CHARLES UNIVERSITY
Faculty of Science

Human Malignancies Associated with Papillomaviruses:

From the Aetiology to Biomarkers

Habilitation thesis

2023 RNDr. Ruth Tachezy, Ph.D.

Content

1. List of publications

1.1 Chapters in monographies

1. Saláková M, Šmahelová J, Hamšíková E, **Tachezy R**. **„**Laboratorní diagnostika lidských papilomavirů.“ Respirační papilomatóza, TOBIÁŠ Medicína hlavy a krku, ed. Viktor Chrobok 2022, pp116-125, ISBN 978-80-7311-207-3.
2. Šmahelová J, **Tachezy R.** „Lidské papilomaviry.“ Respirační papilomatóza, TOBIÁŠ Medicína hlavy a krku, ed. Victor Chrobok 2022, pp60-63, ISBN 978-80-7311-207-3.
3. **Tachezy R**, Šmahelová J. **„**Úloha nízce rizikových lidských papilomavirů v patogenezi nádorů.“ Respirační papilomatóza, TOBIÁŠ Medicína hlavy a krku, ed. Victor Chrobok 2022, pp64-69, ISBN 978-80-7311-207-3.
4. Hamšíková E, **Tachezy R,** Němečková Š. „Profylaktické vakcíny proti lidským papilomavirům: prostředek primární prevence karcinomu děložního hrdla, eventuálně dalších nádorů spojených s infekcí HPV.“ Onkologická imunologie, Mladá fronta a.s., ed. E. Závadová, 2015, pp93-107, ISBN 978-80-204-3756-3.
5. Van Ranst M, **Tachezy R**, Delius H, Burk RD. Classification of the human papillomaviruses based on their molecular evolutionary relationship. In: “Human papillomavirus infection in dermatovenereology”, eds. Von Krogh G. and Gross G., CRC Press, Inc., Florida, U.S.A., 1996.
6. Van Ranst M, **Tachezy R**, Burk RD. Human papillomaviruses: A neverending story? In: “Papillomavirus Reviews: Current Research on Papillomaviruses”, ed. Lacey C., Royal Society of Medicine Services Ltd., Leeds University Press, p. 1-20, 1996.

1.2 Original publications in peer-reviewed journals with impact factor

1. Kadleckova D, **Tachezy R**, Erban T, Deboutte W, Nunvar J, Salakova M, Matthijnssens J.
   The Virome of Healthy Honey Bee Colonies: Ubiquitous Occurrence of Known and New Viruses in Bee Populations.
   mSystems, 7, 3, 2022. (cited 1x) **IF2021=7.328**
2. Kliegr T, Jarkovsky J, Jirincova H, Kuchar J, Karel T, **Tachezy R.**
   Role of population and test characteristics in antigen-based SARS-CoV-2 diagnosis, Czechia, August to November 2021.
   Eurosurveillance, 27, 33, 2200070, 2022. (cited 0x) **IF2021=21.286**
3. Pokryvkova B, Grega M, Klozar J, N, Vencalek O, Nunvar J, **Tachezy R.**
   PD1+CD8+ Cells Are an Idependent Prognostic Marker in Patients with Head and Neck Cancer.
   Biomedicines, 10, 11, 2794, 2022. (cited 0x) **IF2021=4.757**
4. Smahelova J, Hamsikova E, Ludvikova V, Vydrova J, Traboulsi J, Vecalek O, Lukes P, **Tachezy R.**
   Outcomes After Human Papillomavirus Vaccination in Patients With Recurrent Respiratory Papillomatosis: A Nonrandomized Clinical Trial.
   JAMA Otolaryngology-Head and Neck Surgery, 148, 7, 654-661, 2022. (cited 0x) **IF2021=8.961**
5. Rob F, Hugo J, Salakova M, Smahelova J, Gkalpakiotis S, Bohac P, **Tachezy R.**
   Prevalence of genital and oral human papillomavirus infection among psoriasis patients on biologic therapy.
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6. Pokryvkova B, Smahelova J, Dalewska N, Grega M, Vencalek O, Smahel M, Nunvar J, Klozar J, **Tachezy R.**

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2. My career path

My scientific career started in the Department of Parasitology at Charles University in Prague where I worked on my master´s degree thesis and continued for an additional year with a focus on comparing different species and strains of Trichomonads using molecular biological methods (1 paper). In 1989, I joined the Department of Experimental Virology at the Institute of Sera and Vaccines under the leadership of Professor Vladimir Vonka. This was relatively soon after the discovery that herpes simplex virus type 2 (HSV-2) is not the cause of cervical cancer (Vonka et al., 1984; Krcmar et al., 1986), a project which was the central one in the department for many years. It was also at the time of the first detection of human papillomavirus in cervical cancer tissue by the team of Harald zur Hausen (Dürst et al., 1983; Boshart et al., 1984). My research was therefore directed to papillomaviruses. From the beginning of my career, I faced the difficulties of working with clinical samples. One of the first tasks was establishing the *in situ* hybridization method in our laboratory and analysing the material available from the previous prospective study on HSV-2 for the presence of HPV DNA. I undertook a fellowship in the Department of Pathology at the University of Kuopio in Finland under the leadership of Professors Kari and Stina Syrjanen in order to learn the method of *in situ* hybridization and perform the technology transfer. Upon returning to Prague, I was invited to work as a research associate at Albert Einstein College of Medicine in New York. There, during my two-year stay, I got involved in establishing the new polymerase chain reaction technology for the detection and genotyping of a wide variety of human papillomaviruses in different types of clinical materials. As a side project, we were also interested in the detection and characterization of new animal papillomaviruses. After my return to the Czech Republic, I continued this research direction in close collaboration with the Laboratory of Clinical Virology at the Rega Institute at the University of Leuven in Belgium (8 papers). In 1991, the Institute of Sera and Vaccines underwent privatization, and the whole department was moved to the Institute of Hematology and Blood Transfusion (IHBT) where I worked from my return from the US until 2021. In 1998, I became the head of the National Reference Laboratory for Papillomaviruses, which was established in response to the request of the Society for Epidemiology and Microbiology of the Czech Medical Association of J. E. Purkyně. From 2012 to 2015, I was also head of the Laboratory of Molecular Epidemiology of Viruses. The team at the Institute of Hematology and Blood Transfusion participated in the research of haemato-oncological diseases (7 papers), anelloviruses transmitted by blood (2 papers), and viral complications after transplantation (BK polyomavirus, cytomegalovirus (2 papers)). In 2015, the National Reference Laboratory extended its scope and became the National Reference Laboratory for Papillomaviruses and Polyomaviruses. In 2015, I moved my research team to the newly established Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University (BIOCEV) where we continue our work with a focus on discovering new therapeutically and diagnostically relevant markers of malignancies of viral aetiology, while the reference laboratory was based at the IHBT until 2021. Since 2022, it is newly located at the Public Health Institute in Ostrava, still under my leadership. In 2019, I became head of the Department of Genetics and Microbiology at the Faculty of Science, Charles University.

In 2020, during the first wave of the COVID-19 pandemic when we faced the absence of commercially available diagnostic kits and shortage of laboratory capacities, the National Reference Laboratory at the Institute of Hematology and Blood Transfusion designed an in-house method for the detection of SARS-CoV-2 for high-risk haemato-oncological patients. At BIOCEV, in response to the COVID-19 pandemic, the research group under my leadership reorganized a part of the laboratories to meet the highest demands for work with infectious biological samples, made it possible to equip these laboratories, and recruited volunteers, especially PhD and postdoctoral students from BIOCEV and other research institutions in Prague. The whole infrastructure participated in the initiative of academic workplaces with the aim to accelerate the testing of the population for the presence of the SARS-CoV-2 virus in the first wave of the COVID-19 pandemic. High-capacity testing with quality control and management with more than a thousand samples could be analyzed daily. In May 2021, I became a member of the newly established Interdisciplinary Group for Epidemic Situations (MeSES). This expert advisory group of the Ministry of Health of the Czech Republic issued the perspectives, reports, and recommendations based on the newest scientific findings by integrating the views of diverse disciplines (economy, law, human sciences, medicine, biology, etc.). In 2022, I was appointed coordinator for Charles University for the newly started project National Institute of Virology and Bacteriology within the EXCELES program of the National Recovery Plan.

3. Introduction

Cancer, whose name comes from the Greek word karkinos (a giant crab), is nowadays part of the life of modern society. Despite the intensive and long-lasting research and improvements in diagnostic, prevention, and therapeutic methods, one in four people is diagnosed with cancer, and one of three people dies of cancer (http://gco.iarc.fr/today/data/factsheets/cancers/39-All-cancers-fact-sheet.pdf). The first traces of cancer were already documented in ancient Egypt in 1500 B.C. Historically there were numerous theories about the cause of cancers, starting with the humoral theory by Hippocrates, followed by the lymph theory, blasema theory, chronic irritation theory, trauma theory, and parasite theory (Sudhakar, 2009). It was not until the beginning of the 20th century that an infectious cause, the blood fluke *Schistosoma haematobium*, was suggested for the first time as an etiological factor of bladder and liver cancer (Ferguson, 1911).
Nowadays, multiple infectious agents causing or contributing to human cancer have been identified (Jacqueline et al., 2017). It has been estimated that in 2018, infectious agents were responsible for 2.2 million (13%) of 17 million of human malignancies worldwide (without non-melanoma skin cancer). The most prevalent of these malignancies is gastric cancer caused by *Helicobacter pylori* (810 000 cases), followed by many of cancers of different anatomical locations caused by human papillomaviruses (HPVs) (690 000), hepatocellular carcinoma attributed to infection by hepatitis B virus (HBV) (360 000) and hepatitis C virus (HCV) (160 000), and Epstein-Barr virus (EBV)-associated malignancies (156 000). The attributable fraction for infections varies by geographical location and socioeconomic status (Plummer et al., 2016; de Martel et al., 2020).
The majority of carcinogenic infectious agents are oncogenic viruses, with the first one, EBV, identified in 1964 (Epstein et al., 1964) and the last one, Merkel cell polyomavirus (MCV), in 2008 (Feng et al., 2008). The mechanisms of how viruses contribute to the carcinogenic process are very variable. Some act as a direct carcinogen (HPV, MCV, EBV, Kaposi's sarcoma herpes virus (KSHV)). Those are expressing viral oncogenes, which directly contribute to cell transformation. Others can act as indirect carcinogens. They cause chronic infection and inflammation, which leads to carcinogenic mutations in the infected cell. Several oncogenic viruses (HBV, HCV, Human T-lymphotropic virus type 1 (HTLV-1)) cannot be grouped in any categories mentioned above because it is still not known if prolonged infection and chronic inflammation linked to its infection is the only carcinogenic mechanism or if the expression of viral proteins is also necessary for initiation and maintenance of the precancerous or cancerous cell phenotype. For some malignancies, the listed viruses can also act as a cofactor (HPV in cutaneous malignancies) (Pagano et al., 2004; zur Hausen, 2006; Moore & Chang, 2010).
Proving the aetiology of oncogenic viruses in cancer is very difficult since the carcinogenic process is multistep and multifactorial. Furthermore, oncogenic viruses usually infect many people but cause the disease in only a minority of the infected individuals. This suggests that other factors must be involved in the carcinogenic process. Last but not least, cell transformation process is long and involves many genes. In the past, there were numerous

recommendations about the modification of the well-known Koch's and Hill's criteria to accommodate them to the study on the causality relationship of oncogenic viruses in tumors

Table 1 **Estimated numbers of infection-attributable cancer cases in 2018, by infectious pathogen, cancer subsite, and sex** (de Martel et al., 2020).

