Faculty of Medicine and Health Sciences
Laboratory of Cell Biology and Histology

Prognostic value of HPV genotyping in cutaneous warts

Dissertation presented to obtain the degree of Doctor of Medical Sciences at the University of Antwerp to be defended by Nina Redzic

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Faculteit Geneeskunde en Gezondheidswetenschappen
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Prognostische waarde van HPV-genotypering in huidwratten

Proefschrift voorgelegd tot het behalen van de graad van doctor in de medische wetenschappen aan de Universiteit Antwerpen te verdedigen door Nina Redzic

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Abbreviations

AK	Actinic keratosis
ATV	Antwerpse Televisie
AV2	Antiviral 2 a.k.a. Omnivirol (OV)
BCC	Basal cell carcinoma
BD	Bowen's disease
bp	Base pairs
BP	Bowenoid papulosis
DB	Dot blot hybridization
dPCR	Digital PCR
E	Early region
EGF	Epidermal growth factor
EV	Epidermodysplasia verruciformis
GvA	Gazet van Antwerpen
HC	Hybrid capture
HPV	Human papillomavirus
HR-HPV	High-risk HPV
ISH	In situ hybridization
L	Late region
LAMP	Loop-mediated Isothermal Amplification
LCR	Long control region
LR-HPV	Low-risk HPV
LS	Lichen sclerosis
MS	Mass spectrometry
NCR	Non-coding region
NMSC	Non-melanoma skin cancer
OV	Omnivirol a.k.a. Antiviral 2 (AV2)

PDGF	Platelet-derived growth factor
pRB	Retinoblastoma protein
PV	Papillomaviridae
qPCR	Real-time PCR
RT-PCR	Reverse transcription PCR
SA	Salicylic acid
SCC	Squamous cell carcinoma
SK	Seborrhoeic keratosis
SPV	Shope papillomavirus
STH	Southern transfer hybridization
URR	Upstream regulatory region
UZA	Antwerp University Hospital (Universitair Ziekenhuis Antwerpen)
w/v	Weight/volume
α	Alpha
β	Beta
γ	Gamma
μ	Mu
ν	Nu

Chapter 1 Introduction

This chapter illustrates a broad introduction to the human papillomavirus (HPV) discussing i.a. its history, genome structure and life cycle, and classification systems, as well as providing an overview of HPV-related disease with an in-depth discussion on cutaneous warts and their treatment strategies. Lastly, a comprehensive outline for the research rationale is stipulated together with the specific objectives of the thesis.

1.1 History of human papillomavirus

Human papillomavirus (HPV), the most extensively studied virus of the past decade, is composed of a particularly heterogeneous family of DNA viruses, that can infect keratinocytes of the human skin and mucosa (Mammas et al., 2014). Prior to the discovery of HPV, early insights were gathered from observations in rabbits who had developed horns on their bodies and heads (Karamanou et al., 2010). The earliest descriptions of papillomavirus-induced disease in animals date from 1789, as deducted from 'Tableau Encyclopedique et Methodique' (see Figure 1; Bénard et al., 1789). Only in 1933 was Richard Shope able to identify the Shope papillomavirus (SPV) as the cause of this disease (Béziat & Jouanguy, 2021). The link with the cutaneous human disease was established in 1984 when sequencing of both SPV and HPV type 1a originating from human cutaneous warts revealed a strong resemblance (Cladel et al., 2019). Hence, papillomavirus infections have been most early connected to cutaneous diseases in humans, leading to insights of HPV as a 'wart-forming virus.'

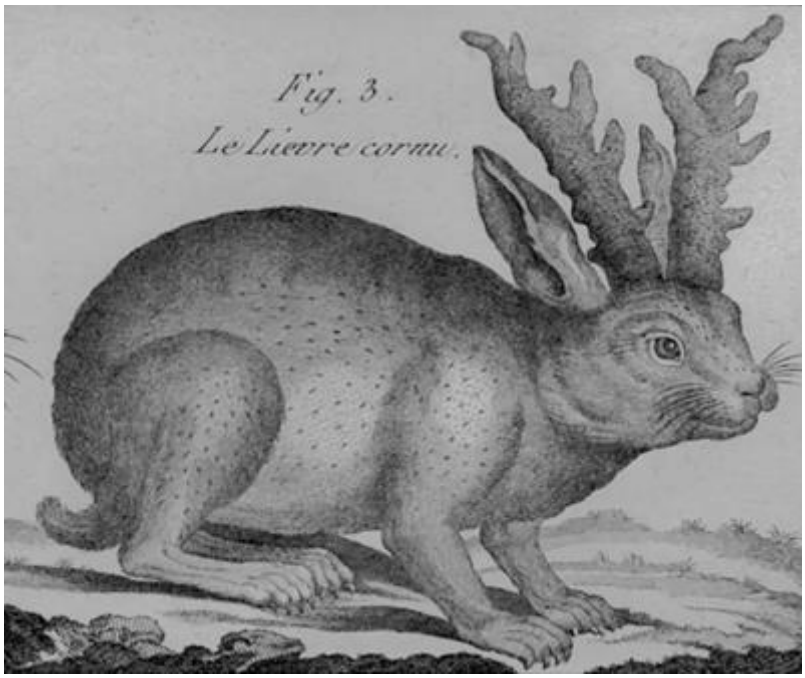


Figure 1. *Image of a rabbit with horns (Lepus cornutus). Adapted from Pierre Joseph Bonnaterre's Tableau Encyclopedique et Methodique from 1789, which offers one of the first descriptions of a papillomavirus-induced disease in animals.*

A few years before, in the 1970s, Harald zur Hausen was the first to suggest that HPV potentially plays a role in the etiology of cervical cancer (Frazer, 2019). Currently, it is a well-known fact that HPV is responsible for the majority of cervical cancer cases worldwide and its presence can be detected in nearly all cervical lesions. Furthermore, HPV was also isolated from multiple other organs and has been proven to be an important source of morbidity and mortality in other mucosal as well as cutaneous disorders. Anno 2021, it was estimated that HPV is responsible for 5% of all cancer cases worldwide (Sung et al., 2021).

1.2 Genome structure and organization

Research into different HPV types demonstrated that high similarities are being observed amongst different HPV genotypes concerning their genomic composition (Doorbar, 2005). Given its high carcinogenic potential, HPV 16 has been extensively studied and hence poses a representative example of genomic structure and organization for other HPV genotypes albeit with minor variations in some aspects such as genome length and organization (Chen et al., 2018). The genome of HPV 16 consists of double-stranded DNA, organized in one circular genome (see Figure 2; de Sanjosé et al., 2018). It comprises 7906 base pairs (bp) with four distinct regions i.e. an early (E) region, a late (L) region, and two non-coding regions (Zheng, 2006). These functional sites are separated by two polyadenylation sites. The designations ‘early’ or ‘late’ refer to the phase in the viral life cycle when these proteins are first expressed (Zheng, 2006).

The early region covers 50% of the entire viral genome and encodes for six early genes (i.e. E1, E2, E4, E5, E6, and E7; Graham, 2010). The E1 and E2 genes are involved in viral DNA replication and regulation of early transcription processes, in which the E1 protein exhibits helicase functionality and E2 functions as a prominent viral gene transcription regulator. The main purpose of the E4 gene is to weaken the host cell cytokeratin fibers, hereby destabilizing the cytoskeleton. The function of E5 is less well understood, but it is known that it can interact with epidermal growth factor (EGF) and platelet-derived growth factor

(PDGF) receptors (Venuti et al., 2011). In contrast to other E-genes, the role of E6 and E7 as viral oncogenes has been extensively investigated. Through combined action, E6 and E7 oncoproteins are potent key factors in host cell cycle disruption, leading to immortalization and transformation of the host cell (Pal & Kundu, 2020). The E6 oncoprotein is known to be involved in the degradation of p53, the most efficient tumor suppressor gene in humans. In addition, E6 is also able to activate telomerase activity thereby facilitating host cell immortalization. The E7 gene interacts with another important cell cycle control gene, the retinoblastoma protein (pRb). Via this dual approach involving both E6 and E7, HPV is capable of stimulating the cell cycle resulting in uncontrolled cell division (Zheng, 2006).

Regarding the late region, this region covers 40% of the viral genome and encodes for the late genes, L1 and L2, the so-called viral capsid proteins. Combined, the major L1 protein and the smaller L2 protein form the viral capsid that encloses the viral genome and enables the transport of the genome to another host cell (Zheng, 2006). Current prophylactic vaccines against HPV infections are based on L1 capsid elements (Stanley, 2006).

The two non-coding regions consist of the non-coding region (NCR) and the upstream regulatory region (URR; Zheng, 2006). The URR is further subdivided into a long control region (LCR), an enhancer region, and the p97 promotor region. The latter promotor is responsible for the expression of all E genes, located upstream of the E6 gene, and is under strict control of various cis-elements. These cis-elements are essential for gene expression regulation, genomic replication, and the formation of viral particles (Mirabello et al., 2012).

For an overview of HPV16 genomic organization and function of each region within the genome see Figure 2. In addition, Figure 3 offers a direct comparison of the viral genome organization of HPVs from several phylogenetic genera including alpha, mu, and beta papillomaviruses. They all share a common genomic organization but vary in size, position, and number of genes they contain (Doorbar et al., 2015). The genes involved in replication (i.e. E1 and E2) and capsid-formation (i.e. L1 and L2) are well conserved in all

papillomaviruses while greater diversity can be seen in the remaining genes (i.e. E6, E7, E5, and E4). For example, beta-HPVs do not have an E5 gene.

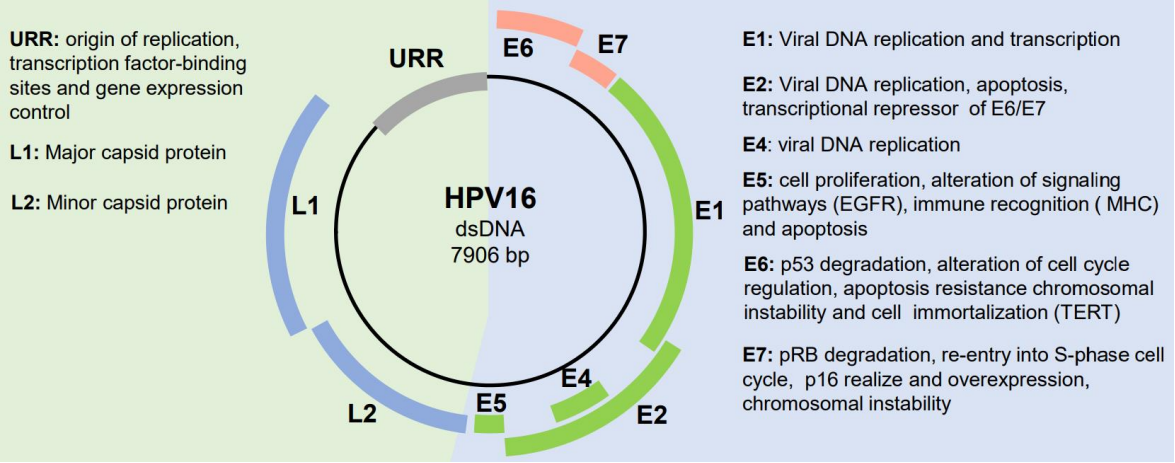


Figure 2. *Human papillomavirus (HPV) 16 viral genome organization. The HPV16 circular genome is depicted together with the different regions/genes and their function within the viral life cycle. The genes that are expressed early in the viral life cycle are denoted with the capital letter "E". The genes that are expressed late are indicated with a capitalized "L". The Upstream Regulatory Region, a non-coding region, is indicated as URR and is located between the stop codon of L1 and the start codon of E6. The non-coding region (NCR, not depicted in the figure) is located between the stop codon of E5 and the start codon of L2. Adapted from de Sanjosé et al., 2018.*

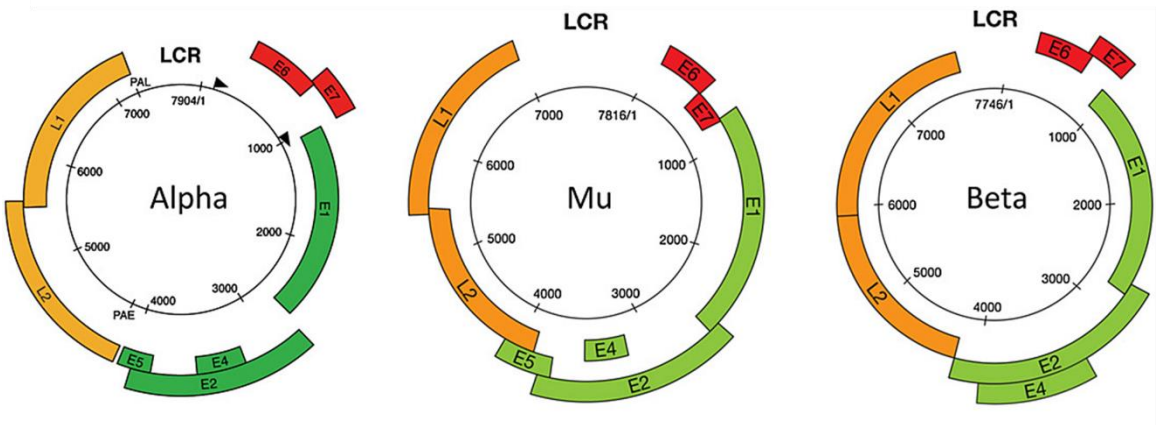


Figure 3. *Papillomavirus viral genome organization. The viral genome of alpha, mu, and beta papillomaviruses exhibit a similar genetic organization with some differences in genome size, position, and number of viral genes. The positions of major promotor regions within the alpha HPVs are marked with arrows. Early and late polyadenylation sites are marked respectively as PAE and PAL. Adapted from Doorbar et al., 2015.*

1.3 Classification

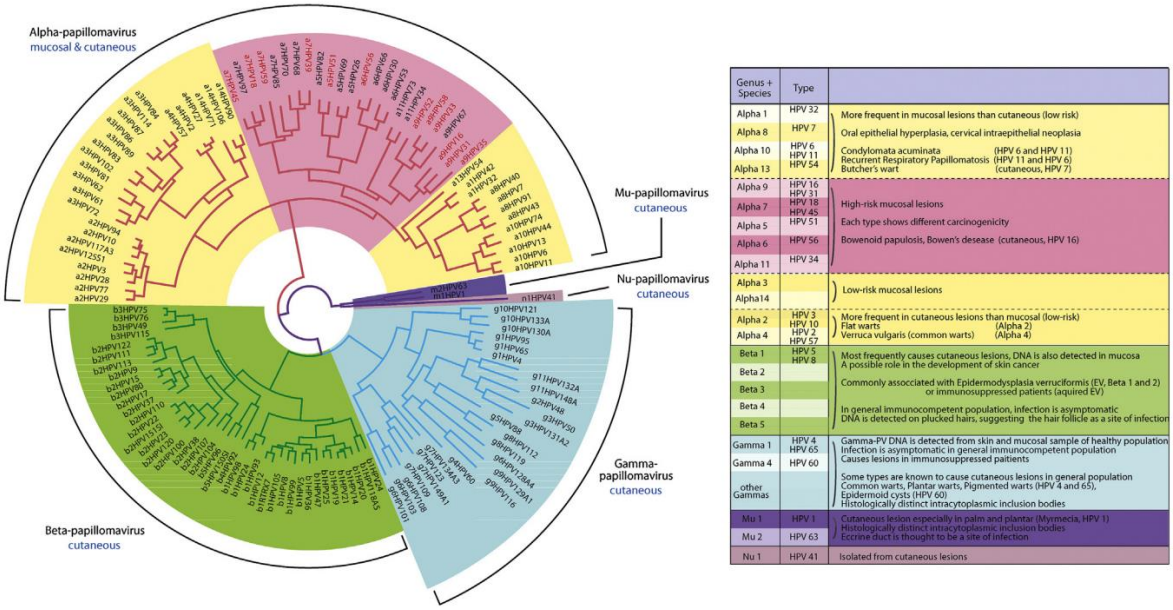
1.3.1 Taxonomic Classification

HPV is part of the Papillomaviridae (PV) family which is classified into various genera and further subdivided into distinct species, each containing one or more types (Zheng, 2006). This taxonomic classification is based on the DNA sequence similarities in the L1 gene and enables the identification of over 200 distinct HPV types (Zheng, 2006).

HPV genera are defined by less than 60% sequence similarity in the L1 gene and are known as alpha (α), beta (β), gamma (γ), mu (μ), and nu (ν; Egawa & Doorbar, 2017). The phylogenetic grouping can sometimes align with biological and/or pathological similarities, where for example various types and species within the same genus exhibit the same tissue tropism or can develop the lesions with similar severities. This however is not always the case and there are often distinct differences (Leto et al., 2011).

Regarding the species level, species within the same genus exhibit a higher degree of sequence conservation (60-70% similarity). In case where there is more than 10% variation in the L1 region as compared to an existing HPV type, a new HPV type is distinguished. A new subtype is identified in the case of 2-10% variation, whereas variation below 2% is considered a variant of an established type (Leto et al., 2011).

Figure 4 provides an overview of the taxonomic classification of HPV with division into different genera, species, and types.



Genus + Species	Type	
Alpha 1	HPV 32	More frequent in mucosal lesions than cutaneous (low risk)
Alpha 8	HPV 7	Oral epithelial hyperplasia, cervical intraepithelial neoplasia
Alpha 10	HPV 6 HPV 11	Condylomata acuminata (HPV 6 and HPV 11)
Alpha 13	HPV 54	Recurrent Respiratory Papillomatosis (HPV 11 and HPV 6) Butcher's wart (cutaneous, HPV 7)
Alpha 9	HPV 16 HPV 31	High-risk mucosal lesions
Alpha 7	HPV 18 HPV 45	Each type shows different carcinogenicity
Alpha 5	HPV 51	
Alpha 6	HPV 56	Bowenoid papulosis, Bowen's desease (cutaneous, HPV 16)
Alpha 11	HPV 34	
Alpha 3		Low-risk mucosal lesions
Alpha14		
Alpha 2	HPV 3 HPV 10	More frequent in cutaneous lesions than mucosal (low-risk) Flat warts (Alpha 2)
Alpha 4	HPV 2 HPV 57	Verruca vulgaris (common warts) (Alpha 4)
Beta 1	HPV 5 HPV 8	Most frequently causes cutaneous lesions, DNA is also detected in mucosa A possible role in the development of skin cancer
Beta 2		Commonly associated with Epidermodysplasia verruciformis (EV, Beta 1 and 2) or immunosuppressed patients (aquired EV)
Beta 3		
Beta 4		In general immunocompetent population, infection is asymptomatic
Beta 5		DNA is detected on plucked hairs, suggesting the hair follicle as a site of infection
Gamma 1	HPV 4 HPV 65	Gamma-PV DNA is detected from skin and mucosal sample of healthy population Infection is asymptomatic in general immunocompetent population
Gamma 4	HPV 60	Causes lesions in immunosuppressed paitients
other Gammas		Some types are known to cause cutaneous lesions in general population Common warts, Plantar warts, Pigmented warts (HPV 4 and 65), Epidermoid cysts (HPV 60) Histologically distinct intracytoplasmic inclusion bodies
Mu 1	HPV 1	Cutaneous lesion especially in palm and plantar (Myrmecia, HPV 1) Histologically distinct intracytoplasmic inclusion bodies
Mu 2	HPV 63	Eccrine duct is thought to be a site of infection
Nu 1	HPV 41	Isolated from cutaneous lesions

Figure 4. *Phylogenetic classification of Human Papillomaviruses. HPV types detected in humans are classified into five different genera i.e. alpha (with cutaneous and low-risk mucosal types depicted in yellow and high-risk mucosal types in pink), beta (green), gamma (blue), mu (purple), and nu (brown). The high-risk alpha HPV types that are marked in red are confirmed as carcinogens based on epidemiological data. The remaining high-risk types are all classified as 'probable' or 'possible' carcinogens. In addition, the table on the right provides a summary of the tropism and pathogenesis of each HPV species. Adapted from Egawa et al., 2017.*

1.3.1.1 *Alpha-papillomavirus*

The α-papillomaviruses typically display an affinity for genital epithelia, although certain members of this genus can infect skin as well (De Villiers et al., 2004). The α-genus is primarily recognized for HPV types 16 (α9) and 18 (α7), which account for 70% of cervical cancer cases worldwide (Shen, 2019). Within the same genus, HPV 6 and 11 (α10) are responsible for causing genital warts, while HPV 3 and 10 (α2), HPV 7 (α8), and HPV 2, 27, and 57 (α9) are associated with various types of cutaneous warts (for more detailed information see section 1.6 Clinical manifestations; Leto et al., 2011).

1.3.1.2 *Beta-papillomavirus*

The β-papillomaviruses exclusively infect the cutaneous epithelium and are a part of the normal human virome from infancy (Antonsson et al., 2003). However, in some cases, β-HPVs can cause cancer (Tommasino, 2017). The majority of such cancers occur in immunosuppressed individuals and in patients with epidermodysplasia verruciformis (EV; for more detailed information see section 1.6 Clinical manifestations). The most researched

β-HPV types are HPV 5 and 8 (β1), frequently found in skin lesions from EV patients (de Jong et al., 2018).

1.3.1.3 *Gamma-papillomavirus*

γ-papillomaviruses are involved in cutaneous infections, with cutaneous warts as the most common manifestations. The most prominent types are HPV 4 and 65 from γ1, and 60 from γ4 species (Leto et al., 2011).

1.3.1.4 *Mu-papillomavirus*

μ-papillomaviruses cause cutaneous warts as well and include only two types i.e. HPV 1 and 63 (De Villiers et al., 2004).

1.3.1.5 *Nu-papillomavirus*

ν-genus includes so far only one type, HPV 41, which can be found in cutaneous warts (De Villiers et al., 2004).

1.3.2 Classification According to Tropism

Specific HPV types seem to have a preference for certain types of epithelial tissue or have been isolated only from specific tissues. As a result, HPVs have been divided into two primary groups: cutaneous and mucosal HPVs (Mistry et al., 2008). While all HPV types share an affinity for keratinocytes within stratified squamous epithelium, there are variations in their tendency to infect different anatomical sites. The majority of α-HPV types exhibit a pronounced preference for mucosal epithelium, while β- and γ-HPVs are primarily responsible for causing cutaneous lesions, earning them the classification of cutaneous HPV types. However, it's important to note that this classification isn't entirely precise, as genital HPV types can be found on the skin, and vice versa (Leto et al., 2011).
In addition, mucosal HPVs are further categorized into low-risk (LR-HPV; e.g., HPV 6) and high-risk groups (HR-HPV; e.g., HPV 16), aligning with their varying potential for oncogenicity in the development of cervical cancer (Burd, 2003).

1.4 Life Cycle

As previously mentioned, HPV is capable of infecting keratinocytes in cutaneous as well as mucosal stratified squamous epithelia. While stratified squamous epithelium is composed of several distinct layers, HPV is only able to infect the lowest epidermal layer i.e. the basal cell layer (Moody, 2017). To this end, a micro-lesion is necessary for HPV to physically reach the basal cell layer, which contains the basal stem cells, the preferred targets of HPV. HPV then exploits the keratinocyte differentiation process of these cells to create a productive viral life cycle (AL-Eitan et al., 2020). During the wound-healing process, active cell division enables the entry of the viral genome in the nucleus and the formation of viral episomes (see Figure 5; McBride, 2017).

The mechanisms used by the viruses to enter the basal stem cells are not clearly understood and are mostly based on research with carcinogenic mucosal HPV types. Once the virus has entered the host cell certain processes take place in a pre-defined order and although these processes are also mostly based on research with HR-HPVs, the broad principles are likely to be common for all HPV types in general (Doorbar et al., 2012; S. V. Graham, 2017a):

- Initiation of the non-productive infection state: Once an HPV particle passes the cell membrane of the host cell, it entirely depends on the host cellular machinery for its DNA replication. The HPV genome is produced at a low copy number in the infected cell and only early viral genes and gene products are expressed during this non-productive infection stage (Harden & Munger, 2017).

- Preservation of the non-productive infection state: an HPV infection can remain in a latent non-productive infection phase for years. This phenomenon is commonly observed in HR-HPV types where the viral genome is maintained in low copy numbers over multiple cell divisions through mechanisms that are not yet completely identified (Harden & Munger, 2017).

- Productive infection stage: When the infected basal cells start to differentiate, the HPV genome hijacks and delays the differentiation cycle to alter the replicative cellular DNA capacity, resulting in the amplification of the viral genome at a high copy number. The late viral genes are then expressed, the viral capsid is formed and new viruses are assembled that will ultimately be released in the upper epithelial layer during shredding of the epithelial cells (Harden & Munger, 2017).

As already mentioned, persistent HPV infection is very rare in LR-HPV types when compared to HR- HPV. HR-HPV types are capable of inducing the proliferation of infected cells already in the lower layers of stratified epithelium which allows them to transition from a non-productive stage to a productive stage infection triggering several mechanisms for the epithelial transformation (Bodily & Laimins, 2011). In some cases, a productive infection can lead to high-grade neoplasia, where overexpression of viral genes leads to subsequent genetic changes in the host cell and possible integration of the viral genome into the cellular chromosome (Doorbar et al., 2015b). It is hypothesized that the increased oncogenic capacity of the HR-HPV types is related to E6 and E7 oncoprotein activity, seeing that E6 and E7 proteins of LR-HPV and HR-HPV types have different functions, which is reflected in their oncogenic potential (Doorbar et al., 2015b). The ability of the HR-HPV types to drive cell division in neoplasia is related to the ability of their E7 protein to bind and degrade multiple members of the pRb protein family, as well as the ability of E6 to efficiently degrade p53 and interact with other cellular proteins, making HPV capable of avoiding the host immune system and allowing the infection to persist and eventually progress to pre-malignant or malignant lesions (AL-Eitan et al., 2020; Nunes et al., 2018).

