011	89	12	16.4%
	Castillo, A. (99)	2006	36	10	28.0%
	Fan, X. (63)	2015	262	22	8.4%
	Garcia Falcone, M.M. (100)	2017	40	10	25.0%
	Gatta, L.B. (43)	2012	50	2	4.0%
	Goto, A. (64)	2011	304	24	7.9%
	Halimi, M. (65)	2011	30	3	10.0%
	Hartley, C.P. (66)	2015	20	0	0%

	Henning, E.M. (45)	1999	22	3	13.6%
	Hirayasu, T. (67)	1996	73	44	60.3%
	Hiroshima, K. (68)	1999	22	1	4.5%
	Ilahi; N. E. (69)	2016	9	1	11.1%
	Isa, S.I. (70)	2015	96	1	1.0%
	Jafari, H. (73)	2013	50	9	18.0%
	Kato, T. (75)	2012	42	7	16.7%
	Kawaguchi, T. (76)	2016	876	3	0.3%
	Lee, J. E. (78)	2016	233	0	0%
	Lin, T. S. (80)	2005	57	29	50.9%
	Lu, Y. (81)	2016	72	33	45.8%
	Miasko, A. (47)	2001	40	4	10.0%
	Miyagi, J. (82)	2001	121	41	33.9%
	Nadji, S. A. (83)	2007	129	33	25.6%
	Pillai, R. N. (104)	2013	208	32	14.9%
	Rezazadeh, A. (105)	2008	16	4	25.0%
	Sarchianaki, E. (51)	2014	100	19	19.0%

	Spandidos, D. A. (53)	1996	99	15	15.0%
	Suh, J. H. (107)	2010	48	1	2.0%
	Van Boerdonk, R. A. A. (55)	2013	211	0	0%
	Welt, A. (57)	1997	38	0	0%
	Xing, L. Q. (89)	1993	49	7	14.2%
	Yanagawa, N. (108)	2013	336	5	1.5%
	Yang, Y (90)	1998	50	13	26.0%
	Yu, Y. (91)	2015	180	100	55.6%
	Zhang, J. (139)	2010	104	18	17.3%
Total			**4476**	**599**	**13.4%**

Table 22: Included studies using fresh frozen tissue

	Author	Year	No. Cases	No. pos. Cases	HPV prevalence
	Coissard, C.J. (40)	2005	218	4	1.8%
	Galvan, A. (42)	2012	85	0	0%
	Guliani, L. (44)	2007	78	10	12.8%
	Iwakawa, R. (72)	2010	297	0	0%
	Jain, N. (74)	2005	40	0	0%
	Joh, J. (101)	2010	30	5	16.7%
	Ogura, H. (84)	1993	29	3	10.3%
	Robinson, L. A. (106)	2016	70	9	30.0%
	Sagerup, C. M. T. (50)	2014	334	13	3.9%
	Shamanin, V. (52)	1994	85	0	0%
	Thomas, P. (109)	1995	31	5	16.0%
	Wang, Y. (87)	2008	313	138	44.1%
	Wang, Y. H. (88)	2010	45	19	42.2%
	Zafer, E. (58)	2004	40	2	5.0%
	Zhang, M. (92)	2009	68	30	44.1%
Total			**1763**	**238**	**13.5%**

Table 23: Included studies conducted in the 1990s

	Author	Year of publication	Time of research	No. Cases	No. pos. Cases	HPV prevalence
	Baba, M. (60)	2010	1994 - 1996	57	11	19.3%
	Hirayasu, T. (67)	1996	1993	73	44	60.3%
	Hiroshima, K. (68)	1999	1995 - 1999	22	1	4.5%
	Miasko, A. (47)	2001	1998 - 1999	40	4	10.0%
	Miyagi, J. (82)	2001	1995 - 1997	121	41	33.9%
	Park, M. S. (85)	2007	1995 - 1998	112	60	53.6%
Total				**425**	**161**	**37.9%**

Table 24: Included studies conducted in the 21st century

	Author	Year of publication	Time of research	No. Cases	No. pos. Cases	HPV prevalence
	Argyri, E. (37)	2017	2013 - 2015	67	2	3.0%
	Badillo-Almaraz, I. (95)	2013	2006 - 2009	39	16	41.0%
	Fan, X. (63)	2015	2004 - 2010	262	22	8.4%
	Garcia Falcone, M.M. (100)	2017	2006 - 2016	40	10	25.0%
	Gatta, L.B. (43)	2012	2005 - 2010	50	2	4.0%
	Giuliani, L. (44)	2007	2002 - 2006	78	10	12.8%
	Halimi, M. (65)	2011	2009 - 2011	30	3	10.0%
	Hartley, C.P. (66)	2015	2004 - 2013	20	0	0%
	Ilahi; N. E. (69)	2016	2012 - 2014	9	1	11.1%
	Ito, N. (71)	2014	2012 - 2013	901	2	0.3%
	Jafari, H. (73)	2013	2006 - 2009	50	9	18.0%
	Jain, N. (74)	2005	2003 - 2004	40	0	0%
	Joh, J. (101)	2010	2006 - 2008	30	5	16.7%
	Kato, T. (75)	2012	2007 - 2008	42	7	16.7%

	Kawaguchi, T. (76)	2016	2012 - 2013	876	3	0.3%
	Lu, Y. (81)	2016	2012 - 2014	72	33	45.8%
	Pillai, R. N. (104)	2013	2002 - 2007	208	32	14.9%
	Podsiadlo, L. (49)	2012	2011	33	1	3.0%
	Robinson, L. A. (106)	2016	2000 - 2013	70	9	30.0%
	Sagerup, C. M. T. (50)	2014	2006 - 2011	334	13	3.9%
	Suh, J. H. (107)	2010	2003 - 2008	48	1	2.0%
	Syrjanen, K. (54)	2012	2008 - 2010	77	4	5.2%
	Wang, Y. H. (88)	2010	2008 - 2010	45	19	42.2%
	Yanagawa, N. (108)	2013	2001 - 2008	336	5	1.5%
	Yu, Y. (91)	2015	2012 - 2014	180	100	55.6%
	Zhang, M. (92)	2009	2006 - 2007	68	30	44.1%
Total				**4005**	**339**	**8.5%**

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A paper is currently being updated to include the newest data and will then be submitted for publication with Julia Karnosky as first author.

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