	Men		Women		Total	
	New cases	New cases attributable to infectious pathogens	New cases	New cases attributable to infectious pathogens	New cases	New cases attributable to infectious pathogens
Helicobacter pylori						
Non-cardia gastric cancer	550 000	490 000	300 000	270 000	850 000	760 000
Cardia gastric cancer	130 000	27 000	46 000	8900	180 000	36 000
Non-Hodgkin lymphoma of gastric location	12 000	8700	10 000	7600	22 000	16 000
Human papillomavirus						
Cervix uteri carcinoma*	..	..	570 000	570 000	570 000	570 000
Oropharyngeal carcinoma	110 000	34 000	26 000	8100	140 000	42 000
Oral cavity cancer	190 000	3900	91 000	2000	280 000	5900
Larynx cancer*	150 000	3600	22 000	≤1000	180 000	4100
Anus squamous cell carcinoma	9900	9900	19 000	19 000	29 000	29 000
Penis carcinoma*	34 000	18 000	..	..	34 000	18 000
Vagina carcinoma*	..	..	18 000	14 000	18 000	14 000
Vulva carcinoma*	..	..	44 000	11 000	44 000	11 000
Hepatitis B virus						
Hepatocellular carcinoma	490 000	270 000	170 000	90 000	660 000	360 000
Hepatitis C virus						
Hepatocellular carcinoma	490 000	100 000	170 000	40 000	660 000	140 000
Other non-Hodgkin lymphoma	260 000	8700	210 000	7200	480 000	16 000
Epstein-Barr virus						
Nasopharynx carcinoma*	92 000	76 000	35 000	29 000	130 000	110 000
Hodgkin lymphoma*	46 000	24 000	33 000	17 000	80 000	40 000
Burkitt lymphoma	7800	4100	3800	2500	12 000	6600
Human herpesvirus type 8						
Kaposi sarcoma*	28 000	28 000	14 000	14 000	42 000	42 000
Schistosoma haematobium						
Bladder carcinoma*	420 000	4000	120 000	1900	550 000	6000
Human T-cell lymphotropic virus						
Adult T-cell leukaemia and lymphoma	1900	1900	1700	1700	3600	3600
Opisthorchis viverrini and *Clonorchis sinensis*						
Cholangiocarcinoma	69 000	2100	56 000	1300	130 000	3500
All cancer types related to infection	..	1 100 000	..	1 100 000	..	2 200 000

The number of cases has been rounded to two significant digits. * Cancer site for which estimates were available in, and extracted directly from, GLOBOCAN 2018 via the Cancer Today website.

Table 1: **Estimated numbers of infection-attributable cancer cases in 2018, by infectious pathogen, cancer subsite, and sex**

(Rivers, 1937; Huebner, 1957; Evans, 1982; Vonka et al., 1987; zur Hausen, 1991). As recommended by Vonka (Vonka, 2000), the criteria proposed by Evans and zur Hausen were modified to reflect the progress in the biological and medical technologies and discoveries in the virology field. Vonka emphasized the epidemiological category as the most relevant one and classified the proposed evidence into direct (meaning more important), and indirect (meaning less important) categories, in terms of evaluating the hypothesis of an etiological

relationship between a virus and a tumour. The direct approaches include epidemiological testing for the prevalence of the virus in cases and controls, for epidemiological characteristics of tumour occurrence and virus spread, time sequence, and the effect of intervention against the virus on tumour incidence. The second approach involves immunological testing of virus-specific antibodies in patients with the virus detected in tissues and controls and/or patients without the presence of the virus in the tumour and antibodies detection in relationship to their clinical condition, and the third approach is based on molecular-biological detection of the virus-specific macromolecules in the tumours. Indirect approaches involve testing of the oncogenicity of the virus in animal models, the transformation capability of the virus on tissue cultures, and detection of persistent viral infection in the body.

4. Papillomaviruses

Human papillomaviruses are estimated to cause 30% of 2.2 million cancers with infectious aetiology, which occur annually. Most are cervical cancer cases (566 000 new cases and 280 000 deaths reported annually worldwide), with 85% occurring in developing countries.

The history of papillomavirus research can be considered a successful "story" from the development and later introduction of a screening test for atypical cervical cells to its implementation into routine practice, which dramatically decreased the incidence of cervical cancer worldwide (Papanicolaou & Traut, 1997) and from the detection of the first human high-risk papillomavirus in premalignant cervical tissue (Dürst et al., 1983) to the development of vaccines, with the first one approved for routine use in 2006. A dramatic decrease in the incidence of cervical cancer or even the elimination of it have recently been reported in some populations thanks to the early start of the routine vaccination and achievement of a high vaccine coverage (Lei et al., 2020; Falcaro et al., 2021; Kjaer et al., 2021).

4.1 Taxonomy, genome organization, and life cycles

Papillomaviruses are a diverse group of small DNA viruses that infect both humans and animals. They have been isolated from almost all species of vertebrates except amphibians (Bravo et al., 2010). Papillomaviruses are monophyletic. They co-evolved slowly with their hosts, causing mostly chronic asymptomatic infections. Except for documented recombination between polyomaviruses and papillomaviruses in bandicoot papillomatosis carcinomatosis virus types 1 and 2, no other cases of recombination inbetween papillomaviruses have been documented. All known papillomaviruses are host species-specific. The taxonomy and classification of papillomaviruses was first based on restriction patterns and genomic cross-hybridizations. Later, thanks to advances in the molecular biological techniques, the sequence of the conserved L1 gene was used to define a new isolate (de Villiers et al., 2004; Bernard et al., 2010). Novel HPV types are each assigned a unique number after the whole genome has been cloned and deposited at the International Human Papillomavirus (HPV) Reference Center at the Karolinska Institute in Sweeden (Van Doorslaer et., al, 2018). Currently 226 characterized human papillomaviruses are known. Recently, metagenomics technologies identified many potentially new HPV types, mainly from the Beta and Gamma genera. It is estimated that about 400 HPV types exist (de Villiers, 2013; Bzhalava et al., 2015). The nomenclature at the species level and above is determined by the Papillomavirus Study Group of the International Committee on Taxonomy of Viruses (ICTV). The family Papillomaviridae contains the subfamily Firstpapillomavirinae with an ever growing list of genera and the second subfamily Secondpapillomavirinae which includes only one genus. The subfamilies share less than 45% sequence identity of the L1 ORF, while the genera less than 60%. ICTV currently lists 53 genera and 133 species of papillomaviruses. Each species is recognized based on the whole genome sequence homology which must be less than 70%. In 2010, 69 established animal papillomavirus types were known (Bernard et al., 2010).

However, in the nomenclature of animal papillomaviruses, certain confusions were discovered and therefore
the International Animal Papillomavirus Reference Center was established at the University of Arizona (Van Doorslaer & Dillner, 2019), which will treat the newly discovered animal viruses in the same manner as the human papillomaviruses. The submitted clones and metadata will be analyzed, and the animal papillomavirus reference centre will formally recommend names for the viral isolates and store the reference clones.
Papillomaviruses are double-stranded DNA viruses with a genome length of 5 748 to 8 607 bp enclosed in a non-enveloped capsid of 50-60 nm in size. The genome contains up to nine open reading frames (ORF). The number of expressed viral proteins is larger than the number of ORFs because the genes are transcribed from multiple promotors and undergo splicing. All papillomaviruses have two genes coding for capsid proteins L1 and L2, of which the L1 coat protein is the main target of the host immune system. The antibodies elicited against the hypervariable amino acid loops of the L1 protein exposed on the surface of the capsid are type-specific and only poorly recognize distantly related types. The minor capsid protein has an immediate-early function in the delivery of the viral genome within the cell and, subsequently, in genome packing. All papillomaviruses contain early genes responsible for replication (E1, E2) and cell cycle regulation, immune evasion, and virus release (E6, E7, E5, E4). The long control region contains the origin of replication and binding sites for viral and cellular transcription factors. It is positioned between the end of L1 and the start of the early gene region. The E5 ORF is missing in some PVs from the Beta genera, the E6 ORF is missing in some types from the Gamma genera, and in some animal viruses, a second LCR is present between the end of the early region and the beginning of L2 ORF. PVs are characterized by a considerable heterogeneity in the positions of promoters and the splicing sites (Zheng & Baker, 2006).
Papillomaviruses infect keratinized and mucosal epithelia. In humans, they are ubiquitously and asymptomatically present in many locations in immunocompetent individuals. Their tropism is thought to be controlled by the anatomical site of infection and by the regulatory elements in the long control region of the viral genome. The viruses enter the epithelia via micro-lesions and bind to extracellular heparin sulfate proteoglycans on the basement membrane. After conformational changes of L1 and L2 and cleavage of furin motif present in L2, the virus enters the cell. The replication is, at the start, limited and regulated by E1 and E2. During the second maintenance phase, the viral genome is replicated in low but constant copy numbers in the proliferating cells. The E6 and E7 proteins can change the host environment so that the maintenance phase of viral replication can continue in differentiated cells, and this can last for years. The final phase occurs in the differentiated cells when viral DNA is amplified to high copy numbers, and progeny virions are produced. The virions are released from the cells, which peel off the surface.
For human papillomaviruses, the typical route of transmission is sexual, and HPV is the most common sexually transmitted virus in humans. Because papillomaviruses are non-enveloped, they are stable and resistant to commonly used disinfectants, and therefore, they can also be transmitted through non-sexual routes like self-inoculation, vertical transmission, and nosocomial transmission (Ryndock & Meyers, 2014). The presence of HPV DNA in non-

sexually active girls is still controversial. While some studies suggested the role of non-penetrative sexual contact, others did not (for review, see Hamsikova et al., 2017).

4.2 Diseases associated with human papillomaviruses

As mentioned above, papillomaviruses cause mostly asymptomatic infections in humans. The infection of the mucosa can manifest as a benign or malignant lesion. The typical benign lesions are condyloma acuminata, which mainly occur in the genital area of men and women and are highly contagious. The low-risk HPV types 6 and 11 are detected in over 99% of these lesions, even though a number of other genotypes were identified at these sites. Types HPV 6 and 11 also cause papillomatous lesions in the larynx of children and adults. This disease, called recurrent respiratory papillomatosis (RRP), has a high morbidity and is challenging to treat. **In our study, a series of 25 RRP cases was analysed. All cases were positive for HPV 6 or 11, and there was no difference in the prevalence of HPV-specific antibodies between patients and controls. Significantly higher antibody titres were detected in the patients who underwent 20 or more surgical procedures for the removal of the lesions** (Tachezy et al., 1994)**. Recently we have finished the phase IIIb clinical trial. The Gardasil vaccine was administered to 50 subjects with RRP, and these patients were followed up for five years. RRP recurrences turned out to be significantly lower after HPV vaccination, and the RRP patients thus experienced a reduced burden of disease.** Between patients with a new or recurrent disease, no difference was found in the frequency of recurrent postvaccination lesions. Therefore, vaccination will provide the same benefit to both groups of patients. In conclusion, the earlier the patients with RRP receive the HPV vaccine, the sooner they may show a reduced burden of disease (Smahelova et al., 2022). Additionally, focal epithelial hyperplasia, a rare benign disorder of the oral cavity, is caused by HPV 13 and 32. Of utmost importance are premalignant lesions of the anogenital tract of men and women. The premalignant lesions of the cervical mucosa are classified either cytologically, according to the latest Bethesda system (Wright et al., 2002), as low or high-grade squamous intraepithelial lesions (LSIL, HSIL) or histologically as a low- or high-grade intraepithelial lesions (LGL, HGL). Low-grade lesions often show signs of productive infection and tend to regress spontaneously. High-grade lesions, if not treated, progress to invasive carcinoma. HPV is detectable in 70 to 100% of lesions, with the prevalence of HPV increasing and diversity of HPV types decreasing from low-grade lesions to invasive cancer. Now it is well established that HPVs are responsible for invasive squamous cancer cases in other anatomical locations (Plummer et al., 2016) (Table 2). Unlike cervical cancer, a variable fraction of cancer cases in other locations can be attributed to HPV infection. Basaloid and warty carcinomas of the vulva are usually preceded by vulvar intraepithelial neoplasia (VIN) of the usual type and are positive for HPVs in 90%, while keratinizing squamous cell carcinoma (SCC) is often preceded by dermatosis and VIN of the differentiated type are much more frequent but only occasionally positive for HPV. **Our extensive study of the correlation between the histological features and HPV profile of vulvar non-neoplastic, precancerous, and neoplastic squamous lesions contributed to the discussion about the proper terminology for the classification of vulvar squamous dysplastic lesions** (Skapa et

al., 2007). About 75% of vaginal SCC, 51% of penile squamous cell warty and basaloid carcinomas, and 88% of anal SCC are also linked to HPV infection. In all these cancers, HPV16 is the most prevalent type, and the variability of the HPV types present is much lower than in cervical tumours (Tachezy et al., 2007). Additionally, in the last decade, HPV was recognized, next to smoking and alcohol consumption, as an important risk factor for head and neck SCC. Importantly, recent meta-analyses have shown that the prevalence of HPV-positive head and neck cancers (HNCs) increased from 41% to 72% from 2000 to 2004 (Mehanna et al., 2013).