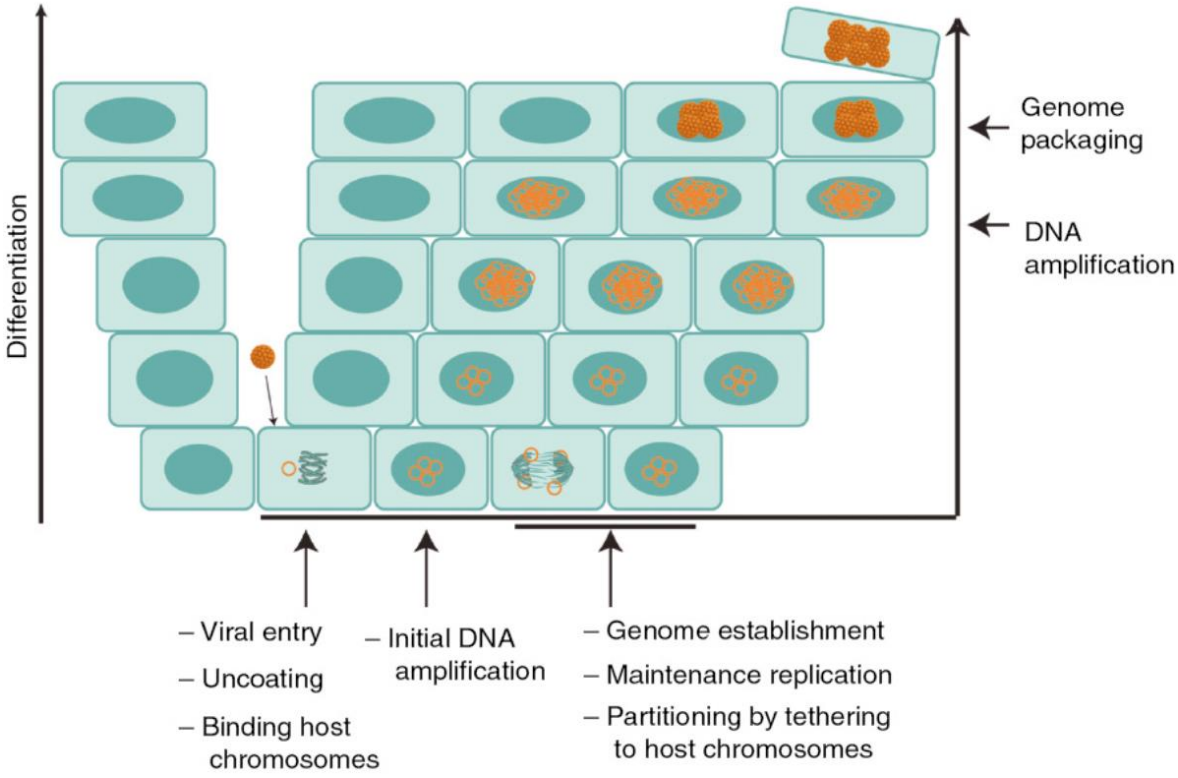


Figure 5. *The life cycle of HPV in squamous stratified epithelium. In the presence of a micro-lesion, HPV is capable of reaching the lower basal epidermal layer and binding to the receptors on the cell membrane from the basal stem cells. Upon cell entry, the virus loses its capsid coating and the viral genome can enter the nucleus at the time of mitosis when the nuclear membrane is dismantled for cell division. Once in the nucleus, the viral genome is amplified in low copy numbers and is attached to the host chromosomes making partitioning in different cells during cell division possible. During cell differentiation, the viral genome is amplified to a high copy number, and viral capsid genes are expressed. The viral capsid is assembled and virions are ready to be released during shredding of the uppermost layer of the epithelium. Relative levels of viral copy number during each stage of viral DNA replication are depicted in brown. Adapted from McBride, 2017.*

1.5 HPV Detection Methods

Testing for the presence of HPV DNA/RNA has mainly gained attention as a cervical cancer screening alternative strategy that started in the late 1980s when it became clear that HPV was involved in cervical carcinogenesis (Saraiya et al., 2013). HPV testing gained further visibility due to its potential to identify precursor lesions before the formation of true malignancies. More recently, efforts were made to standardize molecular testing of exfoliated cervical cells so that they have acceptable screening performance while being

more reproducible and more easily adapted for automated, high throughput testing in clinical practice when compared to other microscopic screening techniques (i.e. cytology and histology; (Arbyn et al., 2016, 2018, 2021; Meijer et al., 2009).

Techniques to detect the presence of HPV in human specimens have considerably evolved in the last four decades and more and more HPV tests are becoming available. Molecular techniques, in particular, are widely used for HPV detection and are categorized into three main groups: (1) direct hybridization assays, (2) signal amplified hybridization assays, and (3) target amplification assays (Zaravinos et al., 2009).

Examples of direct hybridization assay techniques are Southern transfer hybridization (STH), dot blot hybridization (DB), and in situ hybridization (ISH). These techniques do pose several disadvantages such as high DNA input, labor-intensive protocols, and low reproducibility as well as moderate sensitivity for HPV (Zaravinos et al., 2009).

Hybrid capture (HC) assays are based on signal amplified hybridization and have previously been widely used in the HPV world as a comparator and reference test for validation of new upcoming HPV assays, especially the Hybrid Capture 2 High-Risk HPV DNA test (Qiagen, Venlo, Netherlands; Arbyn et al., 2021; Cook et al., 2017; Kulmala et al., 2004). The sensitivity achieved by HC assays is comparable to that of PCR-based assays (i.e. target amplification assays). However, PCR-based assays not only exhibit both high sensitivity and specificity but also require a very limited amount of DNA input and offer the potential for a high degree of automation (Zaravinos et al., 2009).

Furthermore, PCR-based assays can implement a variety of different detection techniques to obtain the end result, with some examples being (1) end-point PCR, such as gel electrophoresis or the most recently developed technique being the digital PCR (dPCR; Lillsunde Larsson & Helenius, 2017; Mahmoodi et al., 2016), (2) real-time PCR (qPCR) based on intercalating dyes such as SYBR green or TaqMan fluorescent probes (Micalessi et al., 2012; Tsakogiannis et al., 2015), (3) reverse transcription PCR (RT-PCR) capable of detecting

HPV mRNA (Andersson et al., 2011), and (4) Luminex PCR based on fluorescent flow cytometry (Geraets et al., 2014).

Other examples of more recently utilized techniques for HPV detection are assays based on sequencing, Loop-mediated Isothermal Amplification (LAMP), and mass spectrometry (MS; Arroyo et al., 2013; Cai et al., 2019; Y. Wang et al., 2020a).

In addition, the introduction of molecular HPV assays for cervical cancer screening has also opened a wide range of opportunities for the diagnosis of other HPV-related conditions. Prominent examples include screening for anal and penile cancers, head-and-neck disease, HPV-related infertility, and most recently also the HPV-induced cutaneous diseases. Although several research laboratories have developed their own HPV assays capable of detecting certain relevant cutaneous HPV types, a fully validated commercial assay for wart-associated HPV types is currently not available (Breznik et al., 2020; de Koning et al., 2010; Michael et al., 2011a; Sasagawa & Mitsuishi, 2012; Schmitt et al., 2011; Y. Wang et al., 2020b).

1.6 Clinical Cutaneous Manifestations

HPV is capable of causing a wide range of diseases from benign lesions to invasive tumors and although it is most commonly known for its involvement in the development of cervical cancer, there are also other mucosal as well as cutaneous disorders where HPV plays a role (see Chapter 2, Table 1). The clinical presentation of HPV infection depends on multiple factors including the viral genotype, the type of epithelium infected (i.e. host cell phenotype), and other host factors such as the status of host immunity (Zhou et al., 2019). Regarding cutaneous disorders, it remains extremely challenging to determine the exact role of HPV in their development (J. Wang et al., 2014). Is HPV responsible for the onset of the disease? Is it a co-factor that acts with other carcinogens to amplify the risk of disease? Or is it merely an innocent bystander without any role in disease pathogenesis?

For more details about the challenges faced during research into an association between HPV and certain cutaneous disorders see Chapter 2: Optimizing the Pre-Analytical Phase for Accurate HPV Detection in Skin Disorders: Insights from a Cutaneous Warts Case Study. A summary of cutaneous clinical manifestations of HPV infection is provided in Table 1.

Table 1. *HPV-related cutaneous disorders. A summary of HPV-related cutaneous disorders is provided with a brief description of the disease state, preferred location of lesions, disease prevalence in the general population, and HPV prevalence in each condition with the most frequently detected HPV types. The exact role of HPV in the disorders marked with an * remains currently unknown (for more detailed information see Chapter 2: Optimizing the Pre-Analytical Phase for Accurate HPV Detection in Skin Disorders: Insights from a Cutaneous Warts Case Study).*

Disorder	Description	Location	Prevalence in the general population	HPV prevalence
Non-malignant skin lesions				
Actinic keratosis (AK)*	AK is characterized by colored spots or plaques with increased keratosis. It is considered a cancer precursor lesion with reported progression rates of 0.03-20% to cutaneous squamous cell carcinoma (SCC; Balcere et al., 2022; Galati et al., 2020).	Sun-exposed skin such as face, lips, ears, scalp, neck, hands and forearms (Marques & Chen, 2023).	Varies from 20-60% depending on geographical location and residence.	Between 50-71%. Mostly beta and gamma HPVs (Galati et al., 2020; Marques & Chen, 2023).
Bowenoid papulosis (BP)	BP is a rare sexually transmitted disease that occurs in both males and females. It is characterized by single or multiple skin-colored papules in the anogenital area and is induced virally by HPV. Clinically BP is similar to genital warts while histologically it has a close resemblance to Bowen's disease (BD; Park et al., 2013).	Anogenital area.	Unknown.	Between 7-56%. Most lesions are associated with oncogenic HPV types with the most prominent being HPV 16 and less frequent HPV18, 31, 33, 34, 35, 39, 42, 48, 51, 52, 53, and 54 (Chamli & Zaouak, 2023).

Table 1 continued

Disorder	Description	Location	Prevalence in the general population	HPV prevalence
Non-malignant skin lesions				
Lichen sclerosis (LS)*	LS is a chronic inflammatory skin disease with symptoms ranging from none to itching sensation and pain, with the possibility of permanent scarring and disfigurement. In addition, patients with LS exhibit a 5% increased risk of SCC in the anogenital area (Hald & Blaakaer, 2018).	Anogenital area.	0,1%. More common in women than in men.	Highly variable, ranging between 0-80%. The most prevalent types are HPV16 and 18, followed by HPV6, 11, 31, 33, 45, 52 and 58 (Hald & Blaakaer, 2018).
Psoriasis*	Psoriasis is a chronic inflammatory disease presenting multiple eruptions of the skin. Psoriasis can arise due to multiple genetic or environmental factors, particularly drugs and infections.	Psoriasis can occur all over the body and is most commonly found on the knees, elbows, trunk, and scalp.	1–5%.	Between 83-92%. Beta types HPV5 and 36 are commonly detected (Cronin et al., 2008; Favre et al., 1998).
Seborrhoeic keratosis (SK)*	SK usually presents as a flat, brownish lesion with a smooth or hyperkeratotic surface and is often confused with warts due to its clinical or histological appearance (i.e. hyperkeratosis, papillomatosis, and acanthosis; Nellessen et al., 2023; Sari et al., 2020).	Various body areas, most typically in heavily sun-exposed skin such as the face and upper body (Sari et al., 2020).	First onset is around the age of 30 years, becoming more frequent with increasing age with 90% of patients older than 60 years exhibiting SK (Jackson et al., 2015).	α-HPV type prevalence in genital SK ranges between 42-72%, while the prevalence in non-genital SK is reported to be more variable (0-91%; Nellessen et al., 2023).

Table 1 continued

Disorder	Description	Location	Prevalence in the general population	HPV prevalence
Non-malignant skin lesions				
Warts	Warts are benign growths on the skin. Cutaneous warts are lesions of the skin, while genital warts commonly manifest in the anogenital region.	Cutaneous warts are most commonly found on hands and feet. Genital warts can be found at the vulva, perineum, perianal area, penis, anus and scrotum.	Cutaneous warts: widespread, affect both children (30%) and adults (1%-13%). Genital warts: 0.1–0.6%, with a peak at 20-29 years (Kombe Kombe et al., 2021; Zhu et al., 2019).	Cutaneous warts: 90%, with most prevalent types being HPV1, 2, 3, 4, 7, 10, 27, 41, 57, 60, 63 and 65. Genital warts: 60%, HPV6 and 11 most frequent (Bruggink et al., 2012; Patel et al., 2013).
Malignant skin lesions				
Keratoacanthoma*	Keratoacanthoma is an epithelial tumor with clinical and histopathological features similar to SCC and a progression rate to malignancy of 15%, that has been classified into low-grade SCC in the recent WHO classification of cutaneous tumors (Murphy et al., 2018; Vîlcea et al., 2022).	Sun-exposed skin.	The incidence ranges between 100-150 cases per 100,000 individuals but is highly underrated due to misclassification as SCC, underreporting, or spontaneous regression before diagnosis (Tisack et al., 2021).	11%. The most prevalent types belong to the beta and gamma (Baek et al., 2022; Neagu et al., 2023).

Table 1 continued

Disorder	Description	Location	Prevalence in the general population	HPV prevalence
Malignant skin lesions				
Bowen's Disease (BD)*	BD is SCC in situ that can occasionally progress to invasive carcinoma. The role of HPV in genital BD is well recognized but is not fully clear in extragenital BD (Baek et al., 2020; Kettler, 1990).	BD is common in sun-exposed areas of the skin, but other sites can also be involved such as the anogenital region, palms, and soles(Palaniappan & Karthikeyan, 2022).	Unknown. It is most frequently found in male Caucasians over the age of 60 (Morton et al., 2014).	Higher in genital BD (45%) compared to extragenital BD (18%). Mostly high-risk HPV16, 33, and 58 are detected, but also other mucosal/cutaneous types can be found i.e. HPV2, 6, 11, 27, 54, 58, 61, 62, 73, 76 (Leto et al., 2011).
Non-melanoma skin cancer (NMSC)	NMSC comprises SCC as well as basal cell carcinoma (BCC). The association of HPV with NMSC is observed both in immunocompetent and immunocompromised patients. For example, 40% of kidney transplant patients who had a transplant 15 years ago will develop NMSC (Matinfar et al., 2018).	Sun-exposed skin such as the face, ears, hands, and shoulders.	30% of all cancers worldwide.	Immunocompetent: 55% SCC, 44% BCC. Immunocompromised: 88% SCC, 75% BCC. Mostly EV-types HPV5 and 8, cutaneous types only in immunocompromised individuals (Leto et al., 2011; Reuschenbach et al., 2011).

Table 1 continued

Disorder	Description	Location	Prevalence in the general population	HPV prevalence
Malignant skin lesions				
Epidermodysplasia verruciformis (EV)	EV is an autosomal recessive disease that causes a defect in cellular immunity and alters the way HPV is detected and cleared by the immune system, making the affected individuals highly susceptible to skin cancer induced by HPV. These patients frequently first exhibit warts and plaques of seborrheic keratosis in early childhood. Later in life, premalignant lesions such as actinic keratoses and malignant lesions such as BD and SCC can also develop.	Sun-exposed skin.	Approximately 500 patients have been diagnosed worldwide with no clear predisposition to gender, race, or geographic residence (da Cruz Silva et al., 2019; Myers et al., 2023).	90%. Patients with EV are highly predisposed to infection by EV-HPV i.e. HPV5, 8, 9, 12, 14, 15, 17, 19-25, 28, 29, 36-38, 47, 49, and 50. HPV3 and 10 are commonly detected in warts of EV patients (S. V. Graham, 2017b).
Penile cancer*	Penile cancer is a rare neoplasm most common in men aged 50–70 years old. SCC is diagnosed in approximately 95% of all penile cancer cases, followed by other malignancies such as BCC, penile sarcomas, melanoma, lymphoma, and metastatic disease (Iorga et al., 2019; Yu et al., 2019).	Penile cancer is commonly found at the penile glans, followed by the prepuce, coronal sulcus, and less commonly the penile shaft.	0.6% of malignant cases in the USA and Europe, and 5% in developing countries (Douglawi & Masterson, 2017).	20%. Higher in uncircumcised men. HPV16 and 18 are the most frequently detected.
Vulvar cancer	SCC is diagnosed in approximately 90% of all vulvar cancers (Li et al., 2023; Zhang et al., 2018).	Vulvar cancer commonly develops in labia minora, followed by labia majora and perineum.	6% of all female genital tract malignancies worldwide.	Variable 3-77%. HPV16 and 33 are the most predominant HPV types.

1.6.1 Warts

1.6.1.1 *Etiology*

Cutaneous warts or verrucae are the most common clinical manifestations of HPV infection (Leto et al., 2011). Although benign in nature, they can occasionally be very painful and impair the quality of life depending mostly on their location and size. They exhibit a varying worldwide prevalence of 0.84-12.9% (Hashmi et al., 2015a). The prevalence rate in children and young adults is even higher (15-45%) and reported to reach a peak around 12-16 years of age (Sterling et al., 2014). Although warts rarely occur in early childhood, we do see an increase in incidence among school-aged children (Holder et al., 2019a; Kilkenny et al., 1998). They also exhibit a higher incidence in immunocompromised patients. For example, 90% of kidney transplant patients develop skin warts within 5 years of transplantation (Kombe Kombe et al., 2021). Furthermore, cutaneous warts do occur in all races, but they are observed about twice as often in White people, than in Black people or Asians. They are however equally common in men and women (Al Aboud & Nigam, 2023).

1.6.1.2 *Pathophysiology*

Warts are caused by HPV infection of keratinocytes (the predominant cell type in the epidermis). They can be transmitted by direct as well as indirect contact i.e. skin-to-skin contact with a wart directly or indirectly with HPV infected surfaces. A wart can only develop if the normal epithelial barrier is disrupted due to a lesion of the skin (Moody, 2017). HPV then has a chance to settle in the lower epidermal layers, in keratinocytes, and cause abnormal cell growth. After inoculation, the incubation period varies from 3 weeks to 8 months (Leto et al., 2011). As previously explained in Chapter 1.4 Life cycle, the life cycle of HPV is directly linked to the cell differentiation program of the keratinocytes. Infection begins when the HPV reaches the lower epidermal layer where there is no viral replication and the virus is only present in a low number of copies. In the suprabasal layers, however, replication of the viral genome does occur and viral protein synthesis is established as well (Leto et al., 2011).

1.6.1.3 *Epidemiology*

Cutaneous warts are classified into different types based on their morphological features and location as described in Table 2.

Table 3 provides a summary of HPV types commonly found in different cutaneous wart types. The most prevalent HPV types in cutaneous warts are HPV 1, 2, 3, 4, 10, 27, and 57. Other frequently found types are HPV 7, 41, 60, 63, and 65. Occasionally mucosal types are found, all belonging to the HR- or potentially HR-HPV types that are capable of causing cervical cancer (Brentjens et al., 2002; Bruggink et al., 2012; Bzhalava et al., 2013; Cardoso & Calonje, 2011; Cubie, 2013; de Koning et al., 2010; De Koning et al., 2015; Giannaki et al., 2013; Hashmi et al., 2015b; Leto et al., 2011; Michael et al., 2011b; Schmitt et al., 2011).

***Table 2**. Cutaneous wart types. Cutaneous warts are categorized into different wart types based mostly on their morphological appearance and location. The table provides an overview of different wart types, their most prominent morphological features, histological characteristics, preferred location of lesions, as well as prevalence in the general population.*

Wart type	Appearance	Histology	Location	Prevalence
Common warts *Verruca Vulgaris*	Common warts are rough, gray-brown, dome-shaped skin growths. Lesions may be single or multiple and vary in size. The confluence of lesions can form large masses.	Common warts exhibit distinct histopathological features including papillomatosis, hyperkeratosis, hypergranulosis, acanthosis, and elongated rete ridges. Koilocytes (i.e. cells with a round enlarged basophilic nucleus surrounded by a clear halo) are often observed, as well as vertical rows of parakeratosis and multiple keratohyalin granules (Araújo et al., 2021).	Common warts appear most often on the hands and fingers, but they may appear anywhere on the body. A frequent location in children is the knee.	The prevalence of common warts is estimated to range between 5-20% in children and young adults (Cubie, 2013).
Plantar warts *Verruca Plantaris*	Plantar warts often exhibit small black spots, indicating hemorrhages under the skin. Skin striations go around the wart which distinguishes them from calluses or corns. Two types of plantar warts exist: simple warts with a single capillary feeding the infected cells, and mosaic warts with multiple capillaries, making them highly resistant to treatment.	Mosaic plantar warts exhibit histopathological characteristics similar to common warts. Simple plantar warts are characterized by abundant keratohyalin granules and irregular inclusion bodies in the cytoplasm of keratinocytes (Tyring, 2000).	Plantar warts are located on the soles of feet and toes and are frequently painful when standing or walking.	Plantar warts usually occur in children and young adults with an estimated prevalence of 3-30% (Cubie, 2013).

Table 2 continued

Wart type	Appearance	Histology	Location	Prevalence
Flat warts *Verruca Plana*	Flat warts are smooth, flat, skin- or brownish-yellow-colored raised bumps of the skin. They are mostly circular or oval and limited in size to a few millimeters.	Flat warts show hyperkeratosis and acanthosis. Papillomatosis and areas of parakeratosis are not less prominent, with only a slight elongation of rete ridges being observed. There is diffuse vacuolation and an increase in cell size with centralization of nuclei that become strongly basophilic and pyknotic in the spinous and granular layers (Leto et al., 2011).	Flat warts are most commonly found in the face or back of the hands.	Flat warts are the least prevalent wart types, exhibiting again highest prevalence in children and teenagers (0,3-6%; Cubie, 2013)
Filiform warts *Verruca Filiformis*	Filiform warts are long warts with a slender structure that grow perpendicularly or obliquely in relation to the skin surface and have visible, individual projections that often resemble filaments.	Filiform warts appear similar to common warts, but they may have prominent papillomatosis (Al Aboud & Nigam, 2023).	Filiform warts are commonly found on body parts with thin skin (e.g. lips, eyes, chin, and neck).	The prevalence of filiform warts is reported to range between 4-17% in the diseased population (Ghadgepatil et al., 2016).

Table 3. *HPV type-specific prevalence according to wart type. A summary of HPV type-specific prevalence in different wart types is provided with a distinction being made between cutaneous and mucosal HPV types.*

Wart type	HPV type
Common warts	The most commonly found types in common warts are cutaneous types HPV2 and 7, and less frequently HPV1, 2, 4, 7, 27, 28, 29, 57, 60, 65, 77, 91, 94, 95 as well as mucosal types HPV16, 18, 26, 31, 35.
Plantar warts	Simple plantar warts are commonly caused by HPV1, while mosaic warts are usually caused by HPV2 and 57. Other less frequently found cutaneous types are HPV4, 60, 63, 65, and mucosal types 16 and 66.
Flat warts	HPV types most frequently detected in flat warts are HPV3, 10, 28, and 29. Less frequently found types are HPV2, 16, 26, 27 and 41.
Filiform warts	Common HPV types found in filiform warts are HPV1, 2, 4, 7, 27, and 57.

1.6.1.4 *Treatment*

Although most verrucae will disappear spontaneously, many patients do seek treatment (Holder et al., 2019b). According to a study by Berna et al., the estimated annual cost for cutaneous warts in 2019 in the United States amounted to $846 million with an average per-patient cost of $288.28 (Berna et al., 2022). In addition, over-the-counter costs and in-office procedures and medications that were not billed were not included in this cost estimate suggesting that the per-patient cost may be even higher.

The next section provides an overview of the most utilized treatment strategies when considering the clinical management of cutaneous warts.

1.6.1.4.1 First-line Treatment: destructive over-the-counter treatments

1.6.1.4.1.1 Salicylic acid

Salicylic acid (SA) formulations are the most commonly used preparations in the treatment of viral warts (Sterling et al., 2014). SA is an organic acid that destroys epidermal cells and softens the hyperkeratotic epidermis. These effects are postulated to be able to stimulate host immunity, which can be an additional mechanism of action against warts. Commercially available preparations range from 10-26% SA, with 17% SA being the most commonly used over-the-counter preparation. Higher SA concentrations (20-40%) are also possible but often need to be prescribed by a physician.

SA treatment is commonly conceived as rather difficult due to side effects such as irritation of the surrounding skin and labor-intensive daily appliance often resulting in low treatment compliance (Sterling et al., 2014). In a pooled analysis of 16 studies where SA treatment of cutaneous was examined in a total of 813 patients, 421/813 patients were cured; with a mean cure rate of 52%, with a range of 0–87% (Kwok et al., 2011). In contrast, the placebo arms exhibited a cure rate of 23% (range 5–73%; Gibbs & Harvey, 2006). Another fact to consider is that practically all except very low-strength SA can cause chemical burns and should not be used in areas of poor healing such as neuropathic feet (Truong et al., 2022). Alternative over-the-counter available acids are lactic acid and acetic acid.

1.6.1.4.2 Second-line Treatment: physician-administrated treatments

1.6.1.4.2.1 Cryotherapy

Liquid nitrogen is the most commonly used agent for cryotherapy (Sterling et al., 2001). Cryotherapy can be effective either by simple necrotic destruction of HPV-infected cells, or similar to SA by inducing local inflammation contributing to the development of an effective cell-mediated response. Application techniques differ between practitioners with alterations in freeze times, mode of application and treatment intervals. Most practitioners use a spray, but cotton wool-tipped sticks are also commonly used and can be preferred when treating children (Sterling et al., 2001). Treatment is applied until a halo of frozen

tissue is seen around the wart, highly depending on site and size of the lesion. Standard practice is to repeat the treatment every 2–3 weeks until the warts have cleared, up to a maximum of approximately six treatments (Kwok et al., 2012; Sterling et al., 2014). The available evidence from randomized trials broadly supports the efficacy of cryotherapy, except in the case of plantar warts where no treatments, including cryotherapy, have been convincingly demonstrated to be consistently and significantly effective (Gibbs et al., 2002). The reported cure rate of cryotherapy for warts at all sites from randomized trials (n=17) is highly variable, ranging from 0% to 69% with a mean of 49% (Kwok et al., 2011). Where possible, subgroup analysis of data from these trials suggests that cure rates are generally better for warts located in hands than for plantar warts (Sterling et al., 2001). Furthermore, one study suggested that clearance rates are improved when cryotherapy is combined with local salicylic acid application, however statistical significance was not reached (Bunney et al., 1976).

Common adverse events due to cryotherapy are pain and blistering, which are reported to occur more frequently when shorter-interval treatment regimens are applied (Gibbs et al., 2002). Caution must be used when applying cryotherapy near cutaneous nerves, tendons and the nail apparatus, and also in patients with impaired arterial or venous circulation (Sterling et al., 2001). Another common adverse event when utilizing cryotherapy is hypo- or hyperpigmentation, particularly in patients with darker skin types (Cockayne et al., 2011).

1.6.1.4.2.2 Antimitotic Therapy

Bleomycin is a cytotoxic agent commonly used in systemic chemotherapy, which is being applied as a therapy alternative for warts that have failed to respond to other treatments (Singal & Grover, 2020). Bleomycin solution can be injected into warts either by intralesional injection or by a multi-puncture technique where the surface of the wart is ‘pricked’ multiple times with a needle (Di Chiacchio et al., 2019). The injection of bleomycin into the skin is rather painful, and local anesthesia is often advised (Kaul et al., 2021). The

resulting necrosis can also often cause scarring, changes in skin pigmentation and nail damage (Sterling et al., 2001). This rather obvious response to treatment makes it difficult to conduct double-blinded trials, and many studies have been open studies or have used saline injections in the placebo arm. Several randomized-controlled trials (n=14), investigating the efficacy of bleomycin, reported varying cure rates ranging from 16-97% (Friedman, 2021; Kwok et al., 2012).

1.6.1.4.2.3 Immunomodulators

Immunomodulators such as cimetidine, an H2 receptor antagonist that is commonly used in the treatment of gastroesophageal reflux, have also been suggested as a potential treatment for warts (Yilmaz et al., 1996). Although cimetidine has largely undefined immunomodulatory effects, it is postulated to cause an increase in interleukin-2 and interferon-α expression in T lymphocytes, enhancing cell-mediated immunity (Mitsuishi et al., 2003). The efficacy of cimetidine has been demonstrated in open-label studies where it showed to be more effective at clearing warts in higher doses than lower ones with a reported cure rate of 87% (Gooptu et al., 2000). However, several randomized controlled trials were not able to reproduce these results and statistically significant differences in cure rates between cimetidine and placebo were not observed (Lee et al., 2001; Rogers et al., 1999; Scheinfeld, 2003).

An example of a topical immunomodulator is imiquimod (5% cream) which has been widely used in the treatment of genital and perianal warts (Sterling et al., 2014). Imiquimod is capable of stimulating a proinflammatory response through increased release of interferon-α, tumor necrosis factor-α, and interleukin-12 in T lymphocytes, as well as activating natural killer cells (Ahn & Huang, 2014). The efficacy of imiquimod has not yet been investigated in randomized controlled trials but certain non-controlled studies (n=5) do report a combined cure rate of 44%, ranging from 27 to 89% (Ahn & Huang, 2014). Imiquimod has a quite tolerable adverse event profile with mild itching and erythema being most commonly reported (Kim et al., 2006).

Another example of an often-prescribed immunomodulator is zinc. Zinc is capable of affecting the synthesis of cytokines which regulate both the innate and the adaptive immune response (Song et al., 2022). Oral (zinc sulfate), topical (zinc sulfate or oxide), and even intralesional (2% zinc sulfate) wart treatments with zinc have been described and although not all studies had an adequate study design varying clearance rates were reported ranging from 28- 98% (Song et al., 2022). Noted adverse effects due to oral application of zinc were nausea, dyspepsia, and other gastrointestinal symptoms. Adverse effects due to topical use were erythema, scaling, blacking, and swelling, while intralesional injections most commonly caused pain at the site of injection.

1.6.1.4.2.4 Chemical destructive therapy

Cantharidin is a natural toxin derived from blister beetles that is keratolytic and causes the breakdown of intercellular connections resulting in loss of coherence between keratinocytes and intra-epidermal skin blistering (Vakharia et al., 2018). It is typically used as a topical wart treatment in a concentration of 0.7%. A limited number of randomized controlled trials (n=4) evaluated the use of cantharidin and reported wart clearance rates of 64-91% (Vakharia et al., 2018). Other studies investigated the use of cantharidin in combination with podophyllotoxin and salicylic acid and reported clearance rates of 63-100% (Nguyen et al., 2019; Vakharia et al., 2018). Common side effects due to cantharidin treatment are blistering and hyper-/hypopigmentation.

1.6.1.4.3 **Other Treatments**

Certain folk remedies are still practiced as wart treatments mostly based on different herbal therapies (Sterling et al., 2001). Plants used include mayapple (*Podophyllum peltatum*), greater celandine (*Chelidonium majus*), and thuja (*Thuja occidentalis*; Sterling et al., 2014). Garlic (*Allium sativum*) extract was also shown to be effective against warts in one particular study and although the study was controlled, randomization during treatment allocation was not performed (Dehghani et al., 2005).

Occlusion, for example with duct tape, as treatment of cutaneous warts has also been applied in the past, with one study reporting a 47% cure rate within 2 months (Duthie & McCallum, 1951). Other topical treatments also often use some form of occlusion, but the exact mode of efficacy of this part of treatment is yet to be clarified (Kwok et al., 2012).

Injectable immunotherapy is another promising alternative wart treatment. An antigen is injected either intralesionally or systemically to evoke a general immune response followed by wart clearance. Different kinds of antigens can be used, but the most investigated ones include the Candida antigen, mycobacterial antigens, mumps-measles-rubella vaccines, and HPV vaccines (Friedman, 2021). Primary lesion clearance rates of these therapies are reported to reach 27-94% depending on the study design and antigen used, with general clearance of distant warts also commonly described (Friedman, 2021). The most common side effects are pain at the site of injection but depending on the antigen used other more systemic adverse events have also been documented.

1.6.1.4.4 Treatment Conclusion

A systematic review conducted by the Cochrane Skin Group assessed the effects of different treatments for cutaneous warts and highlighted the uncertainty regarding the lack of an optimal treatment (Kwok et al., 2012). They evaluated 21 trials with placebo groups and found an average clearance rate of 27% (0-73%) in the placebo groups after an average period of 15 weeks (4-24 weeks). These data have led some practitioners to recommend that warts should not be treated at all (Sterling et al., 2001). In addition, the wart recurrence rate amongst these treatments was also quite high as well ranging from 12.5–70% (Kwok et al., 2012).

Furthermore, the British Association of Dermatologists stated in their guidelines for the management of cutaneous warts in 2014, that an expectant approach to wart management is entirely acceptable if the affected individual is immunocompetent (Sterling et al., 2014). Furthermore, a recent study by Kuwabara et al. found that in children 66% of warts resolve within 2 years, while 80% resolve within 4 years, whether or not they have been given any

treatment (Kuwabara et al., 2015). They emphasize that the decision to treat or not to treat warts in children should take into account the natural course of warts, host immunity, and the minimal potential of morbidity, as well as the unproven effectiveness, adverse effects, and costs associated with wart treatment. However, there are currently no reliable means of predicting which warts will clear spontaneously and which will remain for years.

So although in theory, a policy of not treating warts is logical, in practice many people do consult healthcare professionals and are treated due to the social stigma and morbidity associated with visible warts (lesions are often painful, persistent, or interfering with natural function; Kwok et al., 2012; Sterling et al., 2014). Moreover, treatment is necessary to prevent the spread of infection as suggested in a study by Massing et al., where the overall wart incidence in a subset population of 1000 children increased from 18% to 25% in two years when treatment was withheld (Massing & Epstein, 1963).
A study by Bruggink et al. demonstrated that the HPV type influences the natural course and response to treatment for plantar warts (Bruggink et al., 2013). These results implied that HPV genotyping could be used to optimize wart treatment schemes. In another study, several patient- as well as wart-specific characteristics were analyzed and it was concluded that from all characteristics evaluated HPV genotype most strongly predicted treatment response (Hogendoorn et al., 2018). The authors concluded that for the development of new wart therapies, it will be essential to take HPV DNA testing into consideration in order to optimize wart treatment management.

1.7 Aims and Outline of the Thesis

1.7.1 Research Rationale

Certain mucosal HPV types have already been associated with the development of cervical cancer; these types are referred to as HR-HPVs. The role of HPV in the development of warts is well recognized, but treatment of these conditions remains challenging. Preliminary studies have demonstrated that HPV typing of skin warts can be used for the selection of optimal treatment (Bruggink et al., 2013; Hogendoorn et al., 2018). Can these findings be confirmed (i.e. the existence of high-risk cutaneous HPV types) and HPV typing as well as other wart-specific characteristics be used to develop a clinical management tool, where the prognosis is possible on which warts are resistant to standard treatment and thus require more aggressive follow-up for a successful outcome?

1.7.2 Specific Objectives

- To evaluate noninvasive sample collection methods for HPV typing of skin disorders.
- To develop a holistic cutaneous HPV assay.
- To determine the genotype-specific distribution of cutaneous and mucosal HPV types in skin warts.
- To evaluate the clinical significance of HPV infection in cutaneous warts i.e. the prognostic value of HPV typing.
- To evaluate whether the efficacy of treatment depends on the detected HPV type i.e. triage of patients based on results from the Omnivirol-Salicylic acid randomized controlled trial (OVW-SA001).

Chapter 2 Optimizing the Pre-Analytical Phase for Accurate HPV Detection in Skin Disorders: Insights from a Cutaneous Warts Case Study

This chapter provides a step-by-step description of the development and optimization of a laboratory workflow for HPV detection in cutaneous disorders, consisting of a non-invasive sampling method and pre-analytical sample processing followed by automated DNA extraction. A detailed summary of the most common hurdles faced during HPV research regarding cutaneous disorders is provided as well.

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Optimizing the pre-analytical phase for accurate HPV detection in skin disorders: insights from a cutaneous warts case study

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Abstract

Background: In previous years, several cutaneous disorders have been associated with human papillomavirus (HPV); however, the exact role of HPV remains largely unknown. The lack of optimization and standardization of the pre-analytical phase forms a major obstacle. The aim of this study was to develop an accurate/patient-friendly sampling method for skin disorders, with cutaneous warts as a case study.

Methods: Various sample processing techniques, pre-treatment protocols and DNA extraction methods were evaluated. Several sampling methods were examined, that is, skin scrapings, swabs and a tape-based method. Quantification of DNA yield was achieved by beta-globin real-time polymerase chain reaction (qPCR), and a wart-associated HPV genotyping qPCR was used to determine the HPV prevalence.

Results: All samples tested positive for beta-globin. Skin scrapings had significantly higher yield than both swab and tape-based methods ($p < 0.01$), the latter two did not significantly differ from each other ($p > 0.05$). No significant difference in DNA yield was found between cotton and flocked swabs ($p > 0.05$). All swabs were HPV positive, and although there were some discrepancies in HPV prevalence between both swabs, an overall good strength of agreement was found [$\kappa = 0.77$, 95% CI (0.71–0.83)].

Conclusion: Although skin scrapings produced the highest DNA yield, patient discomfort was an important limitation of this method. Considering that in combination with our optimized DNA extraction procedure, all samples gave valid results with the less invasive swab methods preferred. Standardization of the pre-analytical phase is the first step in establishing a link between HPV and specific skin disorders and may have significant downstream diagnostic as well as therapeutic implications.

Keywords: cutaneous disorders, cutaneous warts, DNA extraction, HPV detection, sample processing, sampling methods

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Background

The family of human papillomaviruses (HPVs) comprises over 200 different genotypes, classified into different genera according to their DNA sequence. Members of the genus alpha-HPV have a specific tropism for mucosal epithelium, while beta and gamma HPVs most frequently cause cutaneous lesions and are commonly referred to as cutaneous HPV types.[1] HPV is capable of causing a wide range of diseases from benign lesions to invasive tumours and although it is most commonly known for its involvement in the development of cervical cancer, there are also other mucosal as well as cutaneous disorders where HPV plays a role (see Table 1). Regarding cutaneous disorders, it still remains extremely challenging to determine the exact role of HPV in their development. Is HPV responsible for the onset of the disease? Is it a co-factor that acts with other carcinogens to amplify the risk of disease? Or is it merely an innocent bystander without any role in disease pathogenesis?[2]

Table 1. A list of HPV-associated mucosal and cutaneous disorders. [3-5]

Mucosal	**Cutaneous**
Anal cancer	Actinic keratosis[a]
Cervical cancer	Bowen's disease[a]
Condyloma acuminata	Bowenoid papulosis
Conjunctival papilloma	Darier disease[a]
Conjunctiva, eyelid and lacrimal sac cancer	Epidermodysplasia verruciformis
Heck's disease	Keratoacanthoma[a]
Laryngeal cancer[a]	Lichen sclerosus [a]
Oesophageal cancer[a]	Penile cancer[a]
Oral cavity (tongue, mouth, gum, palate), oropharynx and tonsil cancer	Psoriasis[a]
Respiratory papillomatosis	Non-melanoma skin cancer (basal and squamous cell carcinoma)[a]
Vaginal cancer	Vulvar cancer
	Warts

[a] The exact role of HPV in these disorders is currently unknown (causal, co-factor, or coincidental infection).
HPV, human papillomavirus.

To demonstrate that a pathogen causes a disorder, we do not only need a plausible biological mechanism for pathogenesis but also convincing associative epidemiological evidence.[6]

As for the epidemiological evidence of HPV involvement in skin disorders, to date, numerous studies are published with conflicting results. For example, more than 100 studies have investigated the relationship between HPV and cutaneous squamous cell carcinoma (SCC). While some studies have failed to find HPV in SCC, most studies do report HPV infection in some SCCs, although with variable percentages (3.2–85.7%).[2] These contradictory results can be attributed to

several issues (see Table 2), one of the most prominent being a lack of standardization of the pre-analytical phase, that is, different studies usedifferent sampling methods, sample processing protocols, pre-treatments and DNA extraction systems. The use of inconsistent methods results in differences in not only sensitivity but also specificity.[7] This high degree of between-study heterogeneity presents challenges to grouped study analysis and makes it problematic to establish distinct claims about the causal role of HPV in these disorders.[2]

Table 2. Issues causing conflicting results in studies regarding HPV prevalence in skin disorders.

Issue	Example
Pre-analytical phase	Varying sampling methods (e.g. biopsies, swabs, skin shavings, plucked hairs, blood serology),[3] control samples (e.g. peri-lesional vs. site-matched healthy controls), sample handling and extraction protocols (e.g. no prior DNA extraction vs. various DNA extraction systems)[2] all add to study heterogeneity making it difficult to perform grouped study analysis.
HPV detection method	Varying detection methods (e.g. PCR, southern blot, dot blot, reverse hybridisation, in situ hybridization, restriction enzyme digestion, sequencing, Luminex technology, ELISA) exhibit varying assay targets (HPV DNA or antibodies), specificities and sensitivities.[7] While serologic testing detects both current infection and prior viral exposure, DNA detection methods are limited to a specific body site and indicate only current infection. Unambiguous analysis of serology is furthermore complicated considering that seroconversion in cutaneous HPV infections may only appear months after initial contact, not all hosts develop an antibody response to HPV and some antibodies exhibit cross-reactivity between different HPV types.[3]
Holistic HPV test	With the targeted-approach only detection of specific HPV types is possible, unknown or non-targeted HPVs are not detected. This biased approach has led to considerable discrepancy in the prevalence and type of HPV reported in different tissues by different investigators. For a meaningful epidemiological assessment of HPV prevalence in specific lesions, it is crucial to employ a method which is not only sensitive but is also capable of detecting and reliably typing a wide range of HPV genotypes.[8,9]
Sample size	Most studies involve rare cutaneous disorders resulting in small number of subjects available and limited study sample size, which in turn leads to variable HPV prevalence rates.[7] Furthermore small sample sizes can also be attributed to invasive sampling techniques inducing unwillingness of subjects to participate.[10]
Commensal micro-organism	Numerous studies reveal asymptomatic carriage of beta and gamma-HPVs on healthy skin.[3] Cutaneous HPVs have also been found on the skin of new-borns and young children implying that certain HPV types are simply commensal viruses always present on healthy skin.[11]

ELISA, enzyme-linked immunosorbent assay; HPV, human papillomavirus; PCR, polymerase chain reaction.

Following the above-described reasoning, the first step in determining the exact role of HPV in skin disorders is to standardize and optimize the pre-analytical phase. To achieve this objective, there is need for a case study employing a straightforward,easily accessible cutaneous disorder where the causal role of HPV

has already been ascertained,that is, cutaneous warts.[12]

In this study, we performed a head-to-head comparison of different pre-analytic steps, including sampling methods, sample processing techniques, pre-treatment protocols and DNA extraction methods to develop an accurate, patient-friendly sampling method for skin disorders.

Materials and methods

The optimization of the pre-analytical phase included two separate stages: (1) optimization ofthe DNA extraction procedure and (2) development of a patient-friendly sampling method. Theoptimization was performed in preparation for a large clinical trial regarding cutaneous warts, that is, OVW-SA001 trial.[13] Samples were provided by patients visiting the Algemeen Medisch Laboratorium (AML) medical laboratory in December 2017 (Ethical approval number B300201734040).

DNA extraction

Formalin-fixed, paraffin-embedded (FFPE) wart biopsies, skin scrapings, tape and swabs from normal and callous skin were used to optimize the DNA extraction procedure. Multiple target geneswere analysed to determine the optimal DNA quantification method for skin samples. Several manual as well as automatic DNA extraction methods were examined, together with different preservatives, pre-treatment and lysis buffers, and protocol amendments (Table 3). For these latter experiments, multiple skin scrapings from four different patients were used. Skin scrapings were first weighted and divided into equal aliquots before further processing.

Sampling method

Several sampling methods were examined employing cutaneous warts from different individuals, that is, skin scrapings (n = 5), swabs (n = 6) and a tape-based sampling method (n = 6). Skin scrapings were collected by scraping the surface of the affected skin with a sterile scalpel. Abbott Multi-Collect (MC) cotton swabs (Abbott Molecular Inc., Des Plaines, IL, USA) were firmly pressed on the upper layer of the diseased skin while making circular movements during a fixed time period (10s). After sampling, swabs were stored in Abbott MC Specimen medium (Abbott Molecular Inc., Des Plaines, IL, USA). A medical adhesive tape, that is, Opsite Flexifix (Smith & Nephew, London, UK), was applied multiple times (10×) on the surface of the lesion. This ‘tape-lifting’ method is proven to increase DNA yield and is a well-established sampling method in forensic DNA analysis.[17] All samples were stored at 4°C.

Table 3. Summary of various parameters tested during optimization of the DNA extraction procedure.

DNA extraction system	No extraction[14–16]
	QIAamp DNA mini kit: QIAamp DNA Mini and Blood Mini Handbook Third Edition: DNA Purification from Buccal Swabs (Spin Protocol), p. 36-38 (QIAGEN, Hilden, Germany)[17]
	NucliSens easyMAG platform: Generic 2.0.1 protocol (bioMérieux, Boxtel, The Netherlands)
	Medium-Throughput Automation (MTA) system: Genfind DNA Extraction Kit (Hologic, Inc, Marlborough, MA, USA)
	Abbott m2000sp: Abbott mSample Preparation System DNA (Abbott, Wiesbaden, Germany)
Preservative	No preservative, dry sample
	0.9% NaCl[16,18,19]
	Multi-Collect Specimen Collection Kit (Abbott Molecular Inc., Des Plaines, Illinois, USA)
	ThinPrep medium (Hologic, Bedford, MA, USA)
	In-house preservative (50% methanol, 5% diethylene glycol)
Pre-treatment buffer	10mM TrisHCl, 7mM EDTA, 0,5% Tween 20, 1 mg/ml proteinase K, pH 7.5
	30mM TrisHCl, 10mM EDTA, 1% SDS, 1.25 mg/ml proteinase K, pH 8[20]
	20-30mM TrisHCl, 10mM EDTA, 0.5% SDS, 1 mg/ml proteinase K, pH 8
	30mM TrisHCl, 30-36mM EDTA, 5% Tween 20, 0.5% Triton-X-100, 1 mg/ml proteinase K, pH 8
	30mM TrisHCl, 10-100mM EDTA, 0.5% SDS, 1 mg/ml proteinase K, pH 8[21]
	30mM TrisHCl, 30-100mM EDTA, 5% Tween 20, 0.5% Triton-X-100, 800mM GuHCl, 1 mg/ml proteinase K, pH 8[20]
	10mM TrisHCl, 10mM EDTA, 0.9% NaCl, 2% SDS, 6mM Dithiothreitol, 1 mg/ml proteinase K, pH 8[22]
	0.38mM EDTA, 1 mg/ml proteinase K, pH 8[23]
Lysis buffer	NucliSens easyMAG Lysis Buffer (bioMérieux, Boxtel, The Netherlands)
	Aptima Specimen Transfer Kit: for transfer of liquid Pap specimens (Hologic, Bedford, MA, USA)
	Genfind mLysis buffer DNA: Genfind DNA Extraction Kit (Hologic, Inc, Marlborough, MA, USA)
	mLysis DNA: Abbott mSample Preparation System DNA (Abbott, Wiesbaden, Germany)
Protocol amendments	Incubation time: 3-4h, 18h[24]
	Incubation temperature: room temperature, 56°C
	RPM: 0, 500, 1000, 1400
Target gene for DNA quantification	MTCOI
	HMBS
	B-globin

Likewise, the performance of two different swabs, that is, cotton (Abbott MC Specimen Collection) and flocked (FLOQSwab Copan Diagnostics, Murietta, CA, USA), was analysed. FLOQSwabs contain short hydrophilic nylon fibers attached to plastic. Due to their design without an internal absorbent core, they do not disperse and entrap the specimen and should therefore provide better DNA yield.[25] In total, 45 warts were sampled by both types of swabs in an alternating order to account for interpatient variability. All samples were stored at 4°C and subsequently extracted according to the optimized DNA extraction protocol (see further). Quantification of the DNA yield was achieved by beta-globin real-time polymerase chain reaction (qPCR) (cell control)[26] and a newly developed HPV qPCR genotyping assay capable of detecting the most prevalent wart-associated HPVtypes (i.e. HPV1, 2, 3, 4, 7, 10, 27, 41, 57, 60, 63, and 65) was used for HPV detection.[24]

Statistical analysis

Data analysis was conducted using MedCalc version 20.111 (MedCalc Software Ltd, Ostend, Belgium). The paired student's *T*-test was used to compare DNA yields between the different DNA quantification methods as well as for head-to-head comparison of the two swab sampling methods. The Friedman test was used to assess the variation in DNA yield between all the examined preservatives, while the Wilcoxon signed-rank test was employed to compare the two best performing preservatives, that is, the dry and MC stored samples. The Kruskal–Wallis test was used to compare DNA yields between different sampling techniques when comparing more than three methods, that is, skin scraping, swab and tape-based method; while the Mann–Whitney U test was used to compare only two methods, that is, swab and tape-based method. Differences in the HPV-type specific detection between the two swab methods were examined with Pearson's X2 test and kappa analysis. Results were considered statistically significant at $p \leqslant 0.05$.

Results

DNA extraction

The first step to optimize the DNA extraction procedure was to determine the analysis method, that is, DNA quantification method. For this purpose, a total of 21 samples (seven FFPE wart biopsies, seven skin scrapings and swabs from normal skin) were analysed with three different housekeeping genes: two cellular genes, that is, HMBS (hydroxymethylbilane synthase) and beta-globin, and one mitochondrial gene, that is, MTCOI (mitochondrial cytochrome oxidase sub-unit 1). All samples as well as the negative control tested positive for MTCOI. In concordance with previous research (unpublished data), MTCOI seems to be ubiquitous and requires the use of cut-off values for data analysis. This makes unambiguous analysis challenging and MTCOI is therefore not recommended for further cutaneous sample evaluation.

The results of DNA quantification by HMBS and beta-globin are depicted in Figure 1. Although beta-globin consistently exhibits higher DNA yield than HMBS, the DNA yields were only significantly different in biopsy and swab samples (paired student's *T*-test, $p < 0.05$), and not in skin scrapings (paired student's *T*-test, $p > 0.05$). This suggests that beta-globin exhibits superior efficiency in the quantification of samples with lower DNA concentrations. Further analysis will therefore be performed with beta-globin.
The next step was the comparison of several DNA extraction systems. For this purpose, skin-scraping aliquots were used. The results of this experiment are depicted in Table 4. The lowest average DNA yield was obtained via direct polymerase chain reaction without prior DNA extraction with only half of the samples testing positive for b-globin. Although the QIAamp DNA mini kit(QIAGEN, Hilden, Germany) did demonstrate the highest DNA yield, this manual method was considerably labour-intensive and since automated systems are more suitable for high-throughput processing, these methods were preferred. The highest average DNA yield with an automated system was achieved with the Medium-Throughput Automation system (Hologic, Inc, Marlborough, MA, USA).However, only half of the samples tested positive for beta-globin. The only automated system with consistent results and sufficient DNA yield was the NucliSens easyMAG platform (bioMérieux, Boxtel, The Netherlands). Accordingly, further DNA extractions were performed on this system.

The following step was to determine the optimal sample preservative. All samples, that is, the previously described skin scraping aliquots, were stored in their respective preservatives for a period of 5 days at 4°C and extracted using the NucliSens easyMAG platform (bioMérieux, Boxtel, The Netherlands). The results depicted in Table 4 demonstrate that the DNA yield varied significantly according to the preservative used (Friedman test $F_r = 13.76$, $p < 0.01$). The highest average DNA yield was achieved with dry samples and samples stored in MC medium (Abbott Molecular Inc., Des Plaines, IL, USA). Other preservatives exhibited significantly lower yields. The average DNA yield of dry samples and MC samples did not significantly differ (Wilcoxon signed-rank test $p > 0.05$). However, it is still recommended to use a preservative to ensure DNA stabilization until sample processing and prolong sample storage time.

The results of the analysis of several pre-treatment buffers, lysis buffers and protocol amendments are not further discussed in detail. In summary, the final optimized DNA extraction protocol involved sample storage in MC medium(Abbott Molecular Inc., Des Plaines, IL, USA)at 4°C, and overnight digestion in a buffer containing 1 mg/ml proteinase K and 0.38 M EDTA(pH 8) at 56°C and 1400 rpm (Thermo–Shaker TS-100C, Biosan, Riga, Latvia), followed by automated extraction on the NucliSENS® easyMAG® system (Generic 2.0.1 protocol, bioMérieux, Boxtel, The Netherlands) and DNA quantification with beta-globin.

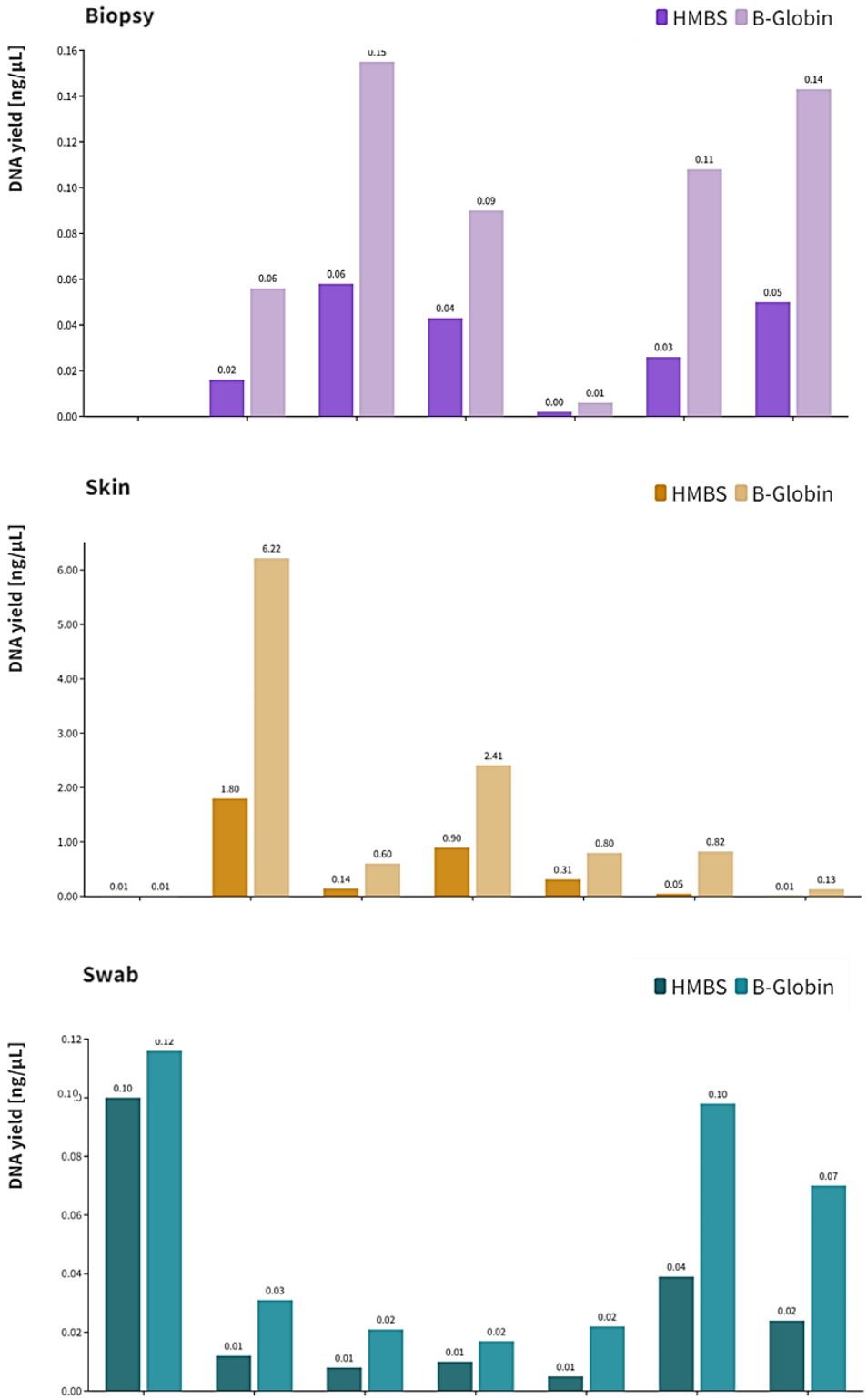

Figure 1. Quantification of the DNA yield of several sample types with two different target genes i.e. B-globin and HMBS. The B-globin DNA quantification was significantly higher in biopsy and swab samples in comparison with HMBS quantification ($p<0.05$). However, the DNA yield in skin scrapings did not significantly differ between the two target genes ($p>0.05$).