Papillomaviruses from the genera Alpha, Gamma, and Mu cause cutaneous diseases in children and immunocompromised individuals. The infection manifests as different types of warts or epidermoid cysts. The role of certain Beta HPV types in non-melanoma skin cancer was suggested in immunocompromised individuals. The involvement of Beta PVs as a cofactor in the early stages of cancer in immunocompetent populations was proposed but has not been proven yet (Aldabagh et al., 2013).

Table 2 Percentages of tumours at diverse anatomical sites attributable to HPV infection (Plummer et al., 2016).

	Number of new cases	Number of new cases attributable to infectious agents	Attributable fraction
Carcinoma			
Non-cardia gastric	820 000	730 000	89·0%
Cardia gastric	130 000	23 000	17·8%
Liver	780 000	570 000	73·4%
Cervix uteri	530 000	530 000	100·0%
Vulva	34 000	8500	24·9%
Anus	40 000	35 000	88·0%
Penis	26 000	13 000	51·0%
Vagina	15 000	12 000	78·0%
Oropharynx	96 000	29 000	30·8%
Oral cavity	200 000	8700	4·3%
Larynx	160 000	7200	4·6%
Nasopharynx	87 000	83 000	95·5%
Bladder	430 000	7000	1·6%

5. Cancers and precancerous lesions of the female genital tract

5.1 Causality of human papillomavirus infection in cervical carcinoma

Prospective studies are high on the hierarchy of evidence of pathogen-disease association. At the Department of Experimental Virology of the Institute of Sera and Vaccines, a prospective study performed from 1975 to 1983 failed to sustain herpes simplex virus type 2 (HSV-2) as an etiological factor of cervical cancer (Vonka et al., 1984; Krcmar et al., 1986). In 1983 and 1984, human papillomavirus types 16 and 18, respectively, were isolated from cervical premalignant and cancer tissues (Dürst et al., 1983; Boshart et al., 1984; Gissmann et al., 1984). Since then, scientific evidence has accumulated very fast, supporting the suggested involvement of HPVs in cervical cancer. However, a single prospective study was published by 1999 (Liaw et al., 1999) and its major limitation was the selection of controls who were only age-matched and follow-up length-matched to cases. In our laboratory, samples from a large prospective study (1975-1983) were available. The immunological analyses of the sera were done first, and the results were published in 1999 (Vonka et al., 1999). In that study, virus-like particles (VLP) corresponding to HPV16, 18, and 33 were used as an antigen as well as synthetic peptides derived from early antigens of HPV 16 E2, E4, and E7. The study showed that healthy women who subsequently would develop cervical neoplasia had at enrolment already significantly higher titres of antibodies against VLPs tested but not against the early proteins. However, upon disease diagnosis, seroconversion against those proteins was detected in 58% of subjects.

Supplement No.1

Tachezy R., Salakova M, Hamsikova E, Kanka J, Havrankova A, Vonka V. Prospective study on cervical neoplasia: presence of HPV DNA in cytological smears precedes the development of cervical neoplastic lesions. Sexually Transmitted Infection, 2003, 79, 191-196. (cited 6x) IF2021=4,199

With the improvement of molecular biological methods, the sensitive detection of HPVs became possible in a variety of clinical materials. For the scope of molecular biological testing, a sample collection from more than 10 000 women aged 25-45 years was available (Suppl. No.1). From all women, data on cytological and colposcopic examination and also very detailed questionnaire data on medical history, socioeconomic status, lifestyle, present health status, and sex and reproduction-associated attributes were available. Controls were matched to patients by age, number of sexual partners, age at first intercourse, smoking habits, and history of treatment. Three groups of patients (N=370) were analysed; 67 patients with disease detected at enrolment, 53 subjects who developed the disease during the follow-up, and 208 controls. Initially, molecular methods were tested for the formalin-fixed paraffin-embedded tissues. However, it turned out that due to fixation in suboptimal conditions, the DNA was degraded and not suitable for the analyses (unpublished data). Nevertheless,

archival cervical smears stained by Giemsa and stored at room temperature were also available, which appeared to be amenable to molecular screening. A fundamental step in this study was the optimization of DNA extraction from cervical smears, followed by polymerase chain reaction (PCR) with general primers targeting the conserved region of the L1 gene to detect multiple HPV types in one step. The sensitivity of the PCR assay was increased by non-radioactive hybridization on dot blots with a cocktail of HPV-specific probes. Furthermore, positive samples on the first PCR assay were further analyzed by type-specific nested PCR assay. To support the results of direct detection of HPVs, serological analyses were done with virus-like particles (VLPs) specific for HPV16, 18, and 33. The results showed a statistically highly significant difference in the presence of HPV DNA between patients and controls. The difference from controls was highly significant not only in the women who had been ill at enrolment but, most importantly, also in those who had developed the disease during the follow-up period (Table 3). Women positive for HPV DNA possessed HPV antibodies to VLP16, 18, and 33 significantly more often than those who were free of HPV DNA.

Table 3 Presence of HPV DNA in women at enrolment (Tachezy et al., 2003).

Table 3 Presence of HPV DNA in women with cervical neoplasia at enrolment, in those who developed the disease in the course of subsequent follow up, and in matched control women

Group	Diagnosis	Total	HPV DNA positive	OR	CI	p Value
A	Cases	67	32 (47.8%)	18.3	7.4 to 44.9	<0.0001
	Controls	147	7 (4.8%)			
B*	Cases	26	12 (46.2%)	97.4	5.4 to 1745.3	<0.0001
	Controls	56	0 (0%)			
C†	Cases	27	10 (37.0%)	7.5	2.1 to 27.1	<0.01
	Controls	55	4 (7.3%)			
B+C‡	Cases	53	22 (41.5%)	18.5	5.9 to 57.6	<0.0001
	Controls	108§	4 (3.7%)			

OR = odds ratio; CI = confidence interval.
Group A = women with cervical neoplasia at enrolment (mean age 35 years); group B = women with slightly suspicious cytological and/or colposcopical findings at enrolment (mean age 34 years); group C = women with normal cytological and colposcopic findings at enrolment (mean age 34 years). Control subjects were matched with the patients by age, age at first intercourse, number of sexual partners, and smoking habit. One to three controls were matched with each patient.
*Relative risk (RR) = not done due to zero value of HPV DNA positive controls.
†RR = 5.0 (CI 1.8 to 14.8).
‡RR = 11.2 (CI 4.1 to 30.9).
§Those controls which were used with both groups B and C have been included only once in the row for groups B+C combined.

The results of this study have shown that patients with cervical HPV infection, healthy at enrolment, have an increased risk of developing the disease (relative risk=11.2). The prevalence of HPV DNA increased with the severity of the disease in women found ill at enrolment. Antibodies specific to HPVs were significantly more often present in women positive for HPV DNA (any VLPs $p<0.05$) and in HPV DNA positive women with the lesions detected at enrolment in comparison to those healthy and HPV DNA positive at enrolment (any VLPs $p<0.05$). **The study confirmed the temporality of HPV infection and cervical lesions development, i.e., one of the most important criteria of causality in medicine.**

5.2 Viral and host risk factors of persistence, progression, and recurrence of disease

Human papillomaviruses are a necessary but not sufficient cause of cervical cancer carcinogenesis (Muñoz et al., 1992; Walboomers et al., 1999). HPV infection is common in sexually active women, but most of the infected women do not develop cervical cancer (Schiffman et al., 2007). The cofactors which influence the persistence of infection and progression or regression of the lesion can be divided into host modifiable, host genetic, and viral.

A large meta-analysis confirmed smoking, long-term use of oral contraceptives, and increased parity as host risk modifiable cofactors (Plummer et al., 2003; Appleby et al., 2007; International Collaboration of Epidemiological Studies of Cervical Cancer, 2007)). The studies of cervical cancer risk in relation to co-infection with other genital infections, such as HSV-2 and *Chlamydia trachomatis*, have so far provided inconclusive data (Cao et al., 2014; Zhu et al., 2016). As in the case of several other HPV non-related cancers, it has been suggested that genetic non-modifiable cofactors play a significant role as well. The accumulation of cervical cancer cases within families and increased risk of the disease in first-degree relatives were reported. The epidemiological studies on the genetic heritability of premalignant lesions and cervical cancer determined the risk to be 37% and 27%, respectively (Martinez-Nava et al., 2016). The research of the past decade has shown that host genetic factors can influence cervical carcinogenesis through interactions of their corresponding protein products with HPV oncoproteins and that polymorphism of some host genetic factors, most notably those involved in the carcinogenic and/or anti-carcinogenic pathways, could contribute to the interindividual differences in susceptibility to cervical cancer (Tan & Ankathil, 2015). Also, modifications in the host genes controlling the immune response have been suggested as a risk factor for cervical cancer, e.g., certain human leucocyte antigen (HLA) genotypes (de Araujo Souza et al., 2009).

HR HPVs encode the E6 protein which binds the cellular p53 tumor suppressor protein, thereby marking it for degradation through the ubiquitin pathway. The degradation of the p53 protein inhibits the arrest of the cell cycle in the G1/S checkpoint in response to DNA damage and allows for the continuous proliferation of cells and accumulation of mutations (Hall & Lane, 1994). The inactivation of the p53 protein is also frequently caused by somatic mutations of the central DNA-binding region of p53. This is common in many tumors but not in cervical cancers. Polymorphism of the p53 protein at codon 72 of exon 4 leads to translation either to arginine or proline residue (Matlashewski et al., 1987). The Arg-p53 allele shows a north-to-south decline (Beckman et al., 1994; Själander et al., 1995), and it was suggested that this isoform has a higher susceptibility to degradation by the HR HPV E6 protein (Storey et al., 1998; Thomas et al., 1999). In 1998, Storey et al. reported a seven times higher risk of cervical carcinoma in women with the Arg/Arg p53 genotype (77% vs. 37% in healthy women).

Last but not least, the viral factors have been shown to influence the outcome of the infection; persistence, progression, and recurrence of the disease.

The search for other risk factors and/or genetic changes that can influence the susceptibility of women to cervical cancer is important because such markers would help to select women at risk for more stringent cervical screening and follow-up and also predict the progression and/or regression of the disease.