HMBS, Hydroxymethylbilane synthase.

Sampling method

After the DNA extraction protocol was optimized, we performed a comparison of the different sampling methods. All samples tested positive for beta-globin and were considered valid. Skin scrapings had significantly higher yield than both swab

Table 4. Summary of the analysis of two DNA extraction parameters (DNA extraction system and sample preservative).

Parameters	**Variables**	**Percentage of B-globin positive samples**	**Average DNA yield (ng/µl)**
DNA extraction system	No extraction[14–16]	50% (2/4)	0.0006
	QIAamp DNA mini kit (QIAGEN, Hilden, Germany)	100% (2/2)	0.0348
	NucliSens easyMAG platform (bioMérieux, Boxtel, The Netherlands)	100% (6/6)	0.0055
	Medium-Throughput Automation system (Hologic, Inc, Marlborough, MA, USA)	50% (3/6)	0.0231
	Abbott m2000sp (Abbott Molecular Inc., Des Plaines, Illinois, USA)	33% (2/6)	0.0030
Preservative	No preservative, dry sample	100% (8/8)	0.0879
	0.9% NaCl[16,18,19]	100% (8/8)	0.0130
	Multi-Collect medium (Multi-Collect Specimen Collection Kit Abbott Molecular Inc., Des Plaines, Illinois, USA)	100% (8/8)	0.0322
	ThinPrep medium (Hologic, Bedford, MA, USA)	63% (5/8)	0.0033
	In-house preservative (50% methanol, 5% diethylene glycol)	75% (6/8)	0.0022

In total 70 skin scrapings from 4 different patients were used for these experiments. The scrapings were weighted and aliquoted in equal proportions prior to testing. B-globin is used as cellular control of a successful DNA extraction and all sample should test positive. The percentage of B-globin positive samples is depicted in the third column together with the total amount of samples tested between brackets. The average DNA yield (ng/µl) per protocol variable is depicted in the fourth column.

and tape-based methods (Kruskal–Wallis test; $p < 0.01$). The latter two did not significantly differ from each other (Mann–Whitney U test; $p > 0.05$; Figure 2(a)). To account for possible interpatient variability, we performed a head-to-head comparison of two types of swabs by sampling each lesion with both swabs (Figure 2(b)). When comparing DNA yield, no significant difference was found between cotton and flocked swabs irrespective of sampling sequence (paired student's T-test; $p > 0.05$). All swabs were HPV positive; however, there were some discrepancies in HPV type-specific detection, but these were not statistically significant and can be attributed to the assay detection limit [Pearson's X2 test $p > 0.05$; $\kappa = 0.77$ (95% CI, 0.71–0.83); see Table 5].

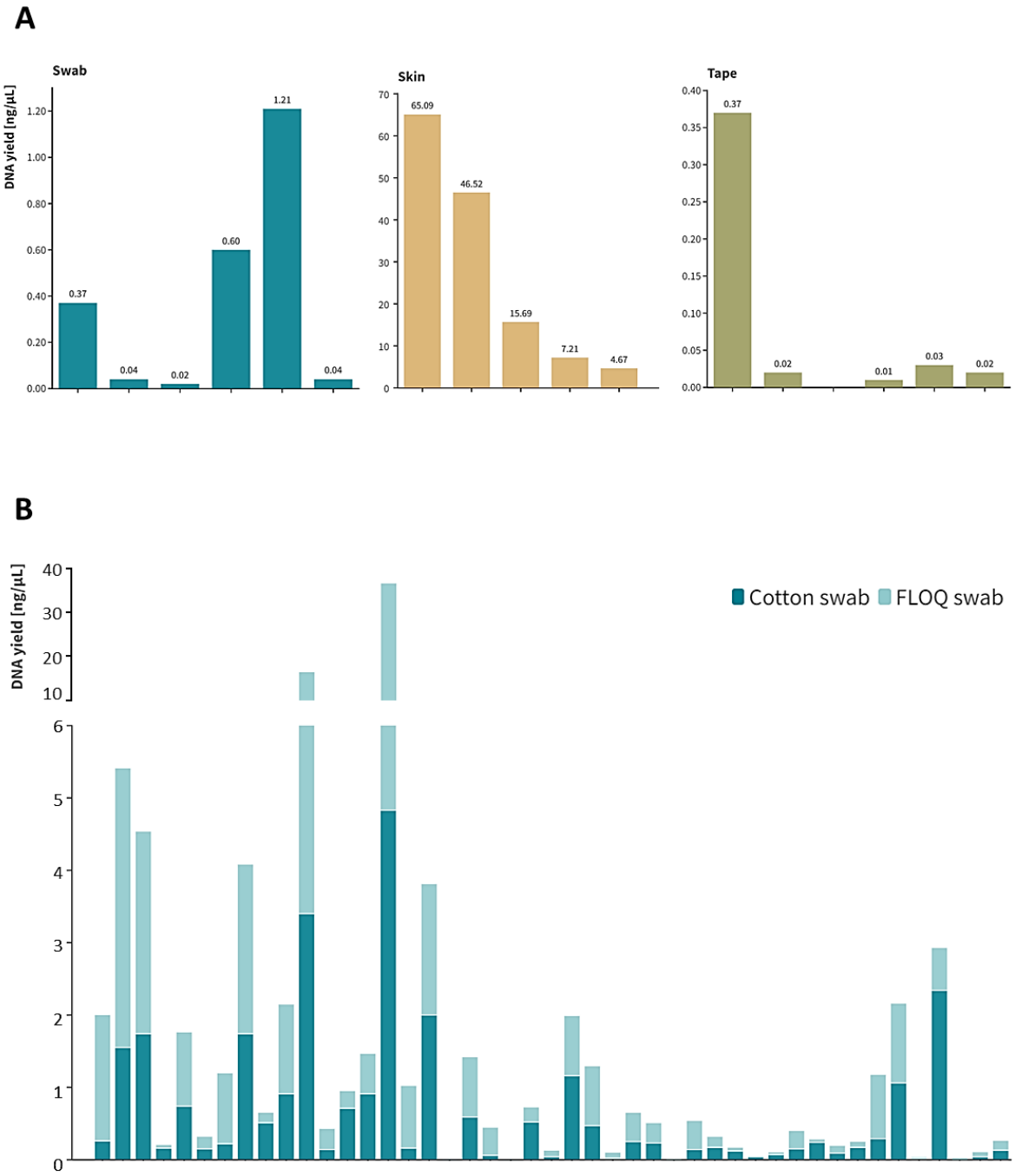

Figure 2. A. Comparison of the DNA yield (ng/µl) of all the examined sampling methods i.e. swab (n=6), skin scrapings (n=5) and tape (n=6). Skin scrapings had significantly higher DNA yield than both swab and tape-based methods ($p<0.01$). The DNA yield did not significantly differ between the latter two methods ($p>0.05$). **B.** Head-to-head comparison of two different swab types i.e. cotton and flocked (n=45). No significant difference in DNA yield was found between the two types of swabs irrespective of sampling order ($p>0.05$).

Discussion

In this study, we describe a comprehensive optimization of the pre-analytical phase of cutaneous samples determined for HPV evaluation. A robust, standardized

protocol for sample processing and DNA extraction of several types of skin samples (i.e. skin scrapings, swabs, tape and FFPE biopsies) was devised. Various head-to-head comparisons between different non-invasive sampling techniques were performed to determine the optimal sampling method.

Table 5: Overview of qualitative comparison between HPV prevalence in cotton and flocked swabs.

Cotton Swab	**Flocked Swab**		
	Positive	*Negative*	***Total***
Positive	140	21	**161**
Negative	32	347	**379**
Total	**172**	**368**	**540**
k = 0.77			

In total 45 warts were sampled with both swabs. These swabs were examined for presence of 12 distinct cutaneous HPV types (i.e. a total of 540 single-plex reactions). The results are shown in the form of frequencies (number of samples that simultaneously satisfy the specific criteria indicated in the column and row). Although there were some discrepancies in HPV prevalence between both swabs, an overall good strength of agreement was found (kappa, k).

HPV, human papillomavirus.

Currently, tissue biopsy is considered the gold standard in sampling skin disorders for HPV detection. Biopsies contain DNA derived from not only superficial but also deeper epithelial layers and can yield information about the infectious HPV reservoir in basal stem cells.[6] In addition, they also provide histological background to a skin disorder, that is, identification of affected skin layers and histological localization of viral particles by immunohistochemistry with HPV-specific antibodies. However, a skin biopsy is a rather invasive procedure, which requires skilled personnel and is accompanied by a moderate cost. These issues often discourage subject participation and make biopsies impractical for large studies.[6] A study by de Koning *et al.*[16] revealed a very high concordance (96%) between the HPV type detected in the superficial wart swabs and wart biopsies. Considering that HPV types identified in wart swabs are representative of the HPV types present in the deeper epithelial layers, less invasive skin sampling methods can be utilized.[16]

While there have been some comparisons between certain skin sampling methods,[16] there have not been, to our knowledge, any direct comparisons between non-invasive sampling techniques. As regards the current study, as far as sampling is concerned, although a somewhat better DNA yield was found in skin scrapings, patient discomfort was an important limitation of this method. Seeing that in combination with the optimized DNA extraction procedure all samples gave valid result, with the less invasive methods preferred. Tape sampling is quick and straightforward; however, the subsequent DNA extraction is more challenging due

to the adhesiveness and rigidityof the tape.[17] According to current literature,tape-based methods also exhibit low reproducibility caused by variable operator sampling techniques.[6] The last non-invasive method analysed was skin swabs. Swabs comprise a very straightforward sampling method, allowing for quick, painless sampling that can be repeated multiple times with little risk and patient inconvenience.[6] An additional advantage of swabs is the option for automated pre-analytical processing, which is not feasible with the alternative methods. Accordingly, the performance of both cotton and flocked swabs was also demonstrated to be equal.

As described in current literature, the main disadvantage of all non-invasive methods is that they only access the superficial epithelial layers, making it difficult to assess if a positive sample represents contamination, carriage, transient or persistent infection.[6] As previously mentioned, a study by deKoning *et al.*[16] already demonstrated that in cutaneous warts superficial swabs showed an equivalent performance to tissue biopsies regarding HPV detection. Correspondingly we were able to detectHPV DNA in all wart swabs in our head-to-headcomparison study of different swab types.

In conclusion, a robust pre-analytical phase is thefirst step necessary to establish an unambiguous link between HPV and certain skin disorders. Furthermore, this optimized sampling technique can also be employed for HPV detection in other mucosal HPV-associated disorders such as head-and-neck, penile as well as anal tumours. Clarification of the viral mechanism in HPV-related disorders may lead to more targeted treatment modalities, reduction in disease burden/healthcare costs, and overall better patient outcomes.[2,6] Future studies should not only examine the effects of specific HPV genotypes and viral loads in patients but also explore the longitudinal, subsequent development of the underlying skin disorders.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from Ethics Committee of Antwerp University Hospital(B300201734040). All participants gave written informed consent prior to entry into the study.

Consent for publication

Not applicable.

Author contributions

Nina Redzic: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Software; Validation; Visualization; Writing – original draft; Writing –review & editing.

A. Rita Pereira: Visualization; Writing – review & editing.

Davy Vanden Broeck: Conceptualization; Supervision; Writing – review & editing.

Johannes P. Bogers: Conceptualization; Supervision; Writing – review & editing.

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 3 Development and Validation of a Wart-Associated HPV Genotyping Assay for Detection of HPV in Cutaneous Warts

This chapter provides a methodical description of the development, optimization, and validation of a real-time PCR assay for the detection of the most prevalent wart-associated cutaneous HPV genotypes. Topics discussed include a literature review of the most prominent HPV types in cutaneous warts, in silico assay development, several phases of assay optimization, analytical validation, and final implementation in a clinical setting in a subset of cutaneous wart biopsies.

The work presented in this chapter was published as a research article in the Journal of Medical Virology (Volume 93).

Reference: Redzic N, Benoy I, Vanden Broeck D, Bogers JP. Development and validation of a wart-associated human papillomavirus genotyping assay for detection of HPV in cutaneous warts. J Med Virol. 2021 Jun;93(6):3841-3848.

Development and validation of a wart-associated humanpapilloma virus genotyping assay for detection of HPV incutaneous warts

Nina Redzic, Ina Benoy, Davy Vanden Broeck, Johannes P. Bogers

Abstract

Cutaneous warts are infectious disorders caused by human papillomavirus (HPV). A recent study revealed that the HPV genotype influences the natural course and response to treatment for plantar warts, suggesting that HPV genotyping could potentially be used to optimize wart treatment schemes. For this purpose, a wart- associated HPV genotyping assay was developed. The assay was subjected to an intensive validation process including, i.a., empiric determination of the annealing temperature, primer-probe optimization, evaluation of the analytical specificity and sensitivity, viral load quantification, and qualitative as well as quantitative analysis of intra-run repeatability and inter-run reproducibility. The newly developed assaywas employed in a small-scale HPV genotyping study of wart biopsies (n = 50). The assay exhibited an analytical type-specific sensitivity and specificity of 100% (95% confidence interval [CI]: 83.9%–100%). The limit of quantification of the tested sequences corresponded to less than 17 viral copies/µl, while the limit of detection was less than 5 copies/µl. Very good to excellent agreements were gained between intra- and inter-run measurements (κ = 0.85–1.00) and coefficients of variation of the quantitative agreements were less then 3%. 22.5% (95% CI: 11%–39%) of the analyzed biopsies were negative for the tested HPV types, while 35% (95% CI: 21%–52%) contained multiple infections. The wart-associated HPV quantitative polymerase chain reaction assay was proven to be highly sensitive and specific. Multiple HPV infections were detected in 35% of lesions, contradicting the current literature claiming that in immunocompetent patients only 4%–16% of warts exhibit multiple HPV infections. This assay is qualified to be implemented in development of future genotype specific wart treatment strategies.

KEYWORDS

PCR, HPV, cutaneous warts, HPV genotyping

Introduction

The family of the human papillomaviruses (HPVs) includes over 200 different genotypes, classified into different genera according to their DNA sequence. Members of the genus alpha-HPV have a specific tropism for mucosal epithelium, while beta-, gamma-, mu-, and nu-HPVs most frequently cause cutaneous lesions and are commonly referred to as cutaneous HPV types.[1] HPV is capable of causing a wide range of diseases from benign lesions to invasive tumors, and although it is most commonly known for its involvement in the development of cervical cancer, there are also other mucosal as well ascutaneous disorders where HPV plays a role, one of the best known being cutaneous warts.

Cutaneous warts are caused by HPV infection of keratinocytes. They are a common, infectious and sometimes very painful problem, with a varying worldwide prevalence of 0.84%–12.9%.[2] The prevalence rate in children and young adults is even higher and reported to reach 30%.[3] Although most warts have a benign nature, they can have a substantial impact on quality of life. This becomes apparent when lesions are painful, persistent or interfering with natural function. An armamentarium of wart treatments is currently at our disposal, starting from folk remedies to over-the-counter medications and more aggressive clinic-based treatments. Unfortunately, none of these treatments seem to produce consistent results and reported efficacies often vary widely depending on several factors (e.g., age, compliance, and immunocompetence).

Interestingly, a study by Bruggink et al. revealed that the HPV genotype influences the natural course and response to treatment in plantar warts.[4] This finding was also confirmed in two additional studies that concluded that from all patient- and wart-specific characteristics analyzed, HPV genotype most strongly predicted treatment response in warts.[5,6] These studies suggested that HPV genotyping could potentially be used to optimize wart treatment schemes by determining which warts will clear spontaneously as opposed to which do need treatment. For this purpose, we developed a wart-associated HPV genotyping polymerase chain reaction (PCR) assay capable of detecting the most prevalent HPV types in cutaneous warts. In this article we describe in detail not only the design of the assay but also the rigorous validation process and employ it in a small-scale pilot study considering HPV-genotyping in wart biopsies.

Methods

Literature review

A profound literature review was performed considering HPV genotype-specific prevalence in cutaneous warts. The HPV detection methods examined included, i.a., general primer-mediated PCR followed by HPV typing by direct sequencing or by restriction enzyme digestion, southern blot, dot blot, reverse hybridization, in situ hybridization, xMAP Luminex technology, and enzyme-linked immunosorbent assay. This review demonstrated that the most prevalent HPV types in cutaneous warts are HPV 1, 2, 3, 4, 10, 27, and 57. Other frequently found types are HPV 7, 41, 60, 63, and 65. [2,6-12]

Assay design

The genomic stability of these types was examined by alignment of all known NCBI sequences of each type and localization of conserved regions and mutational hotspots using a bioinformatic software, that is, CLC Genomics Workbench 9.0 (CLC Bio; Qiagen). Based on this analysis, a wart-associated HPV genotyping quantitative PCR (qPCR) assay was developed, able to detect all the above-mentioned cutaneous HPV types. The assay involves a Taq-Man real-time PCR containing type-specific primers and consensus probes capable of detecting multiple HPV types. The PCR reactions are performed in a 15 µl volume containing LightCycler 480 Probes Master (Roche Applied Science), primers and probes and 3 µl template DNA. The analysis is executed in 5 parallel multiplex or 12 singleplex reactions on the LightCycler 480 system (Roche Applied Science). The assay is not only able to detect multiple HPV types in one reaction due to the unique design of the probes (i.e., consensus probes that can detect two or more types) but can

also be used as a type-specific PCR that only detects one HPV type per reaction due to type-specific primers (see Table 1). The most cost-efficient way of utilizing this unique design is by firstly analyzing each sample with five multiplex reactions. Multiplexing is performed by addition of multiple type-specific primer pairs and one consensus probe. If the sample is found to be positive for a specific multiplex reaction, further HPV typing is performed by two to three singleplex reactions containing one specific primer pair and the consensus probe for which the sample was previously found positive. By this approach the consumption of the most expensive PCR reagent (i.e., probe) is limited, since each sample is only analyzed with a minimum of five multiplex reactions, instead of 12 singleplex reactions. The PCR-program involves (1) pre-incubation: 10 min at 95°C; (2) temperature adjustment: 2 min at 60°C; (3) 50 two-step cycles of amplification: 10 s at 95°C and 30 s at 60°C; and (4) cooling: 10 s at 40°C. The assay was subjected to an intensive validation process including, i.a., empiric determination of the annealing temperature, optimization of primer-probe concentrations, evaluation of the analytical sensitivity and specificity, viral load quantification, and qualitative as well as quantitative analysis of intra-run repeatability and inter-run reproducibility.

Controls and clinical materials

Several types of synthetic controls were used during validation, that is, gBlock gene fragments, containing only target sequences of relevant HPV types (Integrated DNA Technologies [IDT]), and HPV constructs, containing whole genome reference sequences, provided by the World Health Organization and the International HPV Reference Center (Karolinska Institute). Following validation, the wart-associated HPV PCR assay was implemented in a small-scale study considering characterization of genotype-specific HPV prevalence in cutaneous wart biopsies. A total of 50 formalin- fixed, paraffin-embedded (FFPE) biopsies were included in this study. Before and after slicing of the sections (10 × 5 µm) predetermined for DNA extraction, additional sections were made for haematoxylin-eosin (HE) staining to ensure that these were derived from wart epithelium. All samples were extracted according to the previously described optimized DNA extraction protocol.[14] The newly developed wart-associated HPV qPCR assay, together with the in-house HPV Riatol genotyping assay,[15] capable of detecting the most relevant mucosal types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, and 68), was used to determine the HPV prevalence.

Results

Validation of the wart-associated HPV qPCR assay

All primers and probes were designed to be highly specific for their respective targets. The OligoAnalyzer Tool from IDT (Inc. Coralville) was employed to assure that no secondary structures (e.g., hairpins, homo-, and hetero-dimers) would inhibit assay performance in silico. A temperature gradient was performed to empirically evaluate the annealing temperature. Eight different annealing temperatures were analyzed (58.0, 58.4, 59, 59.5, 60, 61, 61.5, and 62°C) on the iCycler system (Bio-Rad). All sequences showed optimal results at the predetermined assay annealing temperature. Subsequently primer and probe concentrations were optimized using several primer and probe concentration ratios (see Table 1). Whole genome HPV constructs were used to evaluate the analytical specificity and sensitivity. The constructs were diluted in different concentrations to create both weak and strong positive

Table 1. Overview of the multiplex PCR assays, the sequences of the primers and probes, amplicon sizes and optimized primer/probe concentrations.

Multiplex	Oligonucleotide	Sequence (5'-3')	Amplicon (bp)	Optimized concentration (nM)
1	HPV1/63 probe	GAA+CCAAGCGTC+CTAGAT		100
	HPV1 FP	GCCAGCACTAAAGGACC	74	200
	HPV1 RP	CCTCCTCGTAACAATAAAGATCTAA		200
	HPV63 FP	GAGCAGCCAAATATAGGTGATT	85	200
	HPV63 RP	CCTCAGCAGGTATATCCTCA		200
2	HPV2/27/57 probe	GGTTGCGTGCAGTTTCGGT		50
	HPV2 FP	CAGATTTCTGCACCCCATC	163	200
	HPV2 RP	CCAAAATCCAAACTCATCGTC		200
	HPV27 FP	TCTGCACCCTATTGTCCTT	141	600
	HPV27 RP	TTGTTGCTGGCACAGC		600
	HPV57 FP	GCACTCTGTAATTGTCCCC	118	200
	HPV57 RP	AGTGTGCTGGCAGCA		200
3	HPV3/10 probe	TGCTGGTCACGATGCACG		100
	HPV3 FP	TCATTGGAGGGGGAGC	108	200
	HPV3 RP	GCATAGTGCAGGGACG		200
	HPV10 FP	AGGGTACTGGAGAGGTAGT	115	200
	HPV10 RP	GCACATTGCATACAGGGATA		200
4	HPV4/60/65 probe	AATAGA+CA+C+CTGTTGC		100
	HPV4 FP	GGAGTCGGTGGTTCCA	78	200
	HPV4 RP	GCAGCATACAATGTAATTCTTACAG		200
	HPV60 FP	GAGGAGTTGTCACCCGA	107	200
	HPV60 RP	GCAATAATAGTAAATCTAACACCTGC		200
	HPV65 FP	AGAGGAGGAGCTTTTTCCT	90	200
	HPV65 RP	CCAAATTCCACAGCAAACAG		200
5	HPV7/41 probe	CA+TAA+CAA+T+G+GCAT		50
	HPV7 FP	GGCTCTCTAGTTACCTCTGATT	162	200
	HPV7 RP	CGATTGTGTAGCAGCACAT		200
	HPV41 FP	TAGCTACTGAGCAGCAGC	114	200
	HPV41 RP	TGGTAGTGTCAACCAATGTTAC		200

Note: Two out of five probes contain Locked Nucleic Acids (LNAs; **+**). LNAs are nucleic acid analogues which are constrained in the ideal conformation for Watson-Crick binding. This conformation makes the pairing with a complementary nucleotide strand more rapid and increases the stability of the resulting duplex. The higher stability causes an increase in the duplex melting temperature of 2-8 °C per LNA monomer, making it possible to use shorter probes but still retain a relatively high melting temperature (Tm) necessary for effective annealing.[13]

Abbreviations: bp, base pairs; FP, forward primer; PCR, polymerase chain reaction; RP, reverse primer.

samples (concentrations from 1×10^5 to 1×10^2 viral copies/µl). Each sequence was evaluated with ten samples positive for the HPV type to be tested and 20 samples positive for other wart-associated HPV types. Each assay exhibited an analytical sensitivity of 100% (95% confidence interval [CI]: 83.9%–100%) for their respective HPV type, and a corresponding analytical specificity of 100% (95% CI:83.9%–100%), that is, cross-reactivity with other HPV genotypes did not occur. Furthermore, absolute quantification was achieved by development of a

standard curve with serial dilutions of gBlock gene fragments with known concentrations (dilutions from 1×10^{-2} to 1×10^{-8} ng/µl were analyzed in triplicate). All standard curves showed acceptable correlation coefficients ($0.990 \leq R2 \leq 1$), slopes ($-3.8 \leq$ slope ≤ -2.9) and PCR efficiencies ($0.830 \leq E \geq 1.210$) in accordance with the guidelines for absolute quantification using qPCR.[16] Further dilutions were made to determine the limit of quantification (LOQ) (the lowest DNA concentration that falls within the linear range) and detection (LOD) (the lowest DNA concentration that can be detected in at least 95% of the cases; see Table 2). To analyze the intra-run repeatability, ten samples positive for the specific type to be tested and ten samples positive for the other types were used. The samples consisted of gBlocks as well as constructs in different dilutions (strong and weak positives) and were tested in duplicate. This run was repeated once more on the same day, and at one day and at seven days after the first run to determine the inter-run reproducibility. Kappa statistics were used to qualitatively analyze the repeatability and reproducibility of each HPV assay. Very good to excellent agreements were gained between intra- and inter-run measurements with kappa coefficients ranging from 0.85 to 1.00. Quantitative agreement was assessed by calculating coefficients of variation (CV) between C_t values as well as between viral copy numbers obtained from different PCR reactions. The obtained CV values were in accordance with predetermined criteria (CV% < 15%; see Table 2).[16]

Table 2. Results of qPCR assay validation.

HPV type	Absolute quantification		Intra-run			Intra-run		
	LOQ (copies/µl)	LOD (copies/µl)	Average CV%		κ	Average CV%		κ
			C_t	log copies		C_t	log copies	
1	4,06	4,06	0,25	0,02	1,00	0,98	0,08	1,00
2	8,13	1,02	0,74	0,10	1,00	1,25	0,13	1,00
3	8,13	2,03	0,69	0,05	1,00	0,69	0,05	0,90
4	8,13	2,03	0,53	0,07	1,00	1,05	0,12	1,00
7	4,06	4,06	0,53	0,06	1,00	2,87	0,30	1,00
10	16,26	2,03	0,32	0,03	1,00	0,54	0,05	1,00
27	8,13	4,06	0,67	0,08	1,00	1,74	0,20	1,00
41	8,13	2,03	0,52	0,06	1,00	2,02	0,22	1,00
57	8,13	2,03	0,78	0,09	1,00	1,67	0,19	0,85
60	2,03	2,03	0,29	0,03	1,00	0,91	0,09	1,00
63	8,13	2,03	0,49	0,04	1,00	0,59	0,06	1,00
65	4,06	2,03	0,46	0,05	1,00	1,29	0,14	1,00

Note: Limits of quantification and detection per type-specific assay are depicted in the table below, together with the average intra- and inter-run coefficients of variation (CV%) and kappa values (κ) per HPV type in their respective linear range.

Abbreviations: C_t, cycle threshold; HPV, human papillomavirus; LOQ, limits of quantification; LOD, limits of detection, qPCR, quantitative polymerase chain reaction.