Supplement No.2

Ho G, Burk R, Klein S, Kadish A, Chang C, Palan P, Basu J, Tachezy R, Lewis R, Romney, S. Persistent genital human papillomavirus infection as a risk factor for persistent cervical dysplasia. Journal of the National Cancer Institute, 1995, 87, 18, 1365-1371. (cited 591x) IF2021=11.816

Unlike some other HPV-associated cancers, cervical cancer is preceded by lesions originally referred to as squamous intraepithelial lesions (SIL) in cytology or cervical intraepithelial neoplasia in histopathology. Some of these lesions have the potential to progress to more advanced stages, while others regress spontaneously. In this paper, the factors which influence the fate of such lesions were explored. Women with cervical lesions were enrolled and followed at 3-month intervals for 15 months (Suppl. No.2). A cervical smear, colposcopy, and HPV DNA detection and typing in exfoliated cervical cells were done at each visit. The HPV DNA detection was performed by Southern blot hybridization and by PCR. In PCR-positive samples, the genotype of HPV was revealed by dot blot hybridization with 39 probes which allowed the detection of type-specific persistent infection. In contrast, Southern blot analyses allowed for typing of 15 different types and semiquantitative analyses of the viral load. Additionally, other factors such as age, ethnicity, education, sexual behaviour, smoking, and oral contraceptive use were evaluated as risk factors for lesion persistence. For the final analyses, data on 70 women with a grade II cervical intraepithelial lesion were available. They came for six visits on average, yielding altogether 532 visits and 350 pairs of observations. The persistent lesion was defined as a lesion being positive at two consecutive visits by HPV DNA detection by Southern blot (regardless of HPV type detected) or by PCR (type-specific persistence, the same type present on both occasions). None of the evaluated potential risk factors correlated with the persistence of lesions. Persistence of lesions was associated with continuous HPV positivity, and the highest risk was for patients **with type-specific persistent infection and a high viral load. The data have shown that the natural history of genital HPV infection is critical for the prognosis of cervical lesions. Therefore, HPV testing should be considered in the management of patients with this disease.**

Supplement No.3

Tachezy R, Mikyskova I, Ludvikova V, Rob L, Kucera T, Slavik V, Bekova A, Robova H, Pluta M, Hamsikova E: Longitudinal study of patients after surgical treatment for cervical lesions: HPV DNA detection and prevalence of HPV-specific antibodies. European Journal of Clinical Microbiology & Infectious Diseases, 2006, 25, 8, 492-500, 2006. (cited 15x) IF2021=5.103

The principal aims of this study were to test whether the persistence of human papillomavirus (HPV) DNA is predictive of recurrent disease in women after surgical treatment for cervical lesions, to distinguish between persistent and newly acquired HPV infection, and to observe the effect of surgical treatment on the levels of HPV-specific antibodies (Suppl. No3). Patients (N=198) surgically treated for low-grade or high-grade squamous intraepithelial lesions and age-matched controls (N=35) were followed up at 6-month intervals for 18 months. Altogether, the presence of HPV DNA in cervical smears was detected using consensus PCR, and serum levels of HPV-specific antibodies to HPV types 16, 18, 31, 33, and 45 were measured. In ten patients positive for HPV type 16 in consecutive samples, the HPV 16 variants were identified using a PCR specific for the long control region (LCR) with the aim to distinguish between persistent and newly acquired infection. Data regarding demographics, risk factors for cervical cancer, and risks related to HPV exposure were collected through a patient questionnaire. Our results have shown that subjects with persistent HPV infection (HPV DNA detection) are at increased risk of developing cytological and/or colposcopic abnormalities after surgery. Also, higher reactivity to HPV-specific antibodies was observed in these women. All ten patients with HPV 16 infection detected in consecutive samples showed persistence of either the same prototype or the same variant during the follow-up period. Analyses of the variants of HPV16 in the consecutive samples suggested that the detected infections were more likely to be reactivation of latent infection than newly acquired infection. Risky sexual behaviour and smoking were more common in patients than in controls. **Persistent HPV infection, as demonstrated by both HPV DNA detection and antibody detection, appears to be a risk factor for the recurrence of pathology after surgery. Recurrences are more likely to be a result of reactivation of a latent HPV infection than of a newly acquired infection.**

Supplement No.4

Zehbe I, Tachezy R, Mytilineos J, Voglino G, Mikyskova I, Delius H, Marongiu A, Gissmann L, Wilander E, Tommasino M. Human papillomavirus 16 E6 polymorphisms in cervical lesions from different European populations and their correlation with human leukocyte antigen class II haplotypes. International Journal of Cancer, 2001, 1, 94, 5,711-716. (cited 98x) IF2021=7.316

Both viral and host factors influence the persistence and progression of cervical lesions. HR HPV infection is the main risk factor for the development and progression of premalignant cervical lesions. One of the variants that increase the risk of persistence of the infection and disease progression is the HPV16 E6 variant E-G350 (L83V). The modification of the amino acid sequence of the E6 protein of this variant can cause an alteration of its immunogenicity and consequently can lead to the evasion of host immune surveillance. The study on the Swedish and Italian populations has shown contradictory results. Our study extended the previously analysed Swedish (N=161) and Italian (N=91) cohorts by analyses of a cohort of Czech women (N=107) (Suppl. No.4). We evaluated the prevalence of the HPV16 E6 prototype and its variants. To correlate the viral risk factors with genetic factors, we analysed human leucocyte antigen (HLA) class II haplotypes in the cases with invasive cervical cancer

(N=143). The most common E6 variant in all cohorts was L83V. Data from the Czech cohort were comparable to the Italian one. The L83V variant was most prevalent in low-grade lesions, and the prevalence decreased with the severity of the disease. However, the trend in the Czech cohort was not linear as in the Italian cohort. In all cohorts, DR04-DQ03 showed a trend for a positive association with the cases with L83V.
These results suggest that cervical carcinogenesis is, at least partly, influenced by genetic polymorphism of both HPV and HLA.

Supplement No.5

Tachezy R, Mikyskova I, Salakova M, Van Ranst M. Correlation between human papillomavirus-associated cervical cancer and p53 codon 72 arginine/proline polymorphism. Human Genetics, 1999, 105, 6, 564-566. (cited 36x) IF2021=5.881

This epidemiological study analysed samples taken from Czech women (N=71) with histologically proven HPV-positive invasive cervical cancer, cervical scrapes from Czech women with normal cytological findings (N=74), and blood samples from healthy Czech blood donors (N=98) (Suppl. No.5). Cervical cancer biopsies and cervical smears from women with normal cytological findings were analysed for the type-specific presence of HPV DNA. DNA was extracted from the peripheral blood cells of healthy Czech donors. The p53 genotype was evaluated using a PCR-based assay that detects explicitly either the Pro-p53 or Arg-p53 allele. Observed genotype frequencies were compared with expected genotype frequencies, assuming the Hardy-Weinberg equilibrium. Between cervical smear controls and blood donor controls, no significant differences in the genotype frequencies of p53 were found. Therefore, the two groups were combined for additional analyses.

Table 4 Genotype distribution of codon-72 p53 genotypes (Tachezy et al., 1999)

	Arg/Arg		Arg/Pro		Pro/Pro	
	Obs (%)[a]	Exp (%)[b]	Obs (%)	Exp (%)	Obs (%)	Exp (%)
Invasive cervical cancer (*n*=71)	**37 (52.1%)**	**36.6 (51.6%)**	**28 (39.4%)**	**28.7 (40.5%)**	**6 (8.5%)**	**5.6 (7.9%)**
HPV-16 positive cervical cancers (*n*=53)	28 (52.8%)	28.0 (52.8%)	21 (39.6%)	21.1 (39.8%)	4 (7.5%)	4.0 (7.5%)
HPV-non-16 positive cervical cancers (*n*=18)	9 (50.0%)	8.7 (48.3%)	7 (38.9%)	7.6 (42.2%)	2 (11.1%)	1.7 (9.4%)
All controls (*n*=172)	**92 (53.5%)**	**87.2 (50.7%)**	**61 (35.5%)**	**70.5 (41.0%)**	**19 (11.0%)**	**14.2 (8.3%)**
Blood donor controls (*n*=98)	52 (53.1%)	50.0 (51.0%)	36 (36.7%)	40.0 (40.8%)	10 (10.2%)	8.0 (8.2%)
Cervical smear controls (*n*=74)	40 (54.1.%)	37.2 (50.3%)	25 (33.8%)	30.5 (41.2%)	9 (12.2%)	6.2 (8.4%)
HPV-negative smear controls (*n*=58)	32 (55.2%)	30.4 (52.4%)	20 (34.5%)	23.2 (40.0%)	6 (10.3%)	4.4 (7.6%)
HPV-positive smear controls (*n*=16)	8 (50.0%)	6.9 (43.1%)	5 (31.3%)	7.2 (45.0%)	3 (18.8%)	1.9 (11.9%)
HPV-16 positive smear controls (*n*=9)	5 (55.6%)	4.0 (44.4%)	2 (22.2%)	4.0 (44.4%)	2 (22.2%)	1.0 (11.1%)
HPV-non-16 positive smear controls (*n*=7)	3 (42.9%)	2.9 (41.4%)	3 (42.9%)	3.2 (45.7%)	1 (14.3%)	0.9 (12.9%)

[a]Observed (Obs) genotype frequencies
[b]Expected (Exp) binomial Hardy-Weinberg proportions

No deviation from the Hardy-Weinberg assumption in the groups of subjects in our study was observed. No difference in the frequencies of p53-Pro (0.282 vs. 0.288) and p53-Arg (0.718 vs 0.712) appeared between patients and controls. No significant difference in the proportion of the different genotypes of p53 at codon-72 between patients and controls was found ($\chi 2=0.56$, $p=0.76$). Homozygosity for Arg-p53 was not associated with an increased risk for

invasive cervical cancer compared with heterozygosity (OR=0.88, 95%CI=0.49-1.58). We did not find any statistically significant difference in the p53 codon-72 genotype in relation to the HPV type present in the cancer tissues and to HPV or cytological positivity of cervical smears.

In our study, the Arg/Arg p53 codon-72 isoform did not increase the risk of cervical cancer in Czech women. Our data contradicted the original report of Storey et al. (1998), and the study was one of the first to refute the suspected association.

Supplement No.6

Klug SJ, Ressing M, Koenig J, Abba MC, Agorastos T, Brenna SM, Ciotti M, Das BR, Del Mistro A, Dybikowska A, Giuliano AR, Gudleviciene Z, Gyllensten U, Haws AL, Helland A, Herrington CS, Hildesheim A, Humbey O, Jee SH, Kim JW, Madeleine MM, Menczer J, Ngan HY, Nishikawa A, Niwa Y, Pegoraro R, Pillai MR, Ranzani G, Rezza G, Rosenthal AN, Roychoudhury S, Saranath D, Schmitt VM, Sengupta S, Settheetham-Ishida W, Shirasawa H, Snijders PJ, Stoler MH, Suárez-Rincón AE, Szarka K, Tachezy R, Ueda M, van der Zee AG, von Knebel Doeberitz M, Wu MT, Yamashita T, Zehbe I, Blettner M. TP53 codon 72 polymorphism and cervical cancer: a pooled analysis of individual data from 49 studies. Lancet Oncology, 10, 8, 772-84, 2009. (cited 123x) IF2021=54.433

Since the first report on the increased risk of cervical cancer in women homozygous compared to heterozygous for the arginine allele at codon 72 of the TP53 tumour suppressor gene, more than 80 studies were published by 2006, but the results were inconsistent. Therefore, a pooled analysis, which is at the top of the hierarchy of evidence of the association of the suspected variable with the disease, was performed (Suppl. No.6). Based on the strict criteria, 49 studies (N=7946 cases, N=7888 controls) were included. For the final analyses, the selection of studies was further restricted to epidemiological studies only. These studies had to show controls in the Hardy-Weinberg equilibrium and had to have the T53 polymorphism determined from white blood cells. Our study was between five studies that fulfilled all of those criteria. No excess risk of cervical cancer was identified for women in the pooled analyses, both for arginine homozygotes compared with heterozygotes (OR=1.22, 95%CI=1.08–1.39) and for arginine homozygotes versus proline homozygotes (OR=1.13, 95%CI=0.94–1.35). Subgroup analyses showed significant excess risks for arginine homozygotes compared with heterozygotes only in the studies where controls were not in the Hardy–Weinberg equilibrium, non-epidemiological studies, and studies where the *TP53* genotype was determined from tumour tissue. No excess risk was noted in studies with sound epidemiological design and conduct (OR=1.06, 95%CI=0.87–1.29) and studies in which the *TP53* genotype was determined from white blood cells (OR=1.06, 95%CI=0.87–1.29).