Implementation of the wart-associated HPV qPCR assay

In regard to the study evaluating the genotype-specific HPV prevalence in cutaneous wart biopsies, the wart diagnosis was confirmed by hematoxylin and eosin staining in 80% (40/50) of the samples. Only confirmed wart samples were included in further analysis. All

samples tested positive for β-globin. Total 22.5% (95% CI: 11%–39%) of the samples were negative for the above mentioned cutaneous as well as mucosal HPV types. 42.5% (95% CI: 27%–59%) were positive for only one HPV type and 35% (95% CI: 21%–52%) contained multiple infections (see Figure 1). Cutaneous HPV types 3, 41, 60, and 63 were not detected. 5% (95% CI: 0.8%–18%] of the samples was infected with mucosal low-risk (HPV11) and high-risk (HPV16) types (see Figure 2).

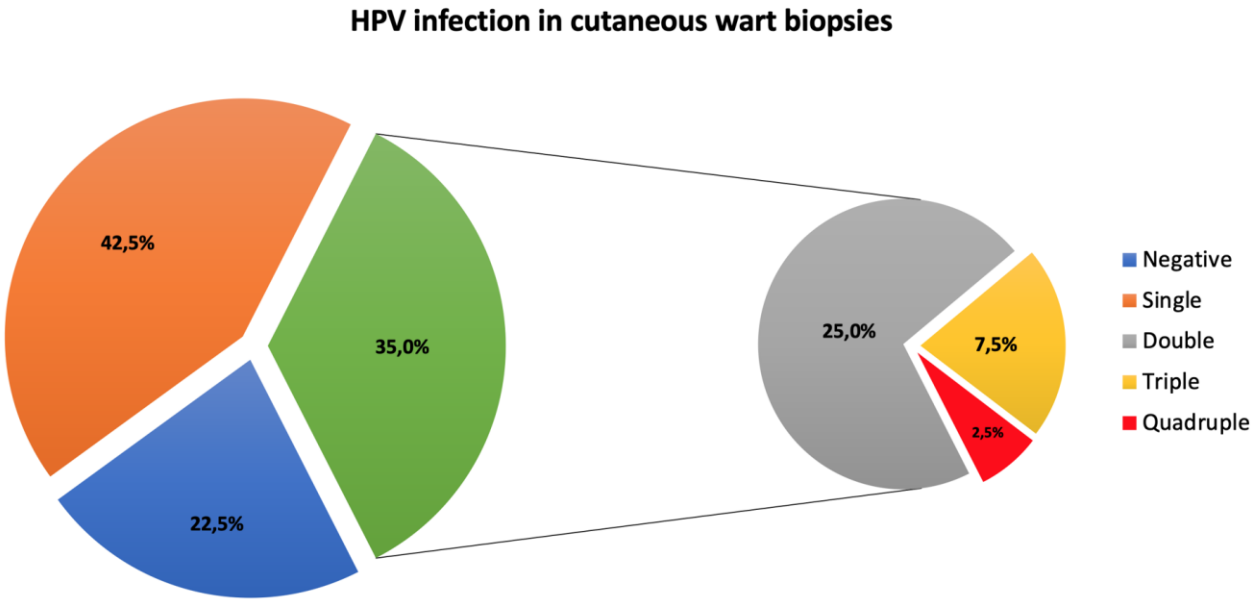

Figure 1. HPV infection in cutaneous wart biopsies. 42.5% [95%CI 27-59%] of the samples exhibited single infections and 35% [95%CI 21-52%] multiple HPV infections. Of the multiple infections, 25% [95%CI 13-42%] were double infections, 7.5% [95%CI 2-21%] triple and 2.5% [95%CI 0.1-15%] quadruple. 22.5% [95%CI 11-39%] of the samples were negative for the tested cutaneous as well as mucosal HPV types.

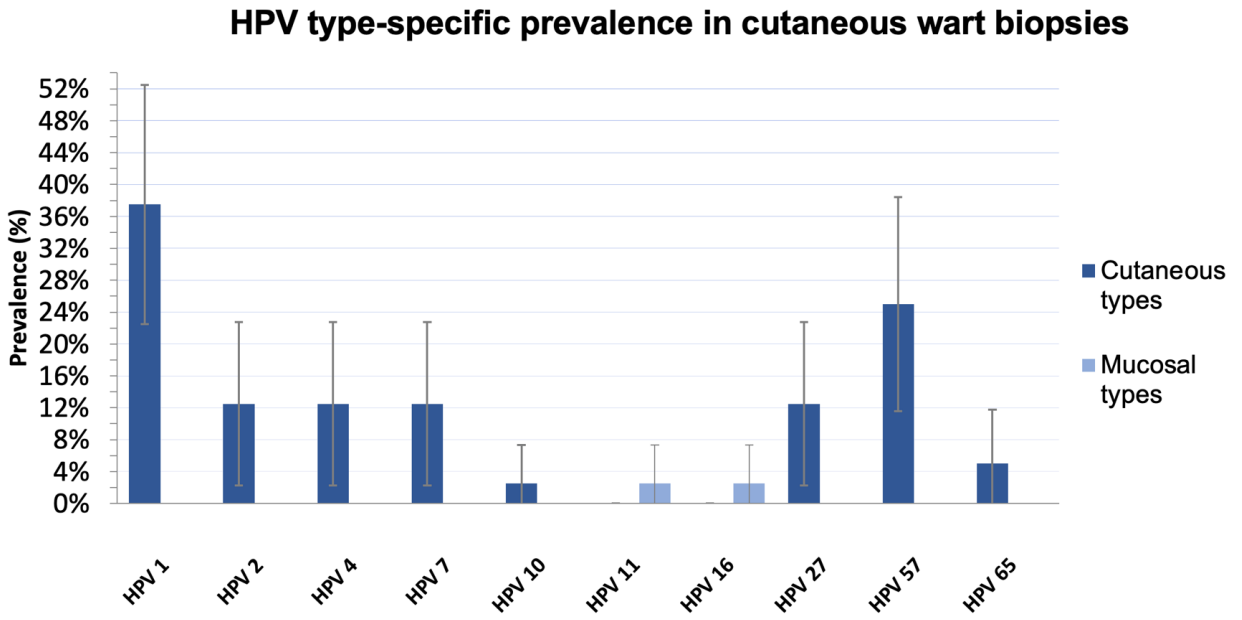

Figure 2. HPV type-specific prevalence in cutaneous warts with 95% confidence intervals. Cutaneous HPV types are depicted in dark grey, mucosal HPV types in light grey. The most prevalent HPV types in the defined population were cutaneous types HPV1 (37.5% [95%CI 23-54%]) and HPV57 (25% [95%CI 13-42%]).

Furthermore, the degree of correlation between the multiplex and singleplex PCR reactions was also evaluated per HPV type in wart biopsies. There were no significant differences in Ct-values between the two reaction types ($p > .05$) and an average CV of ≤5% was observed. This trend was also observed for the most abundant HPV type in samples where multiple HPV types of the same multiplex where present, indicating that the simultaneous co-amplification of different HPV types in biopsies infected with multiple types did not significantly influence the PCR efficiency of the most abundant HPV type. However, the degree of correlation could not be calculated for all HPV types considering that some cutaneous HPV types were not detected in the biopsies (i.e., HPV type 3, 41, 60, and 63).

Conclusion

Although to date several detection methods exist that are able to detect most of the wart-associated HPV types, these methods are generally time-consuming, laborious, and rely on sequencing for HPV genotyping making them rather expensive and therefore not suitable for large epidemiological studies and daily routine diagnostics (e.g., Southern blot hybridization, PCR followed by sequencing).[10] The newly designed wart-associated HPV PCR assay fulfilled all the predetermined validation criteria and was able to amplify HPV DNA from various sources. The assay exhibited an analytical type-specific sensitivity and specificity of 100% (95% CI: 83.9%–100%). The LOQ of the tested sequences corresponded to less than 17 viral copies/µl, while the LOD was less than 5 copies/µl. Very good to excellent agreements were gained between intra- and inter-run measurements ($\kappa = 0.85–1.00$) and CV of the quantitative agreements was less than 3%. In addition, the current assay was also capable of absolute quantification of the type-specific viral load by use of calibration standards, as well as being easily adaptable for automatic processing, making it a high throughput assay and an excellent tool for use in large epidemiological studies.[15]

As regards to the application of the test on a cohort of FFPE wart biopsies, multiple HPV infections were detected in 35% (95% CI: 21%–52%) of biopsies. This is in contrast to the current literature claiming that in immunocompetent patients only 4%–16% of cutaneous warts exhibit multiple HPV infections.[4,10,17–19] The observed occurrence of multiple infections is probably due to the high sensitivity of the newly developed HPV qPCR assay. Our results confirm earlier findings by Schmitt et al., comparing HPV detection rates of two different xMAP Luminex technology based assays in 100 swab samples of cutaneous warts, that is, BSwart and HSL-PCR/MPG HPV genotyping assays.[8] Schmitt et al. demonstrated that the BSwart assay exhibited superior ability of detecting multiple infections in the same sample due to its higher sensitivity. Analogous to our findings BSwart was able to detect 51 (53.6%) single, 34 (35.7%) double, 6 (6.3%) triple, 3 (3.2%) quadruple, and 1 (1.1%) sextuple infections in 95 samples.

It is hypothesized that if multiple HPV types are detected in a wart, usually only one HPV type will be responsible for the development of the wart.[6] This is supported by evidence on the clonal origin of warts and in analogy with cervical HPV infections where it was demonstrated that within a defined cervical intraepithelial neoplastic lesion, only one HPV type is present.[6,20] The most abundant HPV type is biologically most active and proposed to be causing the lesion, while the other types are just transient, passenger infections.[8] Our findings confirm that this theory is also valid for cutaneous types, that is, biopsies with

multiple infections generally exhibit one highly abundant HPV type accompanied by one or more HPV types characterized by a lower viral load. Alternatively, another explanation for some warts displaying multiple HPV types could be the co-infection of single cells with diverse HPV types.[6]

Furthermore, although it is common knowledge that warts are caused by HPV, 22.5% (95% CI: 11%–39%) of the tested biopsies were HPV negative. HPV molecular testing is challenging in FFPE samples due to poor DNA quality in the embedded tissue, resulting in lower HPV detection rates. Especially formalin fixation can cause extensive DNA damage, i.a., cross-linking and fragmentation.[21] In addition, the newly developed assay is limited in detection of only 12 HPV types. It is possible that another type, that is not included in the assay, is responsible for the formation of the wart. Ideally, a more ubiquitous approach to HPV detection should be applied to ensure detection of all HPV types present e.g. sequencing. However, sequencing remains to date rather expensive and cutaneous warts do not pose a serious treat in immunocompetent population. Nevertheless, this is contradicted in immunocompromised subjects, where up to 50% of renal transplant recipients have cutaneous warts at one year post-transplant, and 77%–95% have warts at five years after surgery.[22] Progression of these warts to dysplastic lesions and squamous cell carcinoma (SCC) has been described, and HPV is proposed to have a possible cofactor role in immunosuppression-associated skin cancer. Therefore, information gained from HPV genotyping of warts can also be used for development of prophylactic or therapeutic vaccines.[8] Identification of the wart-associated HPV types is necessary to determine which types should be included in future vaccines, that would make eradication of these types in the population possible, hereby protecting the weakest members, that is, transplant patients and other immunocompromised patients from serious illnesses (e.g., SCC). Nevertheless, before this line of reasoning can be tested we need to ascertain that the same HPV types responsible for the benign lesion in the general population are causing malign lesions in immunocompromised patients. In general, it has already been demonstrated that cutaneous warts from immunocompetent and immunocompromised patients exhibit same genotype distributions.[23–25] However, the number of detected types per lesion seems to differ between the two groups.[26] These findings have to be confirmed in large-scale epidemiological studies comparing the HPV type-specific distribution in the two populations with the same techniques. It should also be interesting to compare the average viral load per HPV type in both groups and examine the potential use of viral load as a biomarker for malignancies. At present, cutaneous warts are again the focus of attention with the increasing number of chronically immunosuppressed patients.[10] A straightforward, highly sensitive, high-throughput HPV genotyping technology, such as that described in this study will be crucial for future research in immunocompromised patients, as well as for the previously mentioned prognosis of natural course and treatment efficacy of warts in immunocompetent individuals.

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Conflict of interests

The authors declare that there is no conflict of interests.

Ethics statement

None required since procedure was part of clinical patient care.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nina Redzic. The first draft of the manuscript was written by Nina Redzic and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability statement

The data that support the findings of this study are available fromthe corresponding author upon reasonable request.

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Chapter 4 Efficacy of AV2-Salicylic Acid Combination Therapy for Cutaneous Warts: Study Protocol for a Single-Centre Randomized Controlled Trial

This chapter provides a comprehensive description of the study protocol of the randomized-controlled clinical trial into the efficacy of Omnivirol (AV2)-Salicylic acid combination therapy as a novel treatment for cutaneous warts. Examples of topics discussed are trial design, patient recruitment strategies, study procedures, follow-up techniques as well as study objectives and outcomes.

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Efficacy of AV2-Salicylic acid combination therapy for cutaneous warts: Study protocol for a single-center randomized controlled trial

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Abstract

Cutaneous warts comprise an extremely common condition caused by infection with the human papillomavirus (HPV). Although most verrucae will disappear spontaneously, many patients do seek treatment. Current wart treatments do not target the cause of the lesion directly, resulting in variable treatment efficacies and high wart recurrence rates. AV2 is a broad-spectrum antiviral drug, that is capable of deactivating HPV. It is however not able to destruct the already infected cells, which raises the need for an additional ablative treatment i.e. salicylic acid (SA). Implementation of AV2-Salicylic acid (AV2-SA) combination therapy would ensure permanent lesion clearance by on the one hand inactivation of HPV by AV2, and on the other hand elimination of the lesion by SA treatment.

The primary aim of this study is to assess the efficacy of AV2-SA treatment versus standard SA treatment, by comparing cure and recurrence rates of cutaneous warts between the two treatment groups (at 12 weeks and six months after randomization). The second aim is to assess the safety and tolerability of AV2-SA therapy. The third aim is to identify subgroups of cutaneous warts that have favorable response to treatment, by comparing cure rates in an HPV genotype-specific manner.

This randomized controlled trial will enroll 260 participants with cutaneous warts who will either receive the AV2-SA combination therapy or SA control treatment. Real time monitoring will be possible by daily photographs sent via WhatsApp™ (a messaging application) as well as online follow-up questionnaires administered on several occasions. HPV genotyping will be performed on swab self-samples.

Keywords:

Cutaneous warts, Antiviral drug, Study protocol, Clinical research, AV2-Salicylic acid combination therapy, HPV genotyping, Randomized controlled trial, Omnivirol

List of abbreviations

Abbreviation	*Explanation*
AML	Algemeen Medisch Laboratorium
AV2	Antiviral 2
AV2-SA	Antiviral 2 – Salicylic Acid
CRF	Case Report Form
GRAS	Generally Recognized As Safe
HPV	Human Papillomavirus
OV	Omnivirol
qPCR	Real-Time Polymerase Chain Reaction
REDCap	Research Electronic Data Capture
SA	Salicylic Acid
UA	University of Antwerp
v/v	Volume/volume percent
w/v	Weight/volume percent

Introduction

Cutaneous warts or verrucae are very common with a varying worldwide prevalence of 0.84–12.9% [1]. The prevalence rate in children and young adults is even higher and reported to reach 30% [2]. Verrucae are caused by infection with the human papillomavirus (HPV) and although most verrucae disappear spontaneously, many patients do seek treatment because their lesion is painful, persistent or interfering with normal function. An armamentarium of wart treatments is currently at disposal, starting from folk remedies to over-the-counter medications and more aggressive clinic-based treatments. Unfortunately, none of these treatments seem to produce consistent results and reported efficacies often vary widely depending on several factors (e.g. age, compliance, immunocompetence). A systematic review conducted by the Cochrane Skin Group assessed the effects of different wart treatments [3]. A modest efficacy of salicylic acid (SA) topical treatment was observed in pooled data of five placebo-controlled trials with average clearance rates of 73% (0–84%) vs. 48% (10–65%) respectively. As to cryotherapy there was inconclusive evidence concerning the efficacy when compared with placebo and other simpler and safer treatments. In total, 21 trials with placebo groups were evaluated and an average clearance rate of 27% (0–73%) in the placebo groups after an average period of 15 weeks (4–24 weeks) [3] was found. These data highlighted the lack of an optimal treatment for verrucae and resulted in some practitioners recommending that warts should not be treated at all [4]. Unfortunately, there is currently no reliable mean of predicting which warts will clear spontaneously and which will remain for years. Although in theory a policy of not treating warts is recommended, in practice many people do consult healthcare professionals and are treated due to the social stigma and morbidity associated with visible warts [2,3]. Furthermore, treatment is also necessary in order to prevent spread of infection in the general population [5].

Contemporary wart treatments intend to simply destroy the infected cells (i.e. physically or chemically ablate warts), and do not have any specific antiviral mode of action. Hence the high wart recurrence rate of 12.5–70% amongst these treatments, due to residual

HPV particles [2]. Currently, there are no specific molecular inhibitors that directly target HPV. By eliminating the source instead of only affecting the lesion, these drugs would make wart recurrence unlikely [6]. Unfortunately, most of these strategies are still in the very early stages of investigation, and since the appearance of vaccines, research into direct antiviral agents against HPV has been suspended [6].

Recently, Cesa Alliance has developed a broad-spectrum antiviral drug, called AV2 (Omnivirol™), that has proven to be highly effective in treatment of cervical lesions caused by HPV [7]. AV2 is a combination of FDA GRAS-label approved organic compounds (natural essential oils: carvone, eugenol, geraniol, and nerolidol) that is postulated to be able to prevent viral entry and proliferation by deactivating the infectious virions before they enter the cell [7].

Although AV2 is capable of deactivating the source of the lesion, it is however not able to destroy the already infected cells. In order to ensure that AV2 is able to reach the epidermal basal layer, which contains the infectious reservoir, and deactivate the viral particles thus preventing future reinfection and recurrence of the lesion, an additional ablative treatment is necessary. Salicylic acid (SA) formulations are the most commonly used preparations in the treatment of warts [2]. SA is an organic acid that destroys epidermal cells and softens hyperkeratotic epidermis. Implementation of AV2-SA combination therapy would ensure permanent lesion clearance by on the one hand inactivation of HPV by AV2, and on the other hand elimination of the lesion by SA treatment.

Furthermore, a recent study by Bruggink et al. revealed that the HPV genotype influences the natural course and response to treatment for plantar warts, hereby suggesting that HPV genotyping could potentially be used to optimize wart treatment schemes [8]. An additional study concluded that from all patient- and wart-specific characteristics analyzed, HPV genotype most strongly predicted treatment response in warts [9]. The authors advised that for development of new wart therapies it is essential to take HPV DNA testing into account in order to determine the most optimal treatment. Therefore, this project not only intends to evaluate the efficacy of standard SA treatment versus AV2-SA combination therapy against cutaneous warts, but also to investigate the predictive value of HPV genotyping regarding treatment response.

Objectives

The primary objective of this study is to assess the efficacy of AV2-SA treatment versus standard SA treatment by comparing: (1) cure rates of the index warts between the two treatment groups at 12 weeks after enrolment; and (2) recurrence rates of index warts between the two treatment groups at six months after enrolment.

The secondary objectives are: (1) to assess the safety and tolerability of AV2-SA therapy and identify the maximum tolerable dosage; (2) to identify HPV type-specific subgroups of cutaneous warts that have a favorable response to treatment; (3) to compare time to clearance and change in size of index wart between the two treatment groups; (4) to

compare clearance of all verrucae, the number of verrucae remaining and potential reinfection at six months after enrolment between the two treatment arms; (5) to determine the genotype-specific distribution of wart-associated HPV types in a Belgian population (according to the age, wart location, postal code, etc.); and (6) to investigate the prevalence of mucosal HPV types in cutaneous warts.

Method and analysis

Trial design and setting

This study comprises two trials i.e. Phase I and Phase II. The Phase I trial is designed to optimize the AV2 treatment dose. The Phase II trial is a double-blind, single-center, two-armed, randomized controlled trial with equal randomization. Participant progress through both trials is shown in Figs. 1 and 2. Specimens will be processed and analyzed at medical laboratory AML, Antwerp, BE. Data management and statistical analysis will take place at the University of Antwerp (UA), Antwerp, BE.

Recruitment and eligibility

Advertising material will be distributed in local community areas frequently accessed by the general public including movie theatres, shopping centers, pharmacies, local dermatology and general practices, hospitals, high schools, and public swimming pools. Individuals responding to an advert will be screened for eligibility by phone. Table 1 provides a list of all the inclusion and exclusion criteria. At the baseline appointment, the study coordinator will again ensure that the patient is eligible, take informed consent, and check that all baseline data have been completed. Subsequently the study coordinator will firstly photograph the lesion and instruct the patient on follow-up procedures, secondly take a sample for HPV genotyping, and thirdly administer the allocated treatment. The recruitment will continue until 260 patients have been enrolled and at least 50% of the study population comprises persistent warts. Persistent warts are defined as warts resistant to previous treatment and/or warts older than six months.

Sample size estimation

The previously mentioned systematic review conducted by the Cochrane Skin Group found six studies comparing SA treatment with placebo, with an average SA cure rate of 70% [3]. This trial is powered to show a 15% difference in effectiveness between standard SA treatment and AV2-SA combination therapy. In order to achieve 80% power, 5% two-sided significance, difference in cure rates of 70% versus 85% at 12 weeks, and allowing for 10% loss to follow-up, a sample size of 130 patients in each treatment group will be required (i.e. 260 patients in total).

Randomization

The study drugs will be randomized at the site of the manufacturer based on a computerized randomization list [11]. The study center will receive sequentially numbered vials, with no knowledge of the allocation table, and randomly assign new study numbers to each vial (this to ensure blinding of the study drug

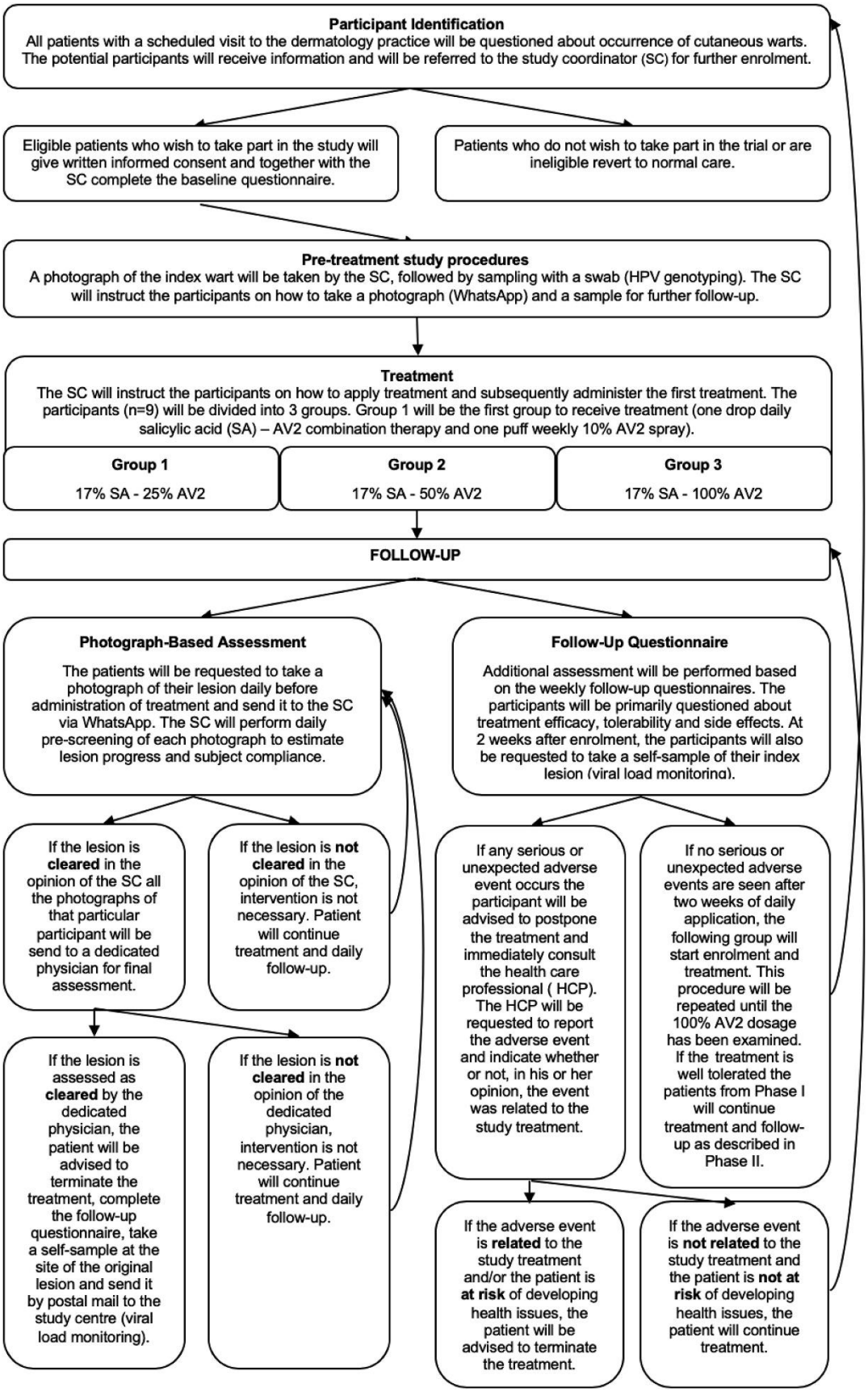

Fig. 1. Flow chart Phase I trial.