This pooled analysis, which allowed for subgroup evaluation, has shown that the increased risk of cervical cancer reported in some previously published studies is attributable to errors in study methods. No association was found between cervical

cancer and *TP53* codon 72 polymorphism when the analysis was restricted to methodologically correct studies.

5.3 Prevalence of HPV-specific antibodies and HPV genotypes in the Czech population

Surveillance of the prevalence of HPV types, HPV-specific antibodies, and genotype distribution is important for the implementation and/or modification of the diagnostic, therapeutic, and preventive strategies, as well as for the evaluation of the efficiency of the modified regimens.
HPVs do not cause viremia, and their life cycle is closely linked to the differentiation of the epithelial cells. The location of the infection in the epidermis is one of the factors which restrict the reaction of the immune system. Depending on the viral load and/or duration of the infection, the antibodies against viral capsid proteins develop in infected individuals with a delay of several months, and the process does not necessarily occur in every infected individual.
(Ho et al., 2004). The antibody response to capsid proteins remains relatively stable over time after the clearance of infection. Therefore, it can be a useful epidemiologic marker that reflects cumulative past infections. Antibodies specific to viral oncoproteins HPV16 E6 and E7 are rarely present in healthy individuals, but their prevalence is high in patients with invasive malignant diseases.
In 2006, the first HPV vaccine became available (Harper, 2009). The assessment of the age-specific anti-HPV antibody prevalence in a particular population is essential for estimating the optimal target age when the implementation of HPV vaccination in cervical cancer prevention is planned. Additionally, it also allows for prospective monitoring of the impact of vaccination.

Supplement No.7

Hamsiková E, Ludvikova V, Stasikova J, Tachezy R. Cross-sectional study on the prevalence of HPV antibodies in the general population of the Czech Republic. Sexually Transmitted Infections, 2013, 89, 2, 133-137. (cited 13x) IF2021=4.199

In our study, sera collected in the scope of serological surveys performed in the Czech Republic in 1996 and 2001 were analysed (N=539, age range 6-20; N=997, age range 9-64 years, respectively) (Suppl. No.7). Additionally, 1614 sera taken from blood donors sampled between 1999 and 2005 were included in the study. The individuals were categorized into 5-year age groups. The highest rates of seropositivity for any of the four vaccine types (HPV 6/11/16/18) were found in 30-39-year-old women and men 60 years of age and older. In children 6-14 years of age, 18% were positive, but in the age category14 years and older, 26% of children had antibodies specific for HPV vaccine types. **The results of our study supported the implementation of HPV vaccination in the Czech Republic before the age of 13. In 2012, routine vaccination started in the Czech Republic, and it is covered by**

health insurance companies for girls 13-14 years of age. In 2018, gender-equal vaccination was approved in the Czech Republic.

Supplement No.8

Tachezy R, Hamsikova E, Hajek T, Mikyskova I, Smahel M, Van Ranst M, Kanka J, Havrankova A, Rob L, Guttner V, Slavik V, Anton M, Kratochvil B, Kotrsova L, Vonka V. Human papillomavirus genotype spectrum in Czech women: correlation of HPV DNA presence with antibodies against HPV-16, 18, and 33 virus-like particles. Journal of Medical Virology, 1999, 58, 4, 378-386. (cited 33x) IF2021=20.693

Supplement No.9

Tachezy R, Smahelova J, Kaspirkova J, Salakova M. Human papillomavirus Type-Specific Prevalence in the Cervical Cancer Screening Population of Czech Women. Plos One, 2013, 8, 11, e79156. (cited 27x) IF2021=3.752

Supplement No.10

Hamsikova E, Smahelova J, Ludvikova V, Salakova M, Rychla J, Skrenkova J, Rob L, Tachezy R. The prevalence of HPV infections in HPV-vaccinated women from the general population. APMIS, 2017, 125, 6, 585-595. (cited 4x) IF2021=3.428

The prevalence of HPV infection and distribution of different genotypes vary across populations and geographical regions (de Sanjosé et al., 2007), with the global HPV prevalence in women with normal cytological findings estimated to be 11.7%. In Europe, the highest prevalence was found in Eastern countries (Bruni et al., 2010). Results of the first study from our laboratory focused on the type-specific prevalence in the Czech Republic were biased for the HPV-type specific prevalence in healthy Czech women because subjects with normal cytological findings were recruited in a hospital-based setting (Tachezy et al., 1999) (Suppl. No.8).
In the study from 2013, samples of women (N=1393, age range 14-79 years) were selected from the bank of samples of the Bioptic Laboratory in Pilsen (Suppl. No.9). Women were from five of 14 districts in the Czech Republic, and both big cities and rural areas were covered. The samples were women who attended the annual gynaecological examination within the cervical screening program. They had no history of atypical or pathological findings on the cervix uteri and no previous treatment for cervical lesions. The results of cytological findings at the time of sampling were available, and the majority of women had normal cytological findings suggesting a proper design of the study. Two methods for HPV DNA detection were used in order to provide internationally comparable data. In the text below, results of the more sensitive PCR-based method, which allows for typing, are reported. In total, 26% were positive for any HPV type, 20% for HR HPV, and 6.0% for LR HPV types. The highest prevalence of any HPV (41%) was observed in 21-25-year-olds and declined rapidly with age, but in the age category of 61-65 years, it was still 14%. HPV 16

was the most common HPV type, but other HR HPV types reported in other studies as the most common were different in the population of Czech women with normal cytological findings. **The knowledge about the distribution of HPV types in our population before the onset of routine vaccination allows for future monitoring of changes in the prevalence of HPV types and for possible type replacement. Since the prevalence of HPV is high in the healthy Czech population and routine HPV detection prior to vaccination is not recommended, the physician should inform the sexually active patients who ask for vaccination that the vaccine might have decreased effectiveness because of an already existing infection. In the study from 2017, we have shown that 11% of women who actively seek HPV vaccination in the Czech Republic are incidentally and/or persistently infected with HPV 16/18 and are therefore likely to have decreased efficiency of the vaccine (Suppl. No.10).**

Over the years, we have performed numerous studies aiming to prove the involvement of HPV in numerous malignancies. These studies provided data on the type-specific prevalence of HPV DNA in HPV-associated malignancies in the Czech population. Historically, the results of such studies were influenced by the available methods for HPV detection and by their improvement. As an example, in the past, the technical limitations even lead to a suggestion that cervical lesions grade I is a disease with a different aetiology than high-grade lesions and invasive carcinoma of cervix uteri. With methodological improvement, these limitations disappeared, and the causal relationship of HPV to precancers and cancers at numerous anatomical locations was proved. With the development of HPV vaccines, the data gained in these studies were also valuable for the calculation of the effect of the implementation of primary prevention against HPV.

Supplement No.11

Tachezy R, Smahelova J, Salakova M, Arbyn M, Rob L, Skapa P, Jirasek P, Hamsikova E. Human Papillomavirus Genotype Distribution in Czech Women and Men with Diseases Etiologically Linked to HPV. PLoS One, 2011, 6, 7, e21913. (cited 18x) IF2021=3.752

In this study, cancer samples from cervical (SCC), vulvar, and anal locations (N=157) and specimens from premalignant lesions (N=695) of the same locations were included (Suppl. No.11). Also, samples of anogenital condyloma accuminatum (N=64) were analysed. HPV was detected in 76% of precancerous lesions. In cancer cases, except for cervical location, only 3 HR and 1 LR HPV type were detected. In cervical carcinomas, 11 HR and no LR HPV types were found. Of SCC, 95% were HPV positive. At that time, in the Czech Republic, the incidence of cancers evaluated in our study was 1 300 cases annually (https://www.uzis.cz/index-en.php?pg=record&id=4424). Based on these data and the results of our study, we have calculated that **approximately 73% of 1 300 incident cancer cases could be attributed to the vaccine HPV types. Additionally, 84% of condyloma acuminatum cases were positive for HPV vaccinal types 6/11. These data supported the fast implementation of routine vaccination in the Czech Republic to achieve a significant**

reduction in the burden of HPV-associated diseases and national healthcare expenditures. However, it is crucial to understand that the effect of vaccination will manifest itself in the long term and only on condition of sufficiently extensive vaccine coverage.
When the new nonavalent HPV vaccine was approved by the European Medicine Agency (EMA) in 2015, we calculated the potential benefit of the application of this vaccine in routine settings. We also included results obtained from our additional studies on HNC. We have calculated that the nonavalent HPV vaccine would be able to protect 943 of 1 300 incident cancer cases (cervical, vulvar, anal) and 300 of 467 annual cases of oropharyngeal cancer in the Czech Republic.

Table 6 The predicted effect of the nonavalent HPV vaccine for the prevention of HPV-associated tumours in the Czech Republic (Hamšíková & Tachezy, 2015).

HPV type in the tumor	Squamous carcinoma of cervix uteri (%)	Vulvar carcinoma (%)	Squamous anal carcinoma (%)	Total anogenital carcinoma (%)	Oropharyngeal carcinoma (%)
HPV16/18*	75.6	24.5	81.8	60.5	61.5
HPV31/45**	8.1	2.0	0.0	5.1	0.0
HPV33/52/58^{+}	8.1	8.2	0.0	7.0	2.7
Total	91.8	34.7	81.8	72.6	64.2

* HPV16 and/or HPV18 positive
** HPV31 and/or HPV45 positive, HPV16, 18, 33, 52, 58 negative
$^{+}$ HPV33 and/or HPV52 and/or HPV58 positive, HPV16, 18, 31, 45 negative

6. Aetiology of prostate cancer and distribution of HPV in the male urogenital tract

Prostate cancer (PC) is the second most common malignancy in men worldwide and the first in Europe. PC can be detected in 80% of men aged over 80 years, but those men usually die for other reasons. Clinically significant PC affects men in their sixties, but since it is likely initiated many years earlier, the primary trigger factor of PC is difficult to establish. In PC, pathogenetic, heritable, and/or environmental factors (age, African American race, family history of PC) may play a role. One of the molecular events suggested as a risk factor for the development of proliferative inflammatory atrophy (PIA), the precursor lesion of PC, is chronic inflammation. PIA can progress to prostate intraepithelial neoplasia (PIN) in which alteration of the cell genome, increased genomic instability, and accumulation of genetic changes are detected. High-grade PIN lesions can eventually progress to invasive PC (De Marzo et al., 2007). Because of the suggested risk of chronic inflammation, different factors which can lead to inflammation were explored, including infection with bacteria or viruses. In a large population-based case-control study, the originally published observation about the association of prostatitis with increased risk of PC was not confirmed (Sutcliffe et al., 2006).