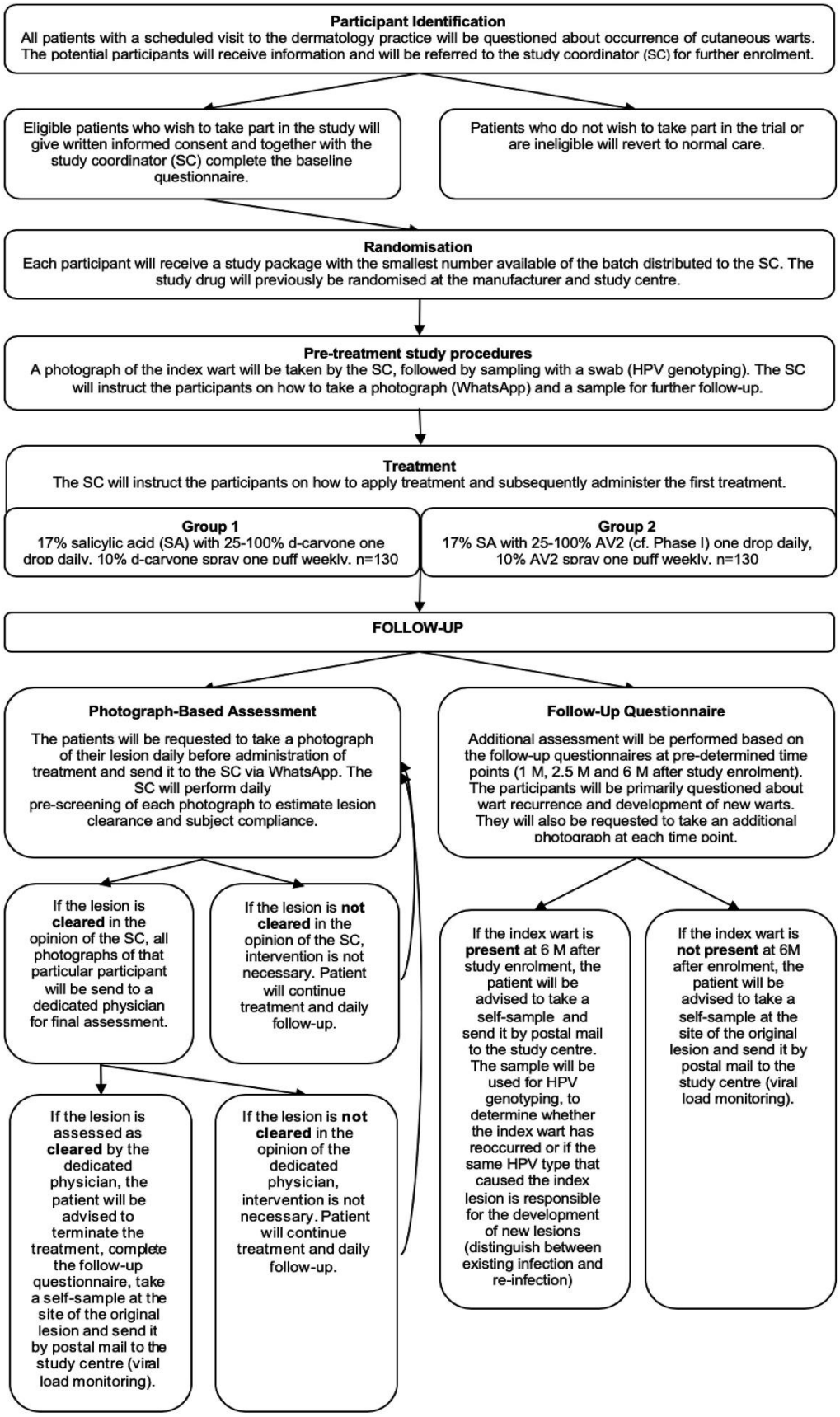

Fig. 2. Flow chart Phase II trial.

manufacturer). The participants will be sequentially assigned a unique subject number, corresponding to the new vial number, at the time of enrolment.

Interventions

Phase I

A dose-finding trial will be conducted on nine patients in total, divided in three groups. The first group will daily apply one drop of 25% AV2 (v/v) – 17% SA (w/v) formulation directly on the lesion, in addition to one puff of 10% AV2 (v/v) spray weekly. The additional weekly spray application is intended to prevent reinfection and formation of new warts in the direct environment of existing lesions. Substance tolerance and adverse events will be assessed via weekly follow-up questionnaires (Table 1). The patients will daily take a photograph of their index lesion (the largest and thickest wart) and send it via WhatsApp™ (a freely available messaging application) to the study coordinator, enabling real-time monitoring of treatment progress (see Fig. 3 for further details). The photographs will also be used as proof of daily treatment and daily reminders will be send in order to improve patient compliance. If no serious or unexpected adverse events are seen after two weeks of application, the second group will start with 50% AV2 (v/v) – 17% SA (w/v) treatment. This procedure will be repeated to a maximum dose of 100% AV2 (group 3) if no issues occur. If the treatment is well tolerated, the patients from Phase I will continue treatment and follow-up as described in Phase II.

Phase II

Participants will be randomized to receive either: (1) the optimal AV2 concentration as determined in Phase I, in combination with 17% SA (w/v) one drop daily and 10% AV2 (v/v) spray one puff weekly; (2) equivalent d-carvone concentration as AV2 concentration in group 1, in combination with 17% SA (w/v) one drop daily and 10% d-carvone (v/ v) spray one puff weekly. D-carvone is a component of AV2 that has no antiviral properties on its own and will be used to provide a fragrance to the SA control treatment in order to ensure blinding. The participants will give the same treatment to all their warts, but only the index lesion will be used for primary outcome assessment. The daily application will be continued for 12 weeks or until the wart is completely cleared. The weekly spray administration, however, will be continued for entire 12 weeks, even after the lesion has cleared (in order to prevent reinfection).

Outcome measures

Baseline data will be collected using a baseline questionnaire at time of enrolment. The main measures will be demographic details and previous wart anamnesis (Table 1).

The primary outcome measures (i.e. presence or absence of index wart) will be assessed via follow-up questionnaires (Table 1) and photographs. A wart is considered cured if it is no longer visible (skin color and skin lines are reestablished) and cannot be palpated anymore by hand. Patients will complete the follow-up questionnaire on three to four occasions (at time of wart clearance, one month, 12 weeks and six months after

enrolment) and take a daily photograph of the index lesion for a maximum period of 12 weeks or until the index wart is cleared, and at six months. The participants will take all photographs according to a standardized protocol (Fig. 3). The final blinded outcome assessment based on photographs will be performed by two dedicated physicians who are unaware of the treatment group to which the patient is allocated. After confirmation of wart clearance by both physicians, the patient will terminate the treatment, complete a follow-up questionnaire, and take a self-sample of the skin surface at the site of the original lesion.

The secondary outcomes (i.e. general treatment efficacy, side effects and compliance) will also be assessed via online questionnaires (Table 1). HPV genotyping will be performed on several occasions (at time of enrolment and/or wart clearance, and at 12 weeks and six months after enrolment). Samples will be collected and stored according to the previously described optimized sampling protocol [13]. The DNA extraction will involve overnight Proteinase K and EDTA digestion, followed by automated extraction on the NucliSENS easyMAG system (bioMérieux) [13]. The samples will be analyzed with two separate in-house PCR assays capable of detecting the most prevalent cutaneous HPV types as well as the most relevant mucosal types, i.e. respectively the newly developed wart-associated HPV qPCR assay and the HPV Riatol genotyping assay [13,14]. In addition, a cellularity control will be performed on every sample by amplification of the beta-globin gene [14].

Adverse events

The patients are instructed to consult the participating healthcare professional immediately at onset of any adverse events. Any adverse events will be reported using the adverse event form. When appropriate an assessment of severity, causality, regularity and intensity will also be performed. The clinical course of each event will be followed until resolution or until it has been determined that the study treatment is not the cause. The possible treatment related adverse events due to salicylic acid treatment are pain, blistering, irritation to the skin, burning sensation, and allergic contact reaction [2]. AV2 has no reported side effects.

Data management

All data will be treated with the strictest confidentiality. The study coordinator will keep a locked subject identification log with the names and allocated subject number of the enrolled patients. The case report forms and samples will only be identified by the subject number. All collected data will be incorporated in the trial master file using the REDCap (Research Electronic Data Capture) data management system [15]. Data quality checks will be undertaken to ensure the accuracy of the data. Paper study documents will be retained in a restricted access archive at the study center. Electronic records will be stored on a secure, password protected server within the UA indefinitely.

Statistical analysis

Baseline data and treatment details will be analyzed using descriptive statistics (i.e. standard deviation, means, percentages). Proportions of patients with complete wart clearance will be compared using chi-square test. To identify subgroups of cutaneous

Table 1. Details of outcome measures and data collection forms. The majority of the outcomes are responses to questions that require a 'Yes' or 'No' answer or open comments, except for pain which will be measured by a 0-4 numeric pain rating scale.

Measure	Source: Content
Eligibility	Inclusion Criteria: patient exhibiting one or more cutaneous warts; aged 12 years or older; agrees to refrain from using prescription or supplemental antiviral medications without first obtaining permission of the coordinating trial dermatologist; able to read Dutch; signed informed consent; able to self-assess and use WhatsApp for follow-up (all Y/N). Exclusion Criteria: patient only exhibiting facial and/or seborrheic warts; not suitable for salicylic acid (SA) treatment due to a medical history of severe diseases (e.g. hepatitis, renal or liver dysfunction, cardiovascular, or gastrointestinal disorders, etc.), impaired healing or neuropathy (e.g. due to diabetes, peripheral vascular disease or any other condition); known or suspected allergic or adverse response to SA, AV2 or its components; immunocompromised patient; patient had already participated in another clinical trial concerning treatment for cutaneous warts within six months before enrolment in this study or is currently in a trial evaluating other treatments for his/her warts (all Y/N).
Demographic Details	Baseline Questionnaire: date of birth; sex (M/F); postal code.
Wart anamnesis	Baseline Questionnaire: General History: number of warts; wart type (verruca vulgaris, verruca plantaris mosaic or simple, verruca plana, verruca filiformis); average size (mm); location; persistence (<6months, >6months); previous treatment (Y/N, if yes specify). Index Wart History: wart type; average size (mm); location; persistence (<6months, >6months).
Efficacy Of Treatment	Follow-Up Questionnaire: Index wart: clearance (Y/N) → if cleared date of clearance; if not cleared potential recurrence Y/N. Other warts: still present (Y/N) → if not present date of clearance; if present number of warts and location (inside a radius of 0.5cm around the original position/another position = 'new warts' → if new warts, inside a radius of 3cm around the index lesion (Y/N; if yes number of warts).
Side Effects Of Treatment	Follow-Up Questionnaire: pain scores (numeric pain rating scale 0=no pain at all – 4=extreme level of pain); another side effects (Y/N, if yes specify).
Treatment Compliance	Follow-Up Questionnaire: use of additional treatments (Y/N; if yes specify); comments about treatment (open text).
Photograph-Based Outcome Assessment	Photograph-Based Assessment Form: Study Coordinator: date at which the photograph is taken; treatment day; index wart cleared (Y/N); size of index wart (mm). Dedicated Physician: photograph interpretable (Y/N); index wart cleared (Y/N); remarks (open text).
HPV Genotyping	Laboratory Form: date at which the sample is taken; treatment day; HPV (pos/neg; if pos specify HPV type(s)).

warts that have favorable response to treatment, cure rates of different treatments will be compared within specific HPV types using 95%CIs, relative risks and risk differences. A logistic regression model will be used to adjust the primary analysis for important prognostic variables (e.g. age, previous treatment, type of wart, persistence). A Cox proportional hazards model will be used to compare the time to clearance of cutaneous warts between the two treatment groups and between different HPV types adjusting for the same covariates as for the primary outcome. Participants will be right censored if they are lost to follow-up or if their verrucae have not cleared. The incidence of all suspected adverse treatment reactions will be summarized by treatment group.

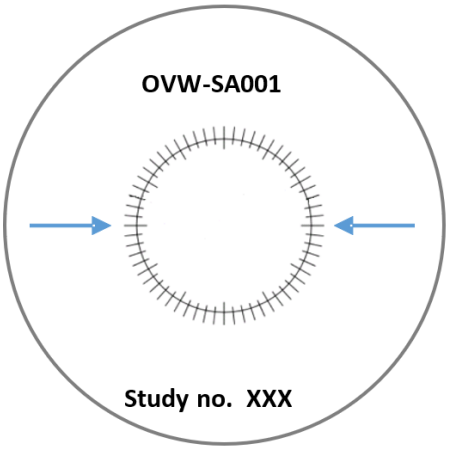

Figure 3. Example of a follow-up sticker that will be used for photograph-based outcome assessment. The circular stickers have an opening at the center and are provided with a measuring scale and a subject number. The photograph will always be taken from the same distance and angle, with the index wart at the center of the sticker. The subject number and the wart measurements will always be clearly legible.

Missing data

The amount of missing baseline data is expected to be minimal as data monitoring will be performed at regular time intervals. If any issues arise subjects will be contacted in order to resolve them. An 'intention to treat' analysis will be used i.e. all patients will be included in their initially randomized groups whether or not they received their allocated treatment. If the status of a patient cannot be verified the patient will be treated as not having a cleared index wart in the primary analysis.

Trial completion

Participants will have the option, at any time, to withdraw from the study. Participants may withdraw for the following reasons: development of safety issues; failure of the participant to adhere to protocol requirements; or the participant wished to exit the trial. In case of a withdrawal the change of circumstances form must be completed to ensure appropriate follow-up.

Ethics

This study has been approved by the Ethics committee of Antwerp University Hospital (B300201734040). The written information that the participants will receive, clearly describes the potential risks and benefits of participation, the voluntary nature of participation, and how confidentiality will be maintained. All participants will give written informed consent prior to entry into the study. The participant will be informed if new information comes to light that may affect the participant's willingness to participate in the trial. The trial will be conducted in full compliance with local regulations governing the conduct of clinical studies.

Dissemination

The results of this study will be published in a peer reviewed medical journal as well as presented at international scientific conferences. Furthermore, participants will be

offered the opportunity to obtain a summary of the findings on completion of the study.

Conclusion

This will be the first randomized controlled trial to evaluate the efficacy of an HPV-targeted treatment for cutaneous warts. The AV2-SA combination therapy is expected to significantly enhance treatment efficacy and substantially decrease wart recurrence, which is the main cause of patient frustration. The results of this study will make further research into efficacy of AV2-treatment against other HPV-related diseases certainly interesting. In addition, if we are able to confirm that HPV genotype indeed influences the natural course and treatment efficacy in cutaneous warts and make additional statements about the response to treatment of warts infected with other HPV types, we could provide physicians with crucial information necessary to determine the optimal treatment for individuals with cutaneous warts (personalized medicine). In other words, this study could prevent patients from receiving unnecessary therapy, sparing them from painful adverse effects and costs of treatment.

Trial status

Recruitment to the study began in March 2018 and has been completed in January 2019. The follow-up is still in progress and will be completed in July 2019. The analysis will be conducted in September 2019.

Bulleted statements

Cutaneous warts are very common infectious disorders caused by human papillomavirus (HPV). Current wart treatments exhibit variable treatment efficacies and high wart recurrence rates. This randomized controlled trial intends to examine the efficacy of a new antiviral drug (AV2) that targets the source of the infection, making wart recurrence unlikely. The unique study design allows for real-time monitoring of treatment progression and patient compliance (via daily photographs). If treatment response to AV2 is proven to be HPV type-dependent, future triage of patients according to HPV type is recommended in order to ensure treatment efficacy and minimalize side effects and treatment costs.

Funding sources

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Declaration of competing interest

The authors have no conflict of interest to declare.
IRB approval status: Reviewed and approved by Ethics committee of Antwerp University Hospital (B300201734040).

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Chapter 5 Execution of the OVW-SA001 Clinical Trial

This chapter describes experienced study-related challenges such as diminished recruitment progress and effectiveness, study withdrawal, and specific investigational product characteristics.

During the conduct of the trial, several challenges were encountered. These were attributed to difficulties with subject recruitment and concerns about product characteristics and stability.

5.1 Recruitment

From the very beginning of the trial, we experienced recruiting problems due to severe underestimation of the advertisement needed to achieve the desired sample size. At first, the recruitment was only done by the participating dermatologist. Due to insufficient recruitment progress other recruiting strategies were employed: (1) distribution of advertising flyers at local pharmacies, and general and dermatology practices; (2) active recruitment by recruiters in public areas that are frequently accessed by the general public; (3) online campaigns trough mailings and social media; (4) visits to Dermatology department of local hospitals; (5) and features by traditional media (i.e. newspaper, radio, TV). For a detailed description of the recruitment process see Figure 6. The recruitment was predicted to end in July 2018, but the proposed sample size was only attained by January 2019. In the end, 628 subjects were identified in total, and 13% of the subjects identified had to be declined from participation without prior screening due to the attainment of the proposed study sample size (see Table 4). The outcome of prolonged recruitment was not only that the study period had to be extended, but several issues with product shelf life also emerged (see Chapter 2.1 Product stability). During monitoring of the study dropout rate, it became clear that one of the main reasons for study withdrawal was a time-consuming protocol (see Table 5). Considering that several subjects who did complete the study also reported that the protocol including daily pictures was too time-consuming, the protocol was adapted to improve study completion rates and disburden the participants. The new protocol included weekly photographs for treatment evaluation, and daily confirmation of treatment by a message via WhatsApp for supervision of patient compliance.

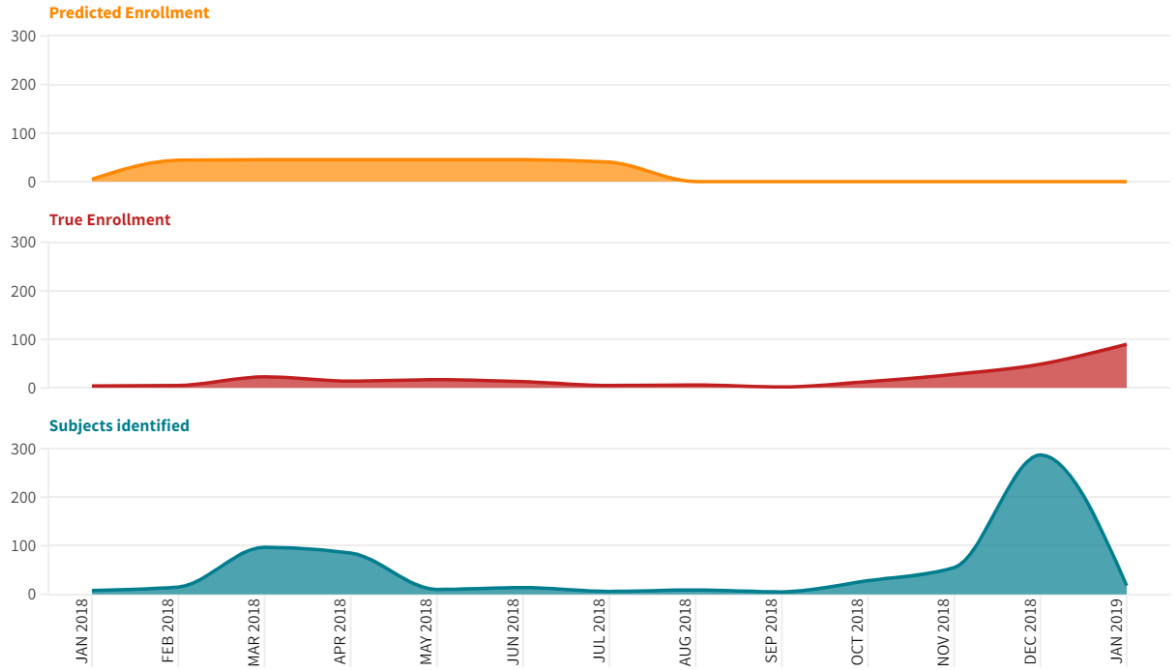

Figure 6. *Recruitment progress of the clinical trial OVW-SA001. The figure depicts the number of subjects per month that were either predicted to enroll in the trial (orange), did truly enroll (red), or were identified as potential participants (blue). Recruitment through participating dermatologists first started in January 2018. Soon it became clear that this type of recruitment was not sufficient and additional advertising flyers were distributed in local pharmacies, and general and dermatology practices. By March 2018, the recruitment still did not proceed as predicted, and active recruitment was employed by trained recruiters in public areas that are frequently accessed by the general public (e.g. shopping centers, train stations, university campuses, and local events). This strategy did increase the number of identified subjects significantly, but due to lack of effectiveness, active recruitment was terminated by May 2018 (see Table 1). During the summer vacation, the recruitment was low and in October 2018 a new strategy of online recruitment was devised. Mailings were sent to all departments of the University of Antwerp, as well as to local hospitals, high schools and swimming pools, dermatology, and pharmacy societies. In addition, an online Facebook campaign was designed, and permission was granted to attend the wart consultation hours at the Dermatology Department of the Antwerp University Hospital (UZA). The online recruitment campaign was eventually noticed by a local newspaper (Gazet van Antwerpen, GvA) which featured an article about the study in December 2018. The study was also broadcast on the local radio channel (Radio 2) and TV station (Antwerpse Televisie, ATV). This resulted in a peak in subject identification in December 2018, followed by a peak in true enrollment by January 2019.*

Table 4. *Recruitment effectiveness and dropout rate per recruitment medium. Recruitment effectiveness is defined as the number of subjects identified by a specific recruitment medium divided by the number of subjects enrolled. Active recruitment is the least effective method with only 10% of identified subjects that eventually enroll. More effective recruitment media are the ones that employ a more targeted approach to the desired population (e.g. recruitment at dermatology practices and pharmacies). The true recruitment effectiveness of some media is even higher than depicted, considering that 13% of the identified subjects were not screened due to the attainment of the sample size criteria (84/628).*

Recruitment Medium	Nr. of Subjects Identified	Nr. of Subjects Enrolled	Proportion of Enrolled Subjects (%)	Recruitment Effectiveness (%)	Nr. of Subjects Not Screened	Nr. of Dropouts (rate, %)
Hearsay	11	9	3%	82%	0	3 (33%)
Participating Dermatologist	18	14	5%	78%	1	0 (0%)
Pharmacy	10	9	3%	90%	0	0 (0%)
General practitioner	24	18	7%	75%	1	3 (17%)
Dermatologist	48	37	14%	77%	2	2 (5%)
Recruiters	153	15	6%	10%	0	6 (40%)
Local hospital Dermatology Department (UZA)	10	8	3%	80%	0	2 (25%)
Mailing (e.g. school, swimming pool, university, etc.)	18	13	5%	72%	0	3 (23%)
Facebook	51	24	9%	47%	5	3 (13%)
Local newspaper (GvA)	191	81	31%	42%	48	3 (4%)
Local radio station (Radio 2)	12	5	2%	42%	3	0 (0%)
Local TV station (ATV)	30	15	6%	50%	7	1 (7%)
Unknown	52	12	5%	23%	17	0 (0%)
Total	**628**	**260**	**100%**	**41%**	**84**	**26 (10%)**

Table 5. *Reasons for study withdrawal. Ten percent (26/260) of participants withdrew from the study on their own accord or were unresponsive for the last follow-up. The main known reasons behind study withdrawal were a time-consuming protocol, issues with product characteristics, and lack of rapid results.*

Reason for study withdrawal	Number of subjects
Time-consuming protocol	3
Product characteristics (e.g. fluidity, odor)	3
Lack of rapidly visible results	3
Illness unrelated to the study	1
Unknown	16
Total	**26**

To conclude, recruitment should ideally be performed before study initiation in order to build an adequate pool of potential participants and conclude the study on time, avoiding future difficulties caused by prolonged study duration. Recruitment via social and traditional media has proven to be most effective. However, it is not sufficient to have a large pool of potential subjects identified, the 'quality' of subjects is also a prominent issue

(see Table 6). Subjects who register to participate in the study on their own initiative are more likely to eventually enroll in the study and finish it accordingly. Furthermore, study dropout rates must be monitored frequently, in order to identify the main cause of withdrawal and develop strategies to prevent it.

Table 6. *Recruitment effectiveness and dropout rate per enrollment initiator. If the initiative to participate is induced by study subjects, they are more likely to enroll in the study (52%) and complete it accordingly. This is demonstrated by the dropout rate in the subject initiative group of 8% and 40% in the study staff initiative. The subjects who contacted the study representatives themselves had more time to think about their participation, consider all the pros and cons (e.g. the amount of time and labor it would include), and are highly motivated to cure their illness.*

Initiative to Participate by	Nr. of Subjects Identified	Nr. of Subjects Enrolled	Recruitment Effectiveness (%)	Nr. of Dropouts (rate, %)
Subject	475	245	52%	20 (8%)
Study staff	153	15	10%	6 (40%)
Total	**628**	**260**	**41%**	**26 (10%)**

5.2 Product Issues

5.2.1 Product Characteristics

Several patients observed that the substance employed during daily treatment was too liquid and difficult to apply. Due to its liquid consistency, the substance not only came in contact with the diseased skin but also with normal skin causing redness, irritation, and an itching sensation. To make the product more user-friendly for future studies an additional substance should be added to increase the consistency. A widely used product in topical wart treatments for this purpose is flexible collodion (Jahromi et al., 2022). It is a solution of nitrocellulose dissolved in ethyl ether and ethanol. The final product is a syrupy fluid, which when applied to the skin and exposed to air forms a protective film over the treated area. This would ensure that SA is contained in the diseased areas of the skin and decrease the risk of adverse events due to contact with normal skin. Three subjects also reported strong fragrance hindrance. One subject reported irritation of the throat and breathing problems during application caused by the strong fragrance.

5.2.2 Product Stability

Prior to clinical trial initiation a solubility study was performed in order to determine the solubility of SA in AV2. Several SA concentrations were analyzed: (1) 40% (w/v) SA; (2) 25% (w/v) SA; (3) 20% (w/v) SA; and (4) 17% (w/v) SA. The indicated amount of SA for each solution was calculated and weighted before being added to 8ml pure AV2, followed by shaking for 5 min. Only the 17% (w/v) SA was able to dissolve completely in AV2, the other SA concentrations remained in a solid state (see Figure 7). All samples were stored at room temperature and protected from light. One year after synthesis the composition of the samples was examined once more. The 17% (w/v) SA remained in the solution, while SA in the other samples still did not change appearance. The same results were observed after two years of storage. It was concluded that SA is poorly soluble in pure AV2 and in order to utilize higher SA concentrations additional substances should be added. Considering that the solubility of 17% (w/v) SA in AV2 was sufficient and this is the proposed SA concentration to be used in the clinical study, the clinical trial could proceed accordingly.

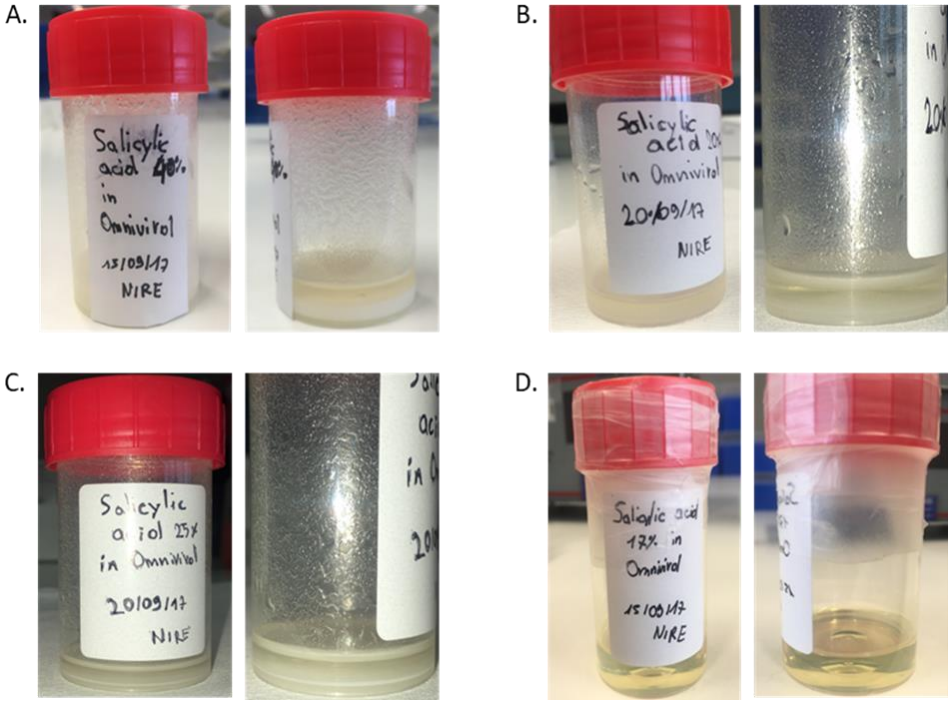

Figure 7. *Salicylic acid (SA) solubility in AV2 (i.e. Omnivirol). Solubility of several SA concentrations in pure AV2 were examined: A. 40% (w/v) SA; B. 25% (w/v) SA, C. 25% (w/v) SA, D. 17% (w/v) SA. Only 17% SA was completely solved, other SA concentrations remained in the solid state. The same results were observed after one and two years of storage.*

During the conduct of the trial, several drug batches were produced. The batch for the Phase I trial was synthesized in December 2017 (Lot no. 17L12/9). The first batch for the Phase II trial was produced in March 2018 (Lot no. 18C07). In all products containing SA, the SA was completely solved. The expiration date of this batch was January 2019. According to protocol, the subjects are supposed to apply the treatment for a maximum period of 12 weeks. Based on this information, the last subject to enter the study should have been enrolled by the 8th of November 2018 in order to complete the treatment phase of the trial by the 31st of January 2019. Unfortunately, due to the above-discussed insufficient recruitment progress, only 98 subjects were enrolled by the 8th of November 2018. The remaining subjects had to receive a new drug batch with a longer expiry date. Due to delivery issues from the manufacturer, the components necessary for the second batch were only delivered by December 2018. This meant that some subjects already enrolled in the study in November 2018 using the first batch (i.e. Lot no. 18C07) and were then requested to continue their treatment with a second batch after January 2019 (i.e. Lot no. 19A21). This was the case for subjects OVW-SA2099-190. Subjects OVW-SA2191-260 were enrolled at the end of January 2019 and only received one batch depending on their allocation i.e. Lot no. 18L24 AV2-SA (study) or 18C07 D-carvone-SA (control).