The role of the interaction of the proteins of numerous pathogens with host genes was investigated since this trigger mechanism of the malignant process was detected in other tumours. The polymorphism of host genes which play a role in cellular defences against inflammation and oxidative stress, can lead to an increased susceptibility to prostate carcinogenesis. Also, mutations of the genes involved in the inflammation process or in those which affect the control of infection can increase PC risk. However, finding the link between PC and a particular infectious agent can be very difficult. Pathogens can initiate the process of malignant transformation or increase the risk of its progression but might not be detectable at the time of PC diagnosis.

HPV is established as a causal factor of several anogenital cancers in women and men. Since the prostate is easily accessible for viruses via the urethra, it was also extensively investigated for the association of HPV infection and cancer development. Since the 1960s, many studies have been published, but they have produced contradictory data. In many cases, it could be attributed to a suboptimal study design and/or to the methods used. In many studies, the clinical material was collected during transurethral surgery and thus could be contaminated by viruses present in the urethra. Also, in many studies, samples from patients with benign prostate hyperplasia (BPH) were used as a negative control without detailed pathological assessment, but in up to 20% of these patients, incidental cancers can be seen. On the other hand, in studies in which small needle-biopsy specimens were used for PC diagnosis, the tumour could be missed. Even the selection of controls matched to patients with PC is not easy since the mean age of patients is relatively high and therefore, traditional controls such as healthy blood donors are mostly not appropriate. Discrepancies in the results of numerous studies can also be attributed to the methods used for HPV DNA detection, which can lead, on the one hand, to false positive results or, on the other hand, to underestimation of the HPV prevalence.

Supplement No.12

Hrbacek J, Urban M, Hamsiková E, Tachezy R, Eis V, Brabec M, Heracek J. Serum antibodies against genitourinary infectious agents in prostate cancer and benign prostate hyperplasia patients: a case-control study. BMC Cancer, 2011, 11, 53. (cited 35x) IF2021=4.638

In this study, we used the indirect serological method to evaluate the possible involvement of nine pathogens in PC and BPH (Suppl. Np.12). In contrast to the direct detection of the pathogen, the presence of antibodies can suggest the involvement of the pathogen in earlier phases of PC carcinogenesis. The study included males who were treated with a radical retropubic or simple transvesical prostatectomy (N=434). All patients were free of urinary tract infection symptoms and had negative urine cultures. Detailed examination of tissues by a pathologist confirmed 105 cases with BPH and 329 with PC. PC patients were more likely to harbour antibodies against *Ureaplasma urealyticum* (OR 2.06; 95% CI 1.08-4.28). Men with BPH were more often seropositive for HPV 18 and *Chlamydia trachomatis* (OR 0.23; 95% CI 0.09-0.61 and OR 0.45; 95% CI 0.21-0.99, respectively) and had higher mean serum CMV

antibody levels than PC patients (p = 0.0004). Among PC patients, antibodies against HPV 6 were associated with a higher Gleason score (p = 0.0305).
In conclusion, antibody seropositivity against the analysed pathogens, except for *Ureaplasma*, did not seem to be a risk factor for PC pathogenesis. The presence or higher levels of serum antibodies against the genitourinary pathogens studied were not consistently associated with PC. Serostatus was not a predictor of disease stage in the studied population.

Supplement No.13

Tachezy R, Hrbacek J, Heracek J, Salakova M, Smahelova J, Ludvikova V, Svec A, Urban M, Hamsikova E. HPV persistence and its oncogenic role in prostate tumors. J Med Virol. 2012,84,1636-1645. IF2019=2.021 (2.373)

Supplement No. 14

Svec A, Mikyskova I, Hes O, Tachezy R. Human papillomavirus infection of the epididymis and ductus deferens: an evaluation by nested polymerase chain reaction. Archives of Pathology & Laboratory Medicine, 2003, 127, 11, 1471-1474. (cited 19x) IF2021=5.686

The possible etiological involvement of HPV in PC was further evaluated in our next study (Suppl. No.13). The second aim of this study was the exploration of the possible role of the male urogenital tract as a reservoir of HPV infection. In order to avoid the drawbacks of numerous studies published before, we have taken the samples of both benign and malignant prostate tissue via open surgical procedures to avoid possible contamination of the samples by HPV from the urethra. Also, during the laparotomy surgery, biopsies from normal tissues from the foreskin, prostate, urinary bladder, seminal vesicles, ductus deferens, and ureter were taken. The tissue samples were analysed by a pathologist to confirm the presence of a tumour, benign hyperplasia, or normal tissue. Besides HPV DNA detection and typing, the detection of the HPV-specific antibodies was done to support the possible etiological relationship between HPV and PC or BPH. For HPV DNA detection, a very sensitive nested PCR method was used since the amount of material gained from the fixed paraffin-embedded tissue sections is always limited. The method used, at the same time, allowed for the detection of many different HPV types. The detection of HPV was done in samples in which the presence of hyperplasia or cancer was histologically confirmed and in biopsies of normal tissues (N=256). HPV was detected in 4% of samples of normal tissues. The highest HPV prevalence rate was detected in the foreskin, followed by the ductus deferens, prostate, and seminal vesicles. No HPV was detected in the healthy urinary bladder and urethral tissue. In four samples, HPV 16 was detected: one sample contained HPV 52 and five samples were positive for an unknown HPV type. In urethral smears of patients with BPH, HPV was detected statistically significantly more often in comparison to patients with PC (P=0.0403). Altogether 17 different HPV types were found, with HPV16 being the most common. In 210 samples from 95 men with BPH and in 90 samples from 51 patients with PC, HPV DNA

presence was found in 2%. There was no concordance between HPV types detected in the tissue and in the penile smear, further confirming the lack of contamination from the urethra. Also, no statistically significant difference in the level of HPV-specific antibodies was observed between patients with PC or BPH compared to healthy blood donors matched to patients by gender and age. Only the prevalence of HPV 6 specific antibodies was significantly lower in cases with PC (P=0.018).

During the evaluation of our samples by a pathologist, a dysplastic lesion of the epididymis was detected, with HPV DNA type 31 found in this sample (Svec et al., 1999). It led us to the hypothesis that HPV might be transmitted sexually together with bacteria. Therefore, we have initiated a nested study of HPV in patients treated for nontuberculous epididymitis caused by sexually transmitted pathogens (Suppl. No.14). In 31% of these patients, HPV DNA was detected in the epididymis or ductus deferens. In one sample, LR HPV 6 was found, and in six samples, DNA of HR HPV types was present. However, the infection was not accompanied by koilocytic atypia or dysplasia.
Our study, as the first one, took a multidisciplinary approach to prove the relationship between HPV and tumours of the prostate, and at the same time, care was taken to avoid the limitations of the numerous other studies. The results indicate that HPV infection is not associated with prostate oncogenesis in men. However, the results of the two other discussed studies imply that multiple tissues of the male genitourinary tract may be important reservoirs for the transmission of some HPV types.

Our data would be even stronger if the detection of viral mRNA were possible because viral mRNA is a marker of active viral infection, not just accidental colonization of the tissue. However, the clinical material was not of sufficient quality to analyse RNA. There is, to my knowledge, only one study that detected viral mRNA in PC tissue (Dodd et al., 1993). This single study is contradicted by an extensive study of transcriptome of 3,775 malignant neoplasms from The Cancer Genome Atlas database (Khoury et al., 2013). In this latter study, no viral transcripts of DNA viruses were detected in PC samples as well as in cancers of many other locations. Furthermore, the risk of PC was not increased in severely immunocompromised individuals (transplant recipients, HIV-infected individuals) while the opposite was true for cancers with established HPV aetiology (Grulich et al., 2007; Vajdic & van Leeuwen, 2009).

Supplement No.15

Hrbacek J, Urban M, Hamsikova E, Tachezy R, Heracek J. Thirty years of research on infection and prostate cancer: No conclusive evidence for a link. A systematic review. Urologic Oncology-Seminars and Original Investigation, 2012, 31 ,7, 951-965. (cited 18x) IF2021=2.954

Due to the conflicting results of many studies regarding the etiology of pathogens in PC, we also performed a detailed evaluation of the published data on infection and PC (Suppl.

No.15). We analysed studies published on MEDLINE until 2011 and included 74 studies in the review. These studies explore the following pathogens: *human papillomavirus, cytomegalovirus, herpes simplex virus, Epstein-Barr virus, BK virus, JC virus, Chlamydia, Mycoplasma, Ureaplasma, Trichomonas, Neisseria, Treponema, Propionibacterium acnes, Xenotropic murine leukemia virus-related virus*, and *Candida albicans*. The studies utilized both molecular biological methods and indirect methods – detection of antibodies. **The results suggested that despite the differences in the design of studies and in methodological approaches that were used, most of the pathogens that were studied were unlikely to be directly involved in prostate carcinogenesis.**
In conclusion, despite the long-lasting research on the role of infection in the etiology of PC, no pathogen can be linked with this malignancy yet.

7. Head and neck cancer as a model of dual aetiology

Head and neck cancers (HNC) are one of the most common cancers worldwide and account for over 878,000 new cases annually. HNC can be found in different anatomical locations, including the lip, oral cavity, salivary glands, nasopharynx, oropharynx, hypopharynx, larynx, nasal and paranasal cavity, and ear, and these tumours also have different clinical and epidemiological characteristics due to the tissue specificity and exposure to risk factors. Most of these tumors are squamous and arise from epithelial cells of the mucosal lining. The incidence and mortality of HNC differ according to gender, cancer location, and country. The differences in the incidence and mortality are due to the variations in the exposure to the main risk factors, smoking and alcohol; 33% of HNC cases are associated with smoking and 4% with alcohol, while 35% are linked to both of these risk factors. Both smoking and consumption of alcohol increase the mutation rates in genes involved in several cellular pathways (Hashibe et al., 2009; Zygogianni et al., 2011; Gaykalova et al., 2014). Tumours in smokers and alcohol consumers are primarily located in the oral cavity, while in non-drinkers and non-smokers, tumours are usually detected in the oropharyngeal area (Rikardsen et al., 2014). While the risk factors of smoking and alcohol consumption have been known for a long time, the possible role of HPV infection was first suggested in 1985, when HPV 16 was detected in HNC (de Villiers et al., 1985; Löning et al., 1985). Since then, HPV DNA has been found in variable proportions of HNC. In 2001, we initiated our first study on the association of HPV with HNC and until today, 23 papers from our team or with our participation have been published. In this chapter, I will discuss the results of just some of them.

Supplement No.16

Tachezy R, Klozar J, Rubenstein L, Smith E, Salakova M, Smahelova J, Ludvikova V, Rotnaglova E, Kodet R, Hamsikova E. Demographic and risk factors in patients with head and neck tumors. Journal of Medical Virology, 2009, 81, 5, 878-887. (cited 70x) IF2021=20.693

We started our research on HPV aetiology in HNC with an epidemiological case-control study, which aimed at the demographic characteristics and risk factors of patients with HNC (Suppl. No.16). HPV DNA detection was performed in the tumour tissue and oral rinses, and the presence of HPV-specific antibodies was tested. HNC patients participated in more risky sexual behaviors (anal-oral contact) (P=0.020) and smoked and drank more than controls (P=0.001; P=0.020, respectively). High-risk HPV DNA was detected in 43% of oral washings of cases and 4% of controls (P<0.0001). Additionally, the association between high-risk HPV DNA in oral rinses and tumor tissues was statistically significant (adjusted P<0.0001). The prevalence of HPV-specific antibodies was significantly higher in cases than in controls (adjusted P<0.0001). **These results provided epidemiological and immunological evidence for HR HPV as a strong risk factor (OR=44.3, P<0.0001) for HNC, even after controlling for age, tobacco, and alcohol use. Additionally, it has been shown that the detection of high-risk HPV DNA in oral rinses and of HPV-specific antibodies in serum could be considered as clinically relevant surrogate markers for the presence of an HPV-associated HNC, with a high sensitivity (83%) and specificity (88%).**

Supplement No.17

Tachezy R, Klozar J, Salakova M, Smith E, Turek L, Betka J, Kodet R, Hamsikova E. HPV and other risk factors of oral cavity/oropharyngeal cancer in the Czech Republic. Oral Diseases, 2005, 11, 3, 181-185. (cited 46x) IF2021=4.068

This study (Suppl. No.17) further showed the more common presence of HPV DNA in tumours located in the oropharyngeal area in comparison to the oral cavity (25% vs. 57%). Patients with HPV-positive tumours were more often non-smokers (100%) and also never alcohol consumers (69%).

Supplement No.18

Klozar J, Kratochvil V, Salakova M, Smahelova J, Vesela E, Hamsikova E, Betka J, Tachezy R. HPV status and regional metastasis in the prognosis of oral and oropharyngeal cancer. European Archives Oto-Rhino-Laryngology, 265, S1, S75-S82, 2008. (cited 73x) IF2021=3.236

In a subsequent study, we have shown that patients with viral etiology of HNC have significantly better overall survival (73 vs. 35%) (P=0.0112) as well as disease-specific (79% vs. 45%) (P=0.0015) (Suppl. No.18) survival rates than patients with tumours not associated with HPV. No difference was found in the classical prognostic factors like pN and extracapsular spread between HPV-positive and HPV-negative tumours. In a multivariate analysis, HPV was confirmed as the most significant prognostic factor in patients with oropharyngeal cancer (HR=0.27; 95%CI=0.12-0.61).
The difference in the prevalence of HPV DNA-positive tumours between cases of the oral cavity and oropharyngeal carcinoma exposed and not exposed to tobacco or alcohol supports the theory that HPV DNA-positive tumours form an aetiologically distinct

subgroup of head and neck tumours. More importantly, HPV has been shown as the most significant favourable prognostic factor for patients with oropharyngeal tumours.

Supplement No.19

Koslabova E, Hamsikova E, Salakova M, Klozar J, Foltynova E, Salkova E, Rotnaglova E, Ludvikova V, Tachezy R. Markers of HPV infection and survival in patients with head and neck tumors. International Journal of Cancer, 2013, 133, 8, 1832-1839. (cited 53x) IF=7.316

Supplement No.20

Simonidesova S, Hamsikova E, Ludvikova V, Klozar J, Vencalek O, Tachezy R, Prognostic value of post-treatment HPV-specific antibodies in patients with oropharyngeal tumours. Journal of Surgical Oncology, 2019, 120, 2, 117-124. (cited 1x) IF2021=2.885

The HPV aetiology in HNC was further confirmed by studies (Suppl. No.19 and No.20) that determined whether the changes in HPV DNA prevalence in oral rinses and/or in HPV-specific antibody levels in the sera of HNC patients have prognostic significance. Most patients positive for HPV-specific antibodies remained positive during the follow-up (1 year after the end of treatment), while 84.8% of those positive for HPV DNA in oral rinse at enrolment became negative. However, the mean titres of HPV16 E6 and/or E7 antibodies at follow-up were significantly lower than at enrolment. No decrease in antibody levels was observed in the majority (5/6) of patients positive for HPV16 E6 and/or E7 antibodies at enrolment who developed recurrent disease. The titres of antibodies specific for HPV16 capsid antigens did not change during the follow-up. In the subsequent long prospective study, we confirmed this data. We showed that the decrease in the level of HPV-specific antibodies taken from patients up to 14 years after the end of the treatment was statistically significant, and the decrease of HPV16 E6 antibodies at 1-year follow-up was associated with better overall survival as well as disease-specific survival.
In conclusion, we have shown that the detection of antibodies specific for the HPV 16 E6 and E7 oncoproteins may serve not only as a marker of HPV-associated malignancy but also as a prognostic indicator in patients with HPV-positive tumours.

Supplement No. 21

Rotnaglova E, Tachezy R, Salakova M, Prochazka B, Koslabová E, Vesela E, Ludvikova V, Hamsikova E, Klozar J. HPV infection in tonsillar cancer: prognostic significance and clinically relevant markers. International Journal of Cancer, 2011, 129, 1, 101-110. (cited 61x) IF2021=7.316

Unlike in cervical cancer where HPV is almost always present and is thought to be etiologically involved in almost all cases, in HNCs, it has been proposed that not all HPV

DNA-positive tumours are etiologically linked to viral infection. To assess the presence of transcriptionally active viruses in HNC patients, different markers were studied (Suppl. No.21). It appeared that the gold standard is the detection of viral mRNA. Since the integrity of RNA is not always preserved in clinical materials, the detection of expression of the p16 protein was also explored as an indirect marker of an active HPV infection.
Our study focused on oropharyngeal tumours whose HPV aetiology was documented. For a subgroup of patients in this study, we analysed the expression of E6 and E2 viral mRNA. In 93% of HPV DNA-positive samples, viral E6 mRNA was detected (active viral infection). The detection of p16 expression and the presence of HPV-specific antibodies correlated well with HPV DNA and RNA presence**. Our study demonstrated that p16 immunostaining and anti-E6/E7 antibodies as a surrogate marker of HPV involvement represent specific, sensitive, and clinically accessible assays for the identification of patients with oropharyngeal tumours who have a considerably better prognosis. The detection of p16 protein expression is now included in the TNM8 staging of oropharyngeal tumours.**

Supplement No.22

Vojtechova Z, Sabol I, Salakova M, Turek L, Grega M, Smahelova J, Vencalek O, Lukesova E, Klozar J, Tachezy R. Analysis of the integration of human papillomaviruses in head and neck tumours in relation to patients' prognosis. International Journal of Cancer, 2016, 138, 2,386-395. (cited 49x) IF2021=7.316

In cervical tumours, it has been shown that two viral oncoproteins, E6 and E7, play an essential role in malignant transformation. They interact with cellular proteins, p53 and pRb, and disrupt cellular growth control pathways. In many cervical tumours, the HPV genome is integrated into the cellular genome. In most cases, the expression pattern of integrated HPV genomes consists of E6-E7 gene transcripts spliced into cell sequences and terminated at polyadenylation sites. The integration leads to the stabilization of native HPV early gene mRNAs and the elimination of viral gene products which downregulate E6/E7 transcription. These changes provide a strong selective growth advantage for cells with an integrated form of HPV.
In the study by Rotnaglova et al. (2011) (Suppl. No.21), we detected 64% of the oropharyngeal HPV-associated tumours with an integrated form of the virus. We further wanted to evaluate the influence of HPV status in HNC on a patient's prognosis (Suppl. No.22). In order to be able to analyse a large number of cases, we have to use formalin-fixed paraffin-embedded (FFPE) tissue samples. During the FFPE procedure, RNA is not well preserved, and a specific method for the evaluation of HPV status is needed. The integration of HPV in fresh tumour tissues can be tested by 3´RACE-based Amplification of Papillomavirus Oncogene Transcripts (APOT), Southern blot, and mapping of E2 integration breakpoint at the mRNA level. To validate the method of mapping E2 integration breakpoint at the mRNA level, we compared the results obtained by APOT and E2 mapping on 20 fresh tissue samples and found them to be in perfect agreement (Kappa value = 0.683). Consequently, using the E2 mapping method on FFPE samples (N=186), we detected integrated HPV in 28% of E6 mRNA-positive tumours and HPV in an

extrachromosomal/mixed form in 73%. Both patient groups with HPV16 E6 mRNA-positive tumours, with an extrachromosomal and/or mixed form of the virus and those with an integrated form only had better disease-specific survival rates (HPV-negative vs. HPV extrachromosomal/mixed $p<0.0001$; HPV-negative vs. HPV integrated $p=0.0037$). The difference in DSS between patients with HPV-positive tumours with an HPV extrachromosomal/mixed vs. integrated form was not statistically significant ($p=0.6742$). The patients with transcriptionally silent HPV (HPV DNA positive, HPV 16 E6 mRNA negative) survived better than HPV-negative patients, but the difference was not statistically significant ($p = 0.2635$). When we divide these patients into HPV DNA positive/p16 positive and HPV DNA positive/p16 negative, these subgroups behaved, in terms of survival, as patients with HPV-positive and HPV-negative tumours, respectively.
In conclusion, we determined the HPV status in tonsillar tumours using two different methods. Furthermore, exact HPV integration sites were identified. The survival analysis in HNC patients did not confirm the HPV integrated genome status as a factor significantly influencing the patients' prognosis. Patients with missing HPV active viral form behaved as those with HPV-negative tumours.

8. In search for diagnostic, therapeutic, predictive and prognostic markers

8.1 miRNAs specific for HPV-associated tumours

New methods and advances in the research of cancer and papillomaviruses extended the possibilities to explore critical disease biomarkers in order to find new targets for diagnostic and therapeutic use as well as new predictive and prognostic markers.
MicroRNAs (miRNAs) as a class of short single-stranded non-coding RNAs are crucially involved in the post-transcriptional regulation of gene expression. The regulation of miRNA expression plays a key role in the development, cell growth, and differentiation processes in eukaryotic organisms. It has also been shown that the expression of miRNAs is deregulated in human cancers, and their expression profiles are specific for tumours of different locations. The miRNAs can act as oncogenes or tumour suppressors and were suggested as promising prognostic and diagnostic biomarkers of human cancers (Jay et al., 2007). Therefore, we focused on miRNA expression profiling in an attempt to identify a set of “HPV core” miRNAs specific for HPV-associated tumours (Suppl. No.23-26).

Supplement No.23

Vojtechova Z, Sabol I, Salakova M, Smahelova J, Zavadil J, Turek L, Grega M, Klozar J, Prochazka B, Tachezy R. Comparison of the miRNA profiles in HPV-positive and HPV-negative tonsillar tumors and a model system of human keratinocyte clones. BMC Cancer, 2016, 16, 382. (cited 28x). IF2021=4.638

In the first miRNAs expression profiling study in HNC, we characterized the expression profiles in a matched set of well-characterized HPV-associated and HPV-negative tonsillar tumours and in immortalized keratinocyte clones to define potential and clinically relevant biomarkers of HNC of different aetiology. The study was performed on fresh frozen tonsillar cancer and non-malignant tissues and cervical tumours and normal cervical tissues. Furthermore, as a model, relative miRNA abundance levels in primary and immortalized human keratinocyte clones were evaluated. A set of formalin-fixed paraffin-embedded tumour samples enriched for the tumour cell fraction by macrodissection were used to confirm differentially expressed miRNAs. We have identified specific up- and down-regulated miRNAs for HPV-associated and non-associated tumours. In comparison with the expression profiles in cervical tumours, we defined a set of "HPV core" miRNAs specific for these two HPV-induced malignancies. Several miRNAs were also shared by the miRNA profiles of HPV-negative tonsillar tumours and the HPV-negative keratinocytes, as well as the HPV-positive tonsillar tumours and HPV-positive keratinocytes. **In this study, the miRNAs characteristics for HPV-induced tumours and tonsillar tumours of different aetiology were identified.**

However, the proposed miRNAs were not in agreement with the only other study published at that time. Therefore, we explored the factors possibly influencing the outcomes of miRNA expression profiling in our subsequent study (Suppl. 24).