All batches were analyzed via gas chromatography by the Pyrenessences Analyses laboratory (Belcaire, France). All study drug batches, those from Phase I as well as Phase II, exhibited correct AV2 concentrations. From the batches of the Phase I trial only batch lot no. 17L12A exhibited the expected SA concentration i.e. 17%SA (see Table 7). The analysis of Phase I batches demonstrated that if the AV2 concentration increases, the SA concentration in the final product decreases. The batches from Phase II exhibited variable SA concentrations. The subjects from group 1 (subjects OVW-SA2001-98, Lot no. 18C07) had comparable SA% in both control and study treatment (i.e. 6.44 and 6.79% SA respectively). The subjects from group 2 (subjects OVW-SA2099-190, Lot no. 18C07 and 19A21) and group 3 (subjects OVW-SA2191-260, Lot no. 18L24 AV2-SA and 18C07 D-carvone-SA) did not exhibit comparable SA% in control and study treatment. Furthermore,

seeing that subjects from group 2 received two different lot numbers with widely varying SA concentrations, the data of this group was not interpretable.

Table 7. *Gas chromatography results from batch analysis. Details of each batch considering composition, salicylic acid (SA) concentration, study phase, and subject application per batch.*

Batch Lot Nr.	Composition	SA Conc. (%)	Study Phase	Applied by Study Subjects
17L12A	25% AV2 - Salicylic acid	17,41	Phase I	1001-1003
17L12B	50% AV2 - Salicylic acid	8,06	Phase I	1004-1006
17L12C	100% AV2 -Salicylic acid	4,37	Phase I	1007-1009
18C07	100% AV2 - Salicylic acid	6,79	Phase II March 2018	2001-2098, 2099-2190
18C07	100% D- Carvone - Salicylic acid	6,44	Phase II March 2018	2001-2098, 2099-2190, 2191-2260
19A21	100% AV2 - Salicylic acid	3,38	Phase II Jan 2019	2099-2190
19A21	100% D-Carvone - Salicylic acid	0,49	Phase II Jan 2019	2099-2190
18L24	100% AV2 - Salicylic acid	0.45	Phase II Jan 2019	2191-2260

5.3 Conclusion

Based on these observations, it is of utmost importance for future studies to develop strong recruitment strategies prior to the onset of enrollment in order to avoid issues with prolonged study duration, drug expiry dates, and the use of multiple drug batches within one trial. Furthermore, in case of limited shelf life, drug production should only start in the final phase of recruitment. The batch analysis must be performed before distribution of each batch to study subjects, ensuring that any discrepancies with drug products can be identified in a timely manner. Detailed and meticulous stability studies on the final study product composition must be performed before the initiation of a clinical trial.

Chapter 6 OVW-SA001 Study Results

This chapter examines the study outcomes of the OVW-SA001 clinical trial such as wart clearance rates at different time points, safety and tolerability of the combination therapy, the study population-specific socio-demographics, wart characteristics, and HPV type-specific prevalences. In addition, a detailed epidemiological profile of a persistent wart is provided that can potentially be employed in the clinical setting as a wart triage tool. Lastly, selected case reports are discussed that suggest a prominent effect of the AV2-SA combination therapy in the treatment of persistent warts.

Part of this chapter was published as a research article in Scientific Reports (Volume 13).

Reference: Redzic N, Pereira AR, Menon S, Bogers J, Coppens A, Kehoe K, Vanden Broeck D. Characterization of type-specific HPV prevalence in a population of persistent cutaneous warts in Flanders, Belgium. Sci Rep. 2023 Oct 15;13(1):17492.

6.1 Primary Outcomes

Due to the complications encountered during the conduct of the trial (described in Chapter 5), primary outcome analysis of the study data could not be performed. However, some general statements about the population could be made.

6.1.1 Clearance at 12 Weeks

15/260 (6%) patients, both from the study (n=9) and control (n=6) treatment arms, were able to clear their index lesions at 12 weeks after enrollment. Only one patient (1/15), participant number OVW-SA2136, experienced a recurrence of the index lesions 6 months after study enrollment i.e. the index lesion was cleared during follow-up at 12 weeks but became visible again at the 6-month follow-up. The participant in question received study drug treatment batch 18C07 and 19A21 (SA concentrations of 6.79-3.38%, see Chapter 5 Tabel 7).

6.1.2 Clearance at 6 Months

233/260 participants completed the study until the last follow-up, resulting in a drop-out rate of 10%. As described in Chapter 5, not all data could be used for the determination of AV2 efficacy as compared to control treatment due to differences in SA concentrations between the two treatment arms (see Chapter 5.2 for more details). Only data from participants who received Lot No. 18CO7 and fully completed the study (n=115), is suitable for this determination. Although the study treatment arm in this subgroup of participants did exhibit greater wart clearance (13/41; 32%) as compared to the control treatment group (16/74; 22%), this difference was not statistically significant ($\chi 2 = 1.411$, $p = 0.235$).

When comparing clearance rates between participants from group 1 (13/41; 32%; subjects OVW-SA2001-98, Lot no. 18C07) and group 3 (4/32; 13%; subjects OVW-SA2191-260, Lot no. 18L24) that received study drug treatment and fully completed the study, again a non-significant variance is found although a trend towards significance could be observed ($\chi 2 = 3.661$, $p = 0.056$). Seeing that the AV2 concentrations in these batches are comparable, the only distinction in the formulation is the SA concentration. These data indicate an almost significant impact of higher SA

concentrations on wart clearance, suggesting that the observed clearance rates could merely be attributed to higher SA concentrations.

A literature review on the efficacy of low-concentration SA treatments in cutaneous warts revealed only one relevant short communication article by Dhar et al. (Dhar et al., 1994). A total of 100 subjects participated in this particular study and treatment consisted of 16.5% SA, applied two times weekly for a total duration of three months. At 3 months 60/100 (60%) participants had cleared their warts and 53/100 (53%) were still wart-clear at 6 months follow-up. These are quite high clearance rates; however, they can be attributed to the study-specific population characteristics, seeing that firstly most patients have not received any treatment before study enrollment (60/100; 60%), secondly wart sizes in this study varied from 1 to 10 mm with a mean wart size of 4.8 mm and thirdly none of the patients had mosaic plantar warts, which are known to be most resistant to treatment. In comparison, in the OVW-SA001 trial the majority of the patients did have previous treatment history (247/269; 92%), the average warts size was 9 mm, and 113/269 (42%) of index warts were verruca plantaris mosaic. Considering the extensive enrichment of the OVW-SA001 study population with highly persistent warts, it can be concluded that the difference in efficacy between the different groups is not merely due to variations in SA concentrations. Furthermore, as previously hypothesized, AV2 seems to indeed require an additional ablative treatment to be effective, reach the basal cell layers, and execute its mode of action (as demonstrated by the higher efficiency of AV2 treatment in combination with higher SA concentrations).

6.2 Secondary Outcomes

Furthermore, due to the complications described in Chapter 5, only certain secondary outcomes could be assessed.

6.2.1 Safety and Tolerability of AV2-SA Therapy

Overall, no serious adverse events were observed during the trial conduct. The most common mild to moderate adverse events with their respective frequencies in the study population are depicted in Table 8. In case of an adverse event, subjects were advised to interrupt treatment for a few days, allowing the affected skin to heal completely followed by afterward resuming treatment as regular. Only one patient (i.e. OVW-SA2130) was advised to stop treatment prematurely at 9 weeks instead of 12 weeks, due to reoccurring adverse events that started mild but increased in severity after resuming treatment. These adverse events included redness of the skin, skin blistering, formation of rough-dry skin, and pain. Considering that this patient received the control treatment, all adverse events could be attributed to the use of SA. There were no substantial differences in the severity or frequency of adverse events between the two treatment arms. Based on this data it can be concluded that the AV2-SA therapy is a safe and well-tolerated topical treatment for cutaneous warts.

Table 8. *Adverse events. An overview of all the adverse events observed in the OVW-SA001 study population during the conduct of the trial, with the number of participants experiencing the event and the frequency of each event in the study population. All adverse events were experienced with mild to moderate severity.*

Adverse event	No. of participants experiencing adverse events (n)	Frequency of adverse events in the study population (%)
Redness	8	3.0
Pain	5	3.0
Itching sensation	6	2.2
Skin blistering	6	2.2
Skin rash	4	1.5
Rough, dry skin	2	0.7
Eyes itching and crying	1	0.4
Fragrance hindrance	1	0.4
Burning sensation	1	0.4
Sensitive skin	1	0.4

6.2.2 Characterization Of Type-specific HPV Prevalence In A Population Of Persistent Cutaneous Warts In Flanders, Belgium

The results of this secondary outcome were published as a research article in Scientific Reports (Volume 13).

Reference: Redzic N, Pereira AR, Menon S, Bogers J, Coppens A, Kehoe K, Vanden Broeck D. Characterization of type-specific HPV prevalence in a population of persistent cutaneous warts in Flanders, Belgium. Sci Rep. 2023 Oct 15;13(1):17492.

Characterization of type-specific HPV prevalence in a population of persistent cutaneous warts in Flanders, Belgium

Nina Redzic, A. Rita Pereira, Sonia Menon, Johannes Bogers, Astrid Coppens, Kaat Kehoe & Davy Vanden Broeck

Abstract

Cutaneous warts are benign skin lesions caused by the human papillomavirus (HPV). Even though they are considered benign, they can have a considerable impact on the quality of life and cause serious illness in certain immunocompromised populations. Studies have shown that the efficacy of wart treatment is dependent on the causative HPV type. Therefore, in this article, we aim to determine the HPV genotype-specific prevalence in cutaneous warts of a Flemish population as part of the Omnivirol- Salycilic acid randomized controlled trial. Swab samples of cutaneous warts (n = 269) were collected during enrollment. The DNA extraction was performed on the automated NucliSENS® easyMAG® system (bioMérieux). The samples were analyzed with two separate in-house PCR assays capable of detecting the most prevalent cutaneous HPV types (i.e. wart-associated HPV qPCR) as well as the most relevant mucosal types (i.e. RIATOL qPCR assay). In total, the type-specific prevalence of 30 distinct HPV genotypes was determined. The beta-globin gene was used as a cellularity control and for viral load quantification. Data concerning wart persistence, previous treatment, wart type, and other relevant wart and patient characteristics was collected through a baseline questionnaire. The study population consisted mostly of persistent warts considering that 98% (n = 263) of the sampled skin lesions were older than six months and 92% (n = 247) had undergone previous treatment. The most prominent wart type was the mosaic verruca plantaris (42%, n = 113). The most prevalent HPV types were cutaneous HPV types 27 (73%, n = 195), 57 (63%, n = 169), and 2 (42%, n = 113). Only 2% (n = 6) of the lesions was HPV negative. The highest median viral loads were observed with HPV27 and 57 (i.e. 6.29E+04 and 7.47E+01 viral copies per cell respectively). The multivariate analysis found significant associations between wart persistence and certain wart types, the number of warts, and HPV genotypes. Based on these findings, persistent warts are more likely to: (1) be verruca vulgaris, verruca plantaris simple or mosaic, (2) to manifest as multiple warts, (3) and to be negative for HPV type 2 or 4. These characteristics can be useful in the clinical setting for future risk stratification when considering treatment triage and management.

Trial registration: NCT05862441, 17/05/2023 (retrospectively registered).

Abbreviations

AIC	Akaike information criterion
BIC	Bayesian Information Criterion
HPV	Human Papilloma Virus
HR-HPV	High-Risk Human Papilloma Virus
IQR	Inter Quartile Range
LR-HPV	Low-Risk Human Papilloma Virus
OVW-SA	Omnivirol-Salicylic acid combination therapy for cutaneous warts
qPCR	Real-Time Polymerase Chain Reaction
VIF	Variance Inflation Factor

Cutaneous warts are benign lesions of the skin, commonly found and widespread among the population, affecting both children (30%) and adults (0.84%-12.9%), with a typically long duration of disease (approx. two years)[1,2]. Clinical presentations of warts show a wide variety, including dome shaped keratotic lesions with exophytic growth, endophytic keratotic papules, and flat-topped papules[3,4]. Depending on their appearance and location, warts are categorized into different types, comprising common warts (verrucae vulgaris), plantar warts (verrucae plantaris simple and mosaic), filiform warts (verrucae filiformis) and plane warts (verrucae plana)[5]. Warts can be found highly prevalent among the population, enriched in children and immunocompromised patients[6–8]. Roughly one-third of schoolchildren have warts, of which the majority clear spontaneously within two years[8,9]. Due to the discomfort they cause, patients often present themselves for treatment rather than waiting for spontaneous clearance[10]. Common first line treatments sought out by affected individuals, include folk remedies (e.g. garlic, thuja tincture, taping) and over-the-counter acids in low concentrations (e.g. salicylic acid, lactic acid, acetic acid). These treatments always require long-term application, often with unsatisfactory results, which is why patients frequently resort to second line treatment administered by a healthcare professional, including cryotherapy, higher concentrations of salicylic acid, excision or locally applied immunomodulatory or antimitotic drugs (e.g. imiquimod, bleomycin, fluorouracil)[11–15]. Despite long-term, intensive treatment schemes, patients are often confronted with the recurrence of warts, inducing frustration and mental fatigue[16].

A systematic review by the Cochrane Skin Group assessed the effects of different treatments for cutaneous warts. It concluded that the rate of clearance remains highly uncertain even after treatment, hereby influencing practitioners to limit treatment efforts and instead opt for a more expectant approach[17]. In concordance with this systematic review, Kuwabara et al. found that 80% of warts do resolve spontaneously within 4 years, regardless if they have been treated or not[18]. However, this still means that the remaining 20% of warts are strongly persistent. Currently, there is no reliable mean of predicting which warts will clear spontaneously and which will remain persistent for years.

Cutaneous warts are caused by infection of the skin with human papillomavirus (HPV), mostly known as the cancer-causing virus, capable of inducing cervical cancer[19]. The HPV family comprises over 200 genotypes, classified into different types according to their DNA sequence[20]. The most prevalent HPV types found in warts are HPV 1, 2, 3, 4, 7, 10, 27, 41, 57, 60, 63 and 65[3,21–24]. A noteworthy study by Bruggink et al. explored the presence of selected HPV genotypes in relation to natural course and treatment response and provided early insights in the causal relationship between HPV genotype and treatment efficacy/lesion resolution[25]. However, the authors were not able to formulate statements about most of the tested HPV types, due to absence of sufficient numbers per HPV type in different treatment groups. Other studies have equally suggested that the efficacy of a wart treatment could be dependent on the causative HPV type, hence indicating a role for HPV genotyping in clinical management and therapy selection[26,27]. Combining insights from these diverse explorations, a single-center randomized controlled trial, i.e. Omnivirol-Salicylic acid combination therapy for cutaneous warts

(OVW-SA), was designed to explore further the predictive value of HPV genotyping in relation to treatment efficacy and time to clearance[28].

This article aims to present baseline findings of the study population included in the OVW-SA trial conducted in Flanders, Belgium, comprising complex interrelationships between patient-specific features as well as wart-specific characteristics, including the most extensive HPV genotyping performed on wart swab samples to date. Based on these findings a detailed epidemiological profile of a typical persistent wart is devised.

Methods

Setting and population

Patients were included as part of the OVW-SA001 clinical trial conducted in a Belgian population between 2018 and 2019[29]. The study consisted of a double-blind, single-centered, randomized clinical trial that was conducted by the University of Antwerp, with Sonic Healthcare BeNeLux (Antwerp, Belgium) operating as the central laboratory. Ethical approval was obtained from the Ethical Review Board of the University of Antwerp (B300201734040). The study was conducted in accordance with applicable national regulations, Good Clinical Practice (2005/28/EC)[30] and the Declaration of Helsinki[31]. Informed consent was obtained from all participants prior to study entry. In case of minors, informed consent was obtained from legal guardians before study participation. Recruitment was done via flyers distributed via dermatologists, pharmacists and via own initiatives aiming to reach an immunocompetent population exhibiting one or more cutaneous warts, aged 12 years or older, and agreeing to sign informed consent written in Dutch. The study area comprised the Flemish region with enrichment in the Antwerp metropolitan area.

Study design

In total 269 patients were included, fulfilling inclusion criteria, hereby reaching precalculated statistical power. At inclusion, data concerning wart duration, previous treatment, wart type and other relevant wart and patient characteristics were collected through a structured baseline questionnaire (Additional File 1). Full details of clinical trial design and methodology are provided in Redzic et al.[28].

Study procedures

At inclusion, a photograph of the index wart (i.e. the largest wart) was taken, followed by sampling with a Floq swab (FLOQSwab Copan Diagnostics, Murietta, California, USA). Sample-taking procedures and processing details were done as described by Redzic et al.[32]. The sample was stored in Abbott multi-Collect medium (Multi-Collect Specimen Collection Kit, Abbott Molecular Inc., Des Plaines, Illinois, USA) and kept at 4 °C prior to further processing. Briefly, DNA extraction was performed according to the optimized method for cutaneous samples on the automated NucliSens easyMAG platform (bioMérieux, Boxtel, The Netherlands). The samples were analyzed for the presence of HPV DNA by the in-house developed wart-associated cutaneous HPV assay, capable of detecting the abovementioned 12 distinct cutaneous HPV types (i.e. HPV 1, 2, 3, 4, 7, 10, 27, 41, 57, 60, 63, 65)[33], as well as the RIATOL HPV genotyping qPCR assay, capable

of detecting mucosal HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 50, 51, 52, 53, 56, 59, 66, 67 and 68[34]. The RIATOL qPCR assay is a fully validated HPV test, used to conduct primary HPV screening for cervical cancer[35,36]. This study used the assay without clinical cut-off at its full analytical potential. Both assays allow for quantitative measurement of viral load after calibration with synthetic gBlock gene fragments (Integrated DNA Technologies, Coralville, Iowa, USA). Sample adequacy (cellularity control) was assessed via amplification of beta-globin, a housekeeping gene, which was similarly applied as a measure of the number of cells present essential for subsequent viral load calculations. In summary, all samples were both qualitatively as well as quantitively tested for the presence of 30 distinct HPV types. A detailed description of assay design and validation can equally be found in Redzic et al.[33].

Data management and analysis

Data management and analysis were conducted using STATA version 17 (Corporation, College Station, Texas, USA). In order to summarize data regarding patient- and wart-specific characteristics descriptive statistics such as numbers and percentages with 95% CIs were applied for categorical variables, and arithmetic mean, standard deviation, and median were used for numerical variables. The presence of any detectable viral load of a specific HPV type was considered diagnostic for infection with that specific type.

Kruskal–Wallis test was used to compare patients' age, number of warts, number of multiple infections and estimated viral loads between different HPV types. Student T-test was used to assesses the number of warts between the two genders and differences in viral load between single and multiple infections. Results were considered statistically significant at P-value ≤ 0.05.

Potential predictors of wart persistence were assessed via a multiple logistic regression model, in which wart persistence was defined as index wart duration of more than 6 months combined with resistance to previous treatment. Several variables were created for analysis. Age was dichotomized into two categories: ≥ 13 years and < 12 years, as patients younger than 12 years are known to have higher rates of wart clearance[37,38]. The number of HPV infections was treated as a categorical variable, with no HPV as the baseline category, one HPV and two or more HPV multiple infections as other categories. The number of warts was categorized as 1 (baseline) versus 2 or more warts. The type of warts variable was divided into four categories, with verruca plantaris mosaic as the baseline, verruca plantaris simple, verruca vulgaris, and "others" (i.e. verruca plana and filiformis). For the univariate analysis, logistic regression was fitted to measure the strength of the association of potential covariates. For model building, all predictors that had a P-value of less than 0.2 were considered, in addition to the potential confounders hypothesized to be of importance: the type of wart, the number of warts, the number of infections and specific cutaneous HPV types that had more than 10 observations (i.e. HPV 1, 2, 3, 4, 10, 27, 57, 63, and 65).

As no other predictors were identified, a multivariable logistic regression analysis was performed to simultaneously control for potential confounders. For variables that were not dichotomized, interaction terms were fitted, and linear assumptions were tested. Both the Akaike information criterion (AIC) and the Bayesian Information Criterion (BIC) were used as two different measures

of model fit, along with the Variance Inflation Factor (VIF), to detect multicollinearity to ensure that yielded values were within acceptable ranges[39].

To obviate problems related to the stability of parameter estimates that arose for the variable HPV 63 due to the flatness of the likelihood method, the Penalization likelihood method was used instead of the standard Maximum likelihood method[40].

Ethics approval and consent to participate

Ethical approval obtained from ethics committee of Antwerp University Hospital, B300201734040. All participants gave written informed consent prior to entry into the study.

Results

Socio-demographics and wart-characteristics

Figure 1A provides an overview of the geographical locations were all the study samples originated from, with most samples (89%) being collected from the Antwerp metropolitan region. A summary of the population- and wart-specific characteristics can be found in Table 1. Median age of participants was 39 (7–76) years for males and 38 (12–82) years for females, and 60.2% of the population was female. At study enrollment, 66 patients (24.5%) had one wart, 156 (58.0%) had 2–10 warts and 47 (17.5%) had more than 10 warts.

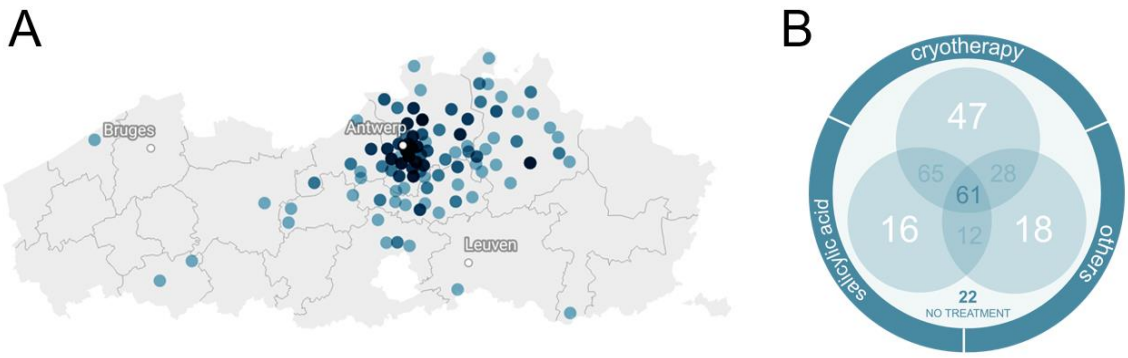

Figure 1. (A) Origin of samples collected during the OVW-SA001 clinical trial. Darker blue colors represent higher density of collections per geographical area. **(B)** Index wart treatment history. The Veen-diagram depicts the number of patients that applied different types of treatments on their index warts. In summary, 22 index warts did not have any previous treatment, while cryotherapy was the primary treatment for most index warts (n=201), followed by salicylic acid (n=154) and various other types of therapy (n=119). The most common treatment combination was cryotherapy combined with salicylic acid (n=65).

All subjects are represented in Fig. 2 by the number of warts at the time of enrollment per age group and gender. There was no significant difference in the number of warts between the two genders (Student T-test $P > 0.05$). However, a significantly larger number of warts was found in the age groups ≤ 15 and 16–25 (Kruskal Wallis $P < 0.005$), suggesting that adolescents and young adults on average, exhibit a higher number of warts. The highest median number of warts was detected in male patients ≤ 15 years old (i.e. 13.5 warts per subject). No significant difference in the number of HPV multiple infections between different age groups was found (Kruskal–Wallis $P > 0.05$).

Considering the location of index warts, 85 (31.6%) were located on the hands, while 176 (65.4%)

were located on the feet. Regarding index wart type, 89 (33.1%) of the index warts were verruca vulgaris, 113 (42.0%) were verruca plantaris mosaic, 60 (22.3%) were verruca plantaris simple, 6 (2.2%) were verruca plana and 1 (0.4%) was verruca filiformis. The average index wart size was 9 mm. Data showed that 263 (97.8%) of the index warts were older than 6 months and 247 (91.8%) were already subjected to prior treatment, indicating enrichment of the study population with highly persistent warts. *In casu*, 154 (62.3%) patients tried previous treatment with salicylic acid, 201 (81.4%) with cryotherapy, and 119 (48.2%) resorted to other treatments (i.e. treatment with other acids, bleomycin injections, fluorouracil creams, chirurgical and laser removal, and more systemic approaches based on cimetidine, zinc or magnesium). Multiple treatments were registered for 166 (61.7%) patients with two or more different treatment types (Fig. 1B).

Table 1. Distribution of patient- and wart-specific characteristics among the study population.

Variable	**Number of patients** (n = 269)	**Percentage** (%)
Sex		
Male	107	39.8%
Female	162	60.2%
Age		
< 12 years	3	1.1%
12-18 years	53	19.7%
> 18 years	213	79.2%
Total number of warts		
1	66	24.5%
2-10	156	58.0%
> 10	47	17.5%
Index wart location		
Hands	85	31.6%
Feet	176	65.4%
Other	8	3.0%
Index wart type		
Verruca vulgaris	89	33.1%
Verruca plantaris mosaic	113	42.0%
Verruca plantaris simple	60	22.3%
Verruca plana	6	2.2%
Verruca filiformis	1	0.4%
Index wart duration		
< 6 months	6	2.2%
> 6 months	263	97.8%
Index wart previous treatment		
Yes	247	91.8%
No	22	8.2%

HPV type-specific prevalence

As regards to sample quality, 261 (97.0%) samples were considered valid as demonstrated by a sufficient B-globin amplification. Moreover, patients showed a high HPV positivity rate where only 6 (2.2%) lesions were HPV negative, while 58 (21.6%) were positive for a single HPV genotype, and 205 (76.2%) contained multiple HPV infections. A high level of multiple infections was observed in 135 (50.2%) cases harboring three or more HPV infections. The maximum number of multiple infections in one patient was seven. Figure 3A provides an illustration off all

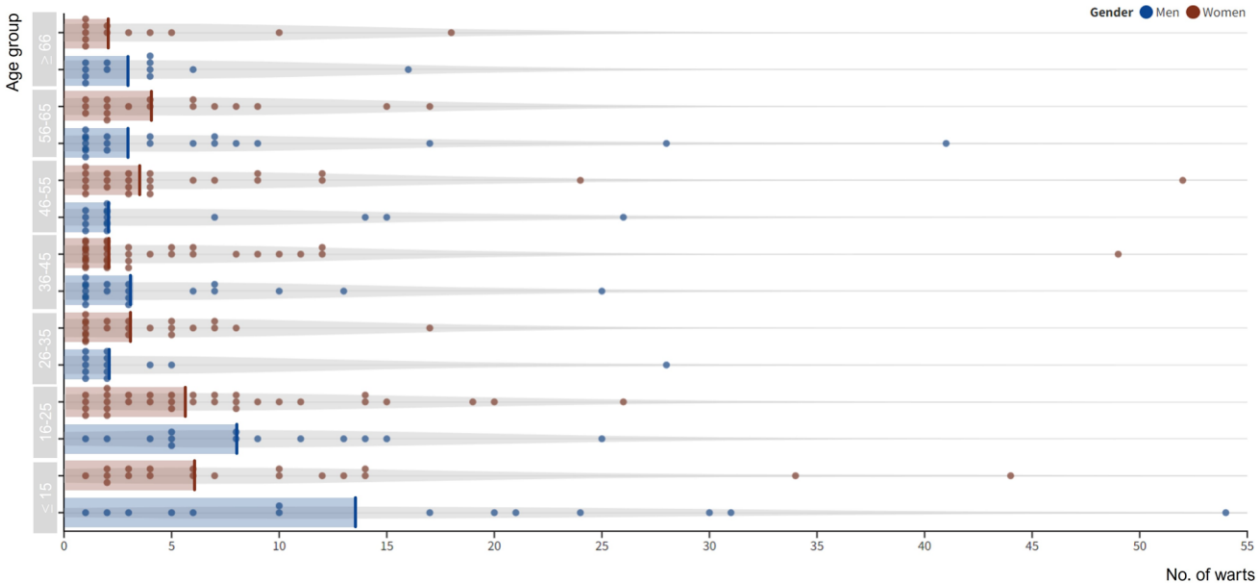

Figure 2. Number of warts per age group in the male and female population. Each study participant is depicted by a colored dot. Male patients are shown in blue, female patients in red. The number of patients per age group was respectively: 33 [≤15], 46 [16-25], 35 [26-35], 55 [36-45], 39 [46-55], 37 [56-65], and 24 [≥66]. The median number of warts per each age group is depicted by a colored line, with the highest median number of warts (13.5) found among patients aged ≤ 15 years. The highest number of warts per patient was found in age group ≤15 years and equaled to 54 warts in total.

subjects based on number of multiple infections per specific index wart type. The HPV multiple infection status is not dependent on wart type, seeing that similar distributions of number of infections can be found in all wart types identified. The most prevalent HPV types were cutaneous HPV types 27 (195/269; 72.5%), 57 (169/269; 62.5%) and 2 (113/269; 42.0%) (Fig. 3B). Only 2% (5/263) of HPV-positive warts were negative for HPV 2, 27 or 57. HPV 65 and 4 were present in 15–23% of samples, while HPV 1 and 10 in 10%, and HPV 3, 41, 60 and 63 in 3–5%. One cutaneous HPV type was not found in our population i.e. HPV 7. In general of all the cutaneous types, HPV 7 has the lowest reported prevalence (0.5%) and is often associated with meat handlers[27,41]. Mucosal HPV types were detected in 7% of lesions (18/269), more specifically high-risk HPV (HR-HPV) types 31, 39, 51, 52, 59, 66, possible HR-HPV 67, and low-risk HPV (LR-HPV) type 6 with respective prevalence ranging from 0.4–1.9% (Fig. 3B). Mucosal HPV types 16 and 18, most commonly associated with cervical cancer, were not found in this population (for more details on HPV type-specific prevalence see Additional file 1).

A full overview of genotype-specific prevalence can be found in Fig. 3B. Overall 26 (45%) warts were HPV 27 positive, 16 (28%) HPV 57, and 8 (14%) HPV 2. Other HPV types found in single infections were HPV 1, 3, 4, 10 and 31, with prevalence ranging from 2–5%. As outlined above, most warts displayed infection with multiple HPV types. Most common coinfections detected included HPV 27 and 57, and were observed in combination with at least one of the HPV types belonging to the alpha 4 (i.e. HPV 2, 27, 57), gamma 1 (i.e. HPV 4, 65) and/or gamma 4 species (i.e. HPV 60; Table 2)[20].

Viral loads ranged from 1.11E−03 to 9.31E+06 copies per cell and showed a type-specific manifestation (Table 3). As for cutaneous HPV types, highest median viral loads were observed

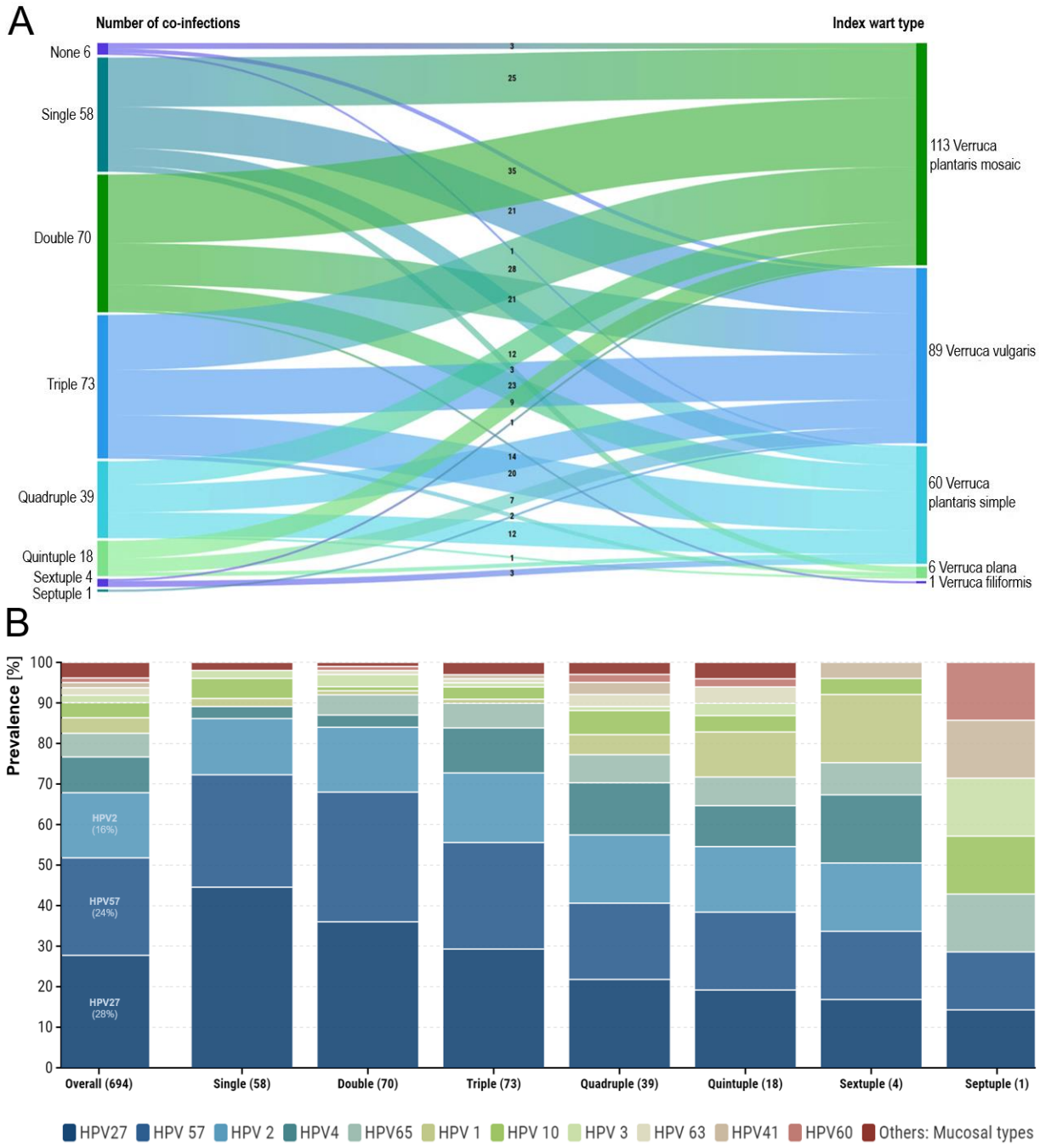

Figure 3. (A) Distribution of subjects based on their multiple infection status per specific index wart type. In summary, 25/58 (43%) of warts infected with a single HPV type were verruca plantaris mosaic, 21/58 (36%) verruca vulgaris, 9/58 (16%) verruca plantaris simple, 3/58 (5%) verruca plana. 35/70 (50%) of warts with double infections were verruca plantaris mosaic, 21/70 (30%) verruca vulgaris, 13/70 (19%) verruca plantaris simple, and 1/70 (1%) verruca filiformis. As regards to warts containing more than two distinct HPV types 50/135 (37%) were verruca plantaris mosaic, 45/135 (33%) verruca vulgaris, 37/135 (27%) verruca plantaris simple, and 3/135 (2%) verruca plana. **(B)** HPV type-specific prevalence in cutaneous warts. A total of 694 HPV infections was detected in the study population. Cutaneous HPV types 27 (28%, 195/694), 57 (24%, 169/694), and 2 (16%, 113/694) were the most commonly found types, with HPV type 7 being the exception, as it was not detected in this population. Certain mucosal HPV types (i.e. HPV 6, 31, 39, 51, 52, 53, 59, 66 and 68) were detected in low percentages (< 2%) and are depicted in group (red). The HPV type-specific distribution according to number of multiple infections is displayed as well with the number of subjects in each group between brackets.

Table 2. Most prevalent multiple infection trends in HPV-positive index warts.

Prevalent multiple infection trends	**Samples** (n=263)	**Percentage in population** (%)
Double infection		
HPV 2 and 27	11	4.1
HPV 2 and 57	8	3.0
HPV 27 and 57	32	11.9
Triple infection		
HPV 2, 27 and 57	25	9.3
HPV 27, 4 and 57	8	3.0
HPV 27, 4 and 65	5	1.9
Quadruple infection		
HPV 2, 4, 27 and 57	8	3.0

for HPV 27 (6.29E+04 copies per cell) and 57 (7.47E+01 copies per cell), and these differed significantly from viral loads of other types (Kruskal–Wallis P < 0.000001). Other genotypes displayed median viral loads ranging from 6.61E−02 to 2.39E+00 copies per cell for cutaneous types, and from 1.98E+01 to 6.09E+04 for mucosal types. The type-specific viral loads found in single infections did not significantly differ from those found in multiple infections within the same HPV type (Student T-test $P > 0.05$).

Predictors of wart persistence

After adjusting for all other covariates in the multivariate model, there were four significant associations with the persistence of the index wart (Table 4). Compared to the verruca plantaris mosaic, warts from the "others" category had 86% (95% CI 0.03–0.83) lesser odds of persistence, although the sample size was small (n = 7). Furthermore, as predicted, having two or more warts was significantly associated with the outcome, with patients harboring two or more warts having 2.65 higher odds (95% CI 1.07–6.55) of persistence than patients with one wart. Concerning HPV genotypes, only HPV 2, and HPV 4 were found to be statistically associated with the outcome, with their presence resulting in a respective decrease of 66% and 70% odds of index wart persistence.

Discussion

In this study, a vast prevalence of HPV was found in cutaneous warts, with only a limited number of warts identified as negative for the analyzed HPV genotypes (2%). From a pre-analytical perspective, our findings confirm the efficient collection of test samples via a non-invasive, swab-based technique. The efficiency of painless collection via surface swabs has been described rendering swab samples a reliable tool to test for viral presence in skin lesions[32,42,43]. It can be expected that by circumventing the need for invasive sample collection, the potential of HPV genotyping in cutaneous lesions will be more easily exploited as patient's reluctance to undergo testing will be substantially reduced as well as the need for highly experienced professionals to apply the technique[43].

Table 3. Viral load quantification of identified HPV types. The table depicts the number of index warts (n) positive for each HPV type and the median viral load per specific type. Viral load is expressed as number of viral copies per cell. Viral load inter quartile range (IQR) per type is likewise described.

HPV type	n	median viral load copy/cell (IQR)
Cutaneous		
HPV 1	27	1.87E-01 (5.16E-02 – 1.08E+00)
HPV 2	112	2.39E+00 (2.43E-01 – 4.64E+04)
HPV 3	12	1.21E-01 (3.59E-02 – 2.75E-01)
HPV 4	60	6.61E-02 (2.36E-02 – 7.63E-01)
HPV 7	-	NA
HPV 10	26	7.18E-01 (8.81E-02 – 3.28E+00)
HPV 27	193	6.29E+04 (7.40E+00 – 2.05E+05)
HPV 41	9	4.47E+01 (9.48E-02 – 3.18E+00)
HPV 57	167	7.47E+01 (3.50E+00 – 9.68E+04)
HPV 60	8	1.10E+00 (2.83E-01 – 3.20E+00)
HPV 63	12	1.11E-01 (6.09E-02 – 7.05E-01)
HPV 65	45	2.85E+00 (5.42E-01 – 2.96E+01)
Mucosal		
HPV 6	1	6.09E+04 (NA)
HPV39	2	3.66E+04 (1.86E+04 – 5.46E+04)
HPV51	4	1.55E+02 (4.79E+01 – 3.54E+02)
HPV52	1	1.47E+03 (NA)
HPV53	5	4.51E+01 (3.79E+01 – 5.20E+01)
HPV59	2	5.14E+01 (3.13E+01 – 7.14E+01)
HPV66	1	1.98E+01 (NA)
HPV67	1	3.69E+04 (NA)

In concordance, the observed high HPV positivity rate also confirms efficiency at the analytical level. The cutaneous wart-associated HPV genotyping assay was carefully designed to ensure maximum sensitivity[33], simultaneously allowing viral load determination. Due to this high sensitivity, HPV types were detected with high accuracy, resulting in the identification of at least one cutaneous HPV type in 97% of all samples, with HPV 27 (73%), 57 (63%) and 2 (42%) being the most prevalent types. This high cutaneous HPV type-specific prevalence further confirms that the most relevant HPV genotypes were selected and included in the cutaneous wart-associated HPV assay. Likewise, the RIATOL qPCR HPV assay, capable of detecting mucosal HPV types, has been exploited at full analytical potential without the use of the previously described clinical cut-offfor primary cervical cancer[35]. This assay is highly sensitive and allows viral load determination even at low cell counts. The high sensitivity of the employed assays correspondingly explains the high prevalence of multiple infections (76.2%), which is in contrast with current literature reporting only 4–46% multiple infections in immunocompetent subjects using similar sampling techniques[27,42,44,45]. However, Schmitt et al. did report the highest prevalence of multiple infections in swab samples currently published (46%), and confirmed that use of a more sensitive method leads to a superior ability to detect multiple HPV infections in the same swab sample.

Table 4. Association of several covariates with wart persistence: multivariable logistic regression analysis.

Variable	Unadjusted odds ratio (95%CI)	p-value	Adjusted odds ratio (95%CI)	p-value
Index wart type	baseline			
Verruca vulgaris mosaic				
Verruca vulgaris	0.50 (0.17-1.46)	0.203	0.75 (0.26-2.20)	0.606
Verruca plantaris simple	0.28 (0.10-0.81)	0.019	0.49 (0.16-1.50)	0.211
Other	0.14 (0.02-0.88)	0.036	0.14 (0.03-0.83)	0.030
Number of warts				
1	baseline			
≥2	2.34 (1.02-5.33)	0.044	2.65 (1.07-6.55)	0.035
Number of infections				
0	baseline			
1	2.12(0.21-21.89)	0.530	3.11 (0.36-26.87)	0.302
>2	1.75(0.19-15.72)	0.612	3.93 (0.31-50.52)	0.293
Cutaneous HPV type				
HPV 1	0.60 (0.19-1.90)	0.390	0.62 (0.18-2.17)	0.459
HPV 2	0.38 (0.17-0.88)	0.024	0.34 (0.12-0.99)	0.048
HPV 3	1.36 (0.17-10.86)	0.770	0.66 (0.08-5.07)	0.693
HPV 4	0.33 (0.14-0.74)	0.007	0.30 (0.11-0.79)	0.015
HPV 10	1.38(0.32-6.12)	0.680	1.61 (0.35-7.46)	0.541
HPV 27	1.36 (0.58-3.18)	0.480	1.36 (0.48-3.85)	0.562
HPV 57	1.40 (0.63-3.13)	0.410	1.26 (0.47-3.35)	0.644
HPV 63	3.20 (0.19-55.94)	0.420	2.20 (0.12-42.03)	0.600
HPV 65	1.04 (0.34-3.18)	0.940	1.30 (0.38-4.51)	0.676

Thus far, the largest study regarding HPV prevalence in cutaneous warts was conducted by Bruggink et al. and comprised 744 warts of 246 immunocompetent patients[27]. The lower prevalence of multiple infections found, can be explained by the fact that in their study individual warts are considered as the unit of analysis instead of patients i.e. multiple warts of the same patients were sampled for further HPV analysis, propagating therefore repetitive results in the population. Furthermore, exclusively 'new' warts were included in their study, defined as warts without any prior treatment from a general practitioner or dermatologist[27]. Considering that in the current study, 92% of subjects were already subjected to prior treatment, the high percentage of multiple infections could be an indicator of persistence. In addition, Bruggink et al. excluded mosaic warts with a diameter of ≥ 1 cm from their study, while this type of warts compromised 20% (n = 55) of our population[27].

To the best of our knowledge, the most comprehensive study exploring HPV genotyping and including a viral load component was the study conducted by Skubic et al.[46]. In contrast, the latter study focused on histological specimens, hereby generating strong evidence on genotype distribution and investigating the role of HPV viral load in cutaneous warts[46]. Our findings, obtained by the collection of cutaneous swabs, largely confirm the findings by Skubic et al., showing the highest prevalence of HPV 27 and 57, with comparable levels of viral load distribution

as well, despite essentially different starting material. Based on observations by de Koning et al. as well as Garcia-Oreja et al., high concordance between cutaneous swabs and biopsies can be expected, thus strengthening findings from this study[42,43].

In this population, HPV 27 and 57 exhibited significantly higher viral loads in comparison to other types, correlating with high viral shedding and associated highest infectious potential, which is translated in highest prevalence in the study population[42,47,48].

The multivariate analysis found significant associations between wart persistence and certain wart types, number of warts and HPV genotypes. Having verruca plana or filiformis significantly decreased the risk of persistence in comparison to verruca plantaris mosaic. The mosaic wart is already recognized as the most persistent wart type and was as well enriched in this study population (42%)[37]. Number of warts being a risk factor for persistence is to be expected, knowing that persistent warts are resistant to treatment and, by definition, have a duration longer than 6 months, which increases the risk of autoinoculation and transmission of the HPV infection from one body site to another. As regards to the role of HPV genotyping, seeing that the high viral load of HPV 27 and 57 results in a subsequent high infectious potential, these types appear to be omnipresent in the population, not only in persistent warts but also in warts that are defined as not persistent[3,27,46]. This finding makes them an inadequate predictor of risk. However, the logistical regression did show that some HPV types are in fact, less likely to cause persistent warts i.e. HPV 2 and 4 (OR 0.38 and 0.31 respectively). HPV 4 has previously been identified by Bruggink et al. as having the most favorable natural course in plantar warts (cure rate 94% (95% CI 73–99%))[25]. HPV 2, however, did not have a promising natural course in their analysis (cure rate 0% (95% CI 0–26) in plantar warts and 3% (95% CI 0–16) in common warts). Claims about cure rates of other HPV types were also not possible, due to a lack of sufficient numbers per HPV type in different treatment groups. Despite certain discrepancies, our findings together with Bruggink et al. do consolidate the future applicability of HPV genotyping in clinical management of cutaneous warts.

Conclusions

Given the high prevalence of HPV types in cutaneous lesions, and their relationship with the clinical manifestation of warts, HPV genotyping with viral load determination can be of added value in daily clinical practice. Combined with the knowledge that the persistence of a lesion is linked to both the HPV genotype, wart type and number of warts, risk assessment can be performed, and therapeutical options can be suggested. In case that the above-defined risk factors are present in a subject, a more aggressive treatment approach can be applied in order to inhibit not only the spread of the infection in the general population but also further autoinoculation. Previous studies are in line with these findings. However, further research is needed to compose a comprehensive tool to guide clinicians in wart treatment and/or follow-up. For a more extensive assessment of HPV-dependent wart persistence, upcoming studies must entail large clinical trials comprising not only different treatment groups (e.g. salicylic acid, cryotherapy and wait-and-see) but also ensuring a sufficient HPV type-specific allocation in each group. Only then, a head-to-head comparison of different genotypes is possible, creating the possibility to make distinct claims about treatment response and natural course based on HPV

type. Nevertheless, the present study already provides certain insights in possible predictors of wart persistence and encourages a more patient-centered and directed approach to wart risk and treatment stratification.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by N.R. Statistical multivariate analysis was performed by S.M. The first draft of the manuscriptwas written by N.R. and D.V.B. All authors commented on previous versions of the manuscript. All authors readand approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Supplementary information

Table 1: Details of outcome measures and data collection forms used in the OVW-SA001 clinical trial. The majority of the outcomes are responses to questions that require a 'Yes' or 'No' answer or open comments, except for pain which was measured by a 0-4 numeric pain rating scale.

Measure	Source: Content
Eligibility	**Inclusion Criteria**: patient exhibiting one or more cutaneous warts; aged 12 years or older; agrees to refrain from using prescription or supplemental antiviral medications without first obtaining permission of the coordinating trial dermatologist; able to read Dutch; signed informed consent; able to self-assess and use WhatsApp for follow-up (all Y/N). **Exclusion Criteria**: patient only exhibiting facial and/or seborrheic warts; not suitable for salicylic acid (SA) treatment due to a medical history of severe diseases (e.g. hepatitis, renal or liver dysfunction, cardiovascular, or gastrointestinal disorders, etc.), impaired healing or neuropathy (e.g. due to diabetes, peripheral vascular disease or any other condition); known or suspected allergic or adverse response to SA, AV2 or its components; immunocompromised patient; patient had already participated in another clinical trial concerning treatment for cutaneous warts within six months before enrolment in this study or is currently in a trial evaluating other treatments for his/her warts (all Y/N).
Demographic Details	**Baseline Questionnaire**: date of birth; sex (M/F); postal code.
Wart anamnesis	**Baseline Questionnaire**: General History: number of warts; wart type (verruca vulgaris, verruca plantaris mosaic or simple, verruca plana, verruca filiformis); average size (mm); location; duration (<6months, >6months); previous treatment (Y/N, if yes specify). Index Wart History: wart type; average size (mm); location; duration (<6months, >6months).
Efficacy Of Treatment	**Follow-Up Questionnaire:** Index wart: clearance (Y/N) → if cleared date of clearance; if not cleared potential recurrence Y/N. Other warts: still present (Y/N) → if not present date of clearance; if present number of warts and location (inside a radius of 0.5cm around the original position/another position = 'new warts' → if new warts, inside a radius of 3cm around the index lesion (Y/N; if yes number of warts).
Side Effects Of Treatment	**Follow-Up Questionnaire:** pain scores (numeric pain rating scale 0=no pain at all – 4=extreme level of pain); another side effects (Y/N, if yes specify).
Treatment Compliance	**Follow-Up Questionnaire:** use of additional treatments (Y/N; if yes specify); comments about treatment (open text).
Photograph-Based Outcome Assessment	**Photograph-Based Assessment Form:** Study Coordinator: date at which the photograph is taken; treatment day; index wart cleared (Y/N); size of index wart (mm). Dedicated Physician: photograph interpretable (Y/N); index wart cleared (Y/N); remarks (open text).
HPV Genotyping	**Laboratory Form:** date at which the sample is taken; treatment day; HPV (pos/neg; if pos specify HPV type(s)).

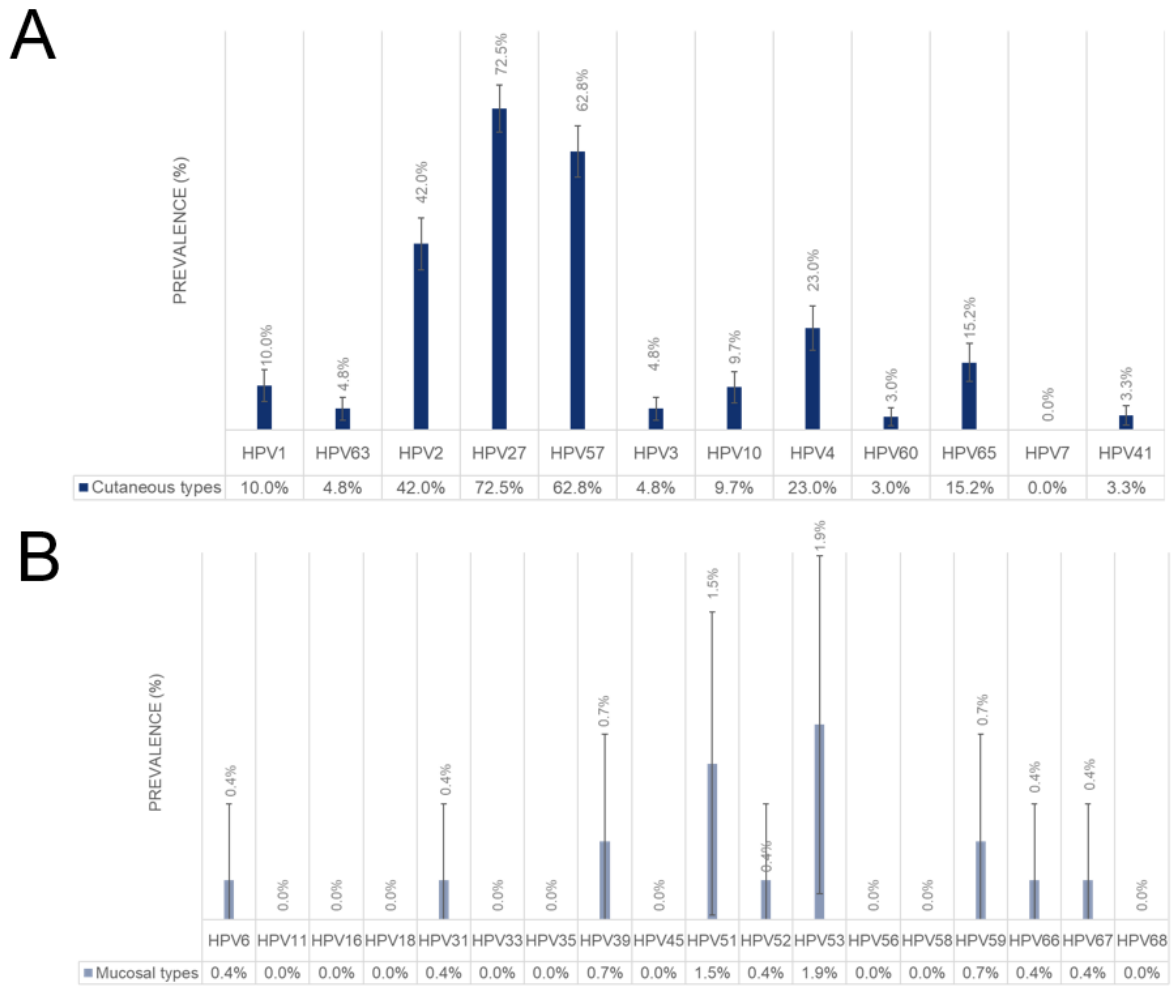