Supplement No.24

Vojtechova Z, Zavadil J, Klozar J, Grega M, Tachezy R. Comparison of the miRNA expression profiles in fresh frozen and formalin-fixed paraffin-embedded tonsillar tumors. PLoS One, 2017, 12, 6, e0179645. (cited 9x) IF2021=3.752

Most of the miRNA profiling studies of tumours were performed on fresh frozen samples (FF) where the RNA is well preserved. However, this type of clinical material is rarely available. Fortunately, it has been shown that the disadvantage of formalin fixation for mRNA analysis is not a drawback for miRNA studies probably due to their small size (Jung et al., 2010). In our study, we performed a comparison of the miRNA expression profiles between FF and macrodissected FFPE tonsillar tumours. Data were processed by different software programs (the SDS 2.4, and the ExpressionSuite v1.0.1 software (Life Technologies, USA), and two different normalization methods (50th percentile shift, and global normalization) were used. We observed a considerable correlation between the miRNA expression profiles of paired FF and FFPE samples; 27-38% of the differentially deregulated miRNAs overlapped between the two types of clinical samples, between the two normalization methods, the overlap was 58–67% of differentially expressed miRNAs, and no influence of the software platform was observed. **Our study has stressed the fact that to be able to compare miRNA expression profiles from different published studies, it is essential to use the same type of clinical material. Furthermore, the selection of the normalization method for data analysis is also important.**

A further advance in molecular biology techniques has shown that also Next generation sequencing methods (NGS) can be used for miRNA profiling. In collaboration with our

colleagues from Croatia, we performed a study using NGS to explore the differentially expressed miRNAs in HNC of different locations and relation to HPV involvement Suppl. No.25).

Supplement No.25

Bozinovic K, Sabol I, Dediol I, Milutin Gasperov N, Vojtechova Z, Tachezy R, Grce M. Genome-wide miRNA profiling reinforces the importance of miR-9 in human papillomavirus-associated oral and oropharyngeal head and neck cancer. Scientific Reports, 2019, 9, 1, 2306. (cited 28x) IF2021=4.997

In this study, miRNA profiling was done by NGS on the fresh tissue of primary oral and oropharyngeal HNC patients. A literature review on similar miRNA studies on HNC populations was performed, and analysis of TCGA miRNA sequencing data for validation of our findings was done. The NGS analyses revealed specific miRNAs in HPV-associated oral HNC and HPV-associated oropharyngeal HNC, with some shared by both groups. The results for selected miRNAs were further evaluated by reverse transcription-quantitative polymerase chain reaction on the whole study population. **Our findings, compared with the literature data, revealed extensive heterogeneity of miRNA deregulation, and only a few miRNAs were consistently affected. One of them was miR-9, being most often, but not exclusively, detected in HPV-associated HNC.**

Supplement No.26

Nunvar J., Pagačová L., Vojtechova Z., de Azevedo NTD., Smahelova J, Salakova M., Tachezy R. Lack of Conserved miRNA Deregulation in HPV-Induced Squamous Cell Carcinomas. Biomolecules, 2021, 11, 5, 764. (cited 2x) IF2021=6.064

Finally, we extended our analysis of differential miRNA expression profiles to other tumours with known HPV association (Suppl. No.26). We analysed HPV-associated or non-associated cervical, anal, vulvar, and tonsillar tumours. One of the surprising observations was the large variability in the total number of deregulated miRNAs between tumours of different locations. The deregulated miRNA pool was specific for tumours in certain anatomic locations, but in tumours of some locations, their total numbers were low. No deregulated miRNA was shared by all four types of HPV-positive tumours. The most significant overlap of deregulated miRNAs was found between tumours that differed both in location and HPV status (HPV-positive cervical tumours vs. HPV-negative vulvar tumours). **This study further shows that HPV infection does not elicit conserved miRNA deregulation in squamous tumours of different locations.**

To summarize our data as well as data from numerous other studies, the research of miRNAs expressed in HPV-related tumours revealed a significant heterogeneity. This can be attributed to the differences in study design (type of clinical samples, data evaluation method, HPV detection method). In individual tumours of the same location,

certain consistency in the deregulation of some miRNAs has been shown. However, HPV core miRNAs were not detected through HPV-associated tumours of different locations. In HNC tumours, differentially expressed miRNAs detected only in HPV-associated vs. HPV-negative tumours might shed some more light on the observed differences in HNC of different aetiology since these miRNAs affect different cancer pathways.

8.2 Analyses of the tumour microenvironment (TME) of HNC

Patients with HPV-associated oropharyngeal tumours have a survival advantage. They show better treatment response and less frequently develop recurrence. This recognition led to the inclusion of the indirect marker of an active viral infection – p16 protein expression - in the scoring system for oropharyngeal tumours and allowed for better risk stratification of patients. The substantially better prognosis of patients with HPV-associated oropharyngeal tumours stimulated the discussion about modification of the therapy with the aim to decrease the side effects of the treatment by lowering the dose of chemotherapy or radiation. Unfortunately, the clinical trial of de-intensification of treatment resulted in inferior survival (Ventz et al., 2019). Therefore, new approaches/markers that will help to improve the risk stratification of HNC patients for alternative treatment regimens are needed.

It has been shown that the immune system plays a vital role in the survival of patients with different types of cancer. A variety of immune markers were explored, and several have been shown useful in clinical settings. Additionally, alternative immunotherapeutic treatments are now available for a variety of malignancies, including HNC, and immune markers that will improve the risk stratification of patients for such types of treatments are needed. The number and phenotype of immune cells infiltrating the tumour microenvironment (TME) were identified as clinically applicable markers. Even though there are already numerous publications dealing with the analyses of TME in HNC, they are burdened by the lack of the unification of methodological approaches (Almangush et al., 2022).

New complex methods for the analyses of tumour immune environments are now available. With the standardization of the techniques and with the help of new complex methods, it might be possible to better describe the complex host-tumour interactions and consequently to develop therapeutic strategies to modify the immune system of the patients to effectively control the growth of the tumour.

In a search for the immune markers, we have implemented and optimized two methods that allow for the complex immune profiling of solid tumours: mass cytometry and multiplex multispectral fluorescent immunohistochemistry (Suppl. No.27-29). The results of clinical testing of HNC samples by the optimized mass cytometry prove the ability to identify various immune cells, including rare subpopulations (Suppl. No.27). This optimized method is now utilized in our ongoing studies.

Supplement No.27

Polakova I, Pelak O, Thürner D, Pokryvkova B, Tachezy R, Kalina T, Smahel M. Implementation of Mass Cytometry for Immunoprofiling of Patients with Solid Tumors. Journal of Immunology Research, 2019, 2019, 6705949. (cited 4x) IF2021=4.493

Unlike mass and flow cytometry, multiplex multispectral fluorescent immunohistochemistry allows the detection of a smaller number of targets. On the other hand, the different populations of immune cells can be analysed *in situ* with respect to the localization in different tumour compartments, and their interactions can be studied in detail. Such an approach can reveal possible influence of the type and number of cells in different compartments on the prognosis. Such an approach focusing on immune cells is a basis for defining the immunoscore. This indicator is used in risk stratification of patients with colon cancer independently of TNM staging (Pages et al., 2018). With the help of this method, we have explored the differences in the TME of HPV-associated and non-associated HNC to explain better prognosis of patients with HPV-positive tumours and to evaluate the populations of cells infiltrating different compartments of tumours, thus providing a possible independent prognostic tool for HNC patients.

Supplement No.28

Pokryvkova B, Smahelova J, Dalewska N, Grega M, Vencalek O, Smahel M, Nunvar J, KlozarJ, Tachezy R. ARG1 mRNA Level Is a Promising Marker in Head and Neck Squamous Cell Carcinomas. Diagnostics, 2021, 11, 4, 628. (cited 3x) IF2021=3.992

In the following study (Suppl. No.28), we focused on detecting tumour-associated macrophages (TAMs) of different polarization, the most abundant family of tumour-infiltrating immune cells with controversial influence on the prognosis. Here, we have detected more TAMs in the stroma of tumours of any aetiology and more pro-tumorigenic macrophages in the stroma of HPV-negative tumours. This finding was supported by the RT-qPCR detection of higher expression of M2 markers, a cluster of differentiation 163 (CD163), ARG1, and prostaglandin- endoperoxide synthase 2 (PTGS2), while in HPV-associated tumours, higher expression of a marker of anti-tumorigenic M1 macrophages, nitric oxide synthase 2 (NOS2), was found. In HPV-negative tumours, non-macrophage arginase 1 (ARG1)-expressing population of cells was detected with higher abundance in comparison to HPV-associated tumours. Regardless of the aetiology of HNC tumours, the expression of ARG1 mRNA was revealed as an adverse prognostic factor for the overall survival of HNC patients. This observation was made for the first time in HNC but has been documented in colorectal cancer and Hodgkin lymphoma by others (Romano et al., 2016; Ma et al., 2019). Inhibition of ARG1 and nitric oxide production via inhibition of phosphodiesterase 5 (Tadalafil) was tested in a phase II clinical trial with promising results showing reversion of the tumour-specific immune suppression in HNC patients (Califano et al., 2015).

Supplement No.29

Pokryvkova B, Grega M., Klozar J, Vencalek O, Nunvar J, Tachezy R. PD1+CD8+ Cells are an Independent Prognostic Marker in Patients with Head and Neck Cancer. Biomedicines, 2022, 10, 11, 2794. (cited 0x) IF2021=4.757

Next, we have focused on the analyses of TILs in TME (Suppl. No.29). As in the previous study on TAMs, we detected more TILs in the stroma of tumours regardless of the aetiology. In HPV-associated tumours, more CD4+, CD8+, and PD1+CD4+, PD1+CD8+ T cells were detected. We did not observe differences in Treg numbers between the HPV+ and HPV- groups, but the levels of the Treg subpopulation, producing the costimulatory molecule ICOS (ICOS+FOXP3+CD4+) was more abundant in the HPV− tumours. Most importantly, we have revealed that higher levels of PD-1+CD8+ T lymphocytes were a favourable prognostic factor for patients in all our models. This finding suggests that the level of PD-1+CD8+ T lymphocytes should be included in the prognostic Immunoscore for patients with OC and OPSCC of different aetiology.

9. Conclusion

Since the seminal discovery of HPV infectious aetiology of cervical cancer, tremendous progress has been achived in the field of epidemiology, diagnostics, and prevention of diseases associated with HPV. Besides cancers of the anogenital tract of women and men, a significant portion of HNC appeared to be partially linked to infection with HR HPV types. The incidence of HPV-associated HNC is increasing in high-income countries, and in the United States it surpassed the incidence of cervical cancer. The development and implementation of sensitive molecular biology methods, in combination with the uncovering of molecular and immunological markers, played a vital role in this progress. Careful genotyping of HPVs based on specific PCR, DNA hybridization, and sequencing in our and other epidemiological studies elucidated which HPV types represent a high risk for cancer. This knowledge was essential for the development and introduction of successful prophylactic vaccines that protect against HPV-associated diseases, most importantly against cancers in different anatomical locations. In recent years, revitalization of immunotherapies opened new possibilities in the individualised treatment of patients and emphasizes the need for new immunological markers. The pre-vaccination studies not only provide the knowledge for vaccine development and implementation but also provide data for future surveillance of HPV infections in the population.

My research was deeply wedded and performed in a very fruitful, mutual collaboration with the clinical institutions, including the education of medical doctoral students in the lab under my supervision.

I am happy and grateful that I had the chance to participate in the research that supported the successful story from the discovery of the HPV aetiology in a number of diseases to the improvement of early diagnosis and the implementation of one of the most successful vaccines in medical history.

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12. Reprints of selected papers

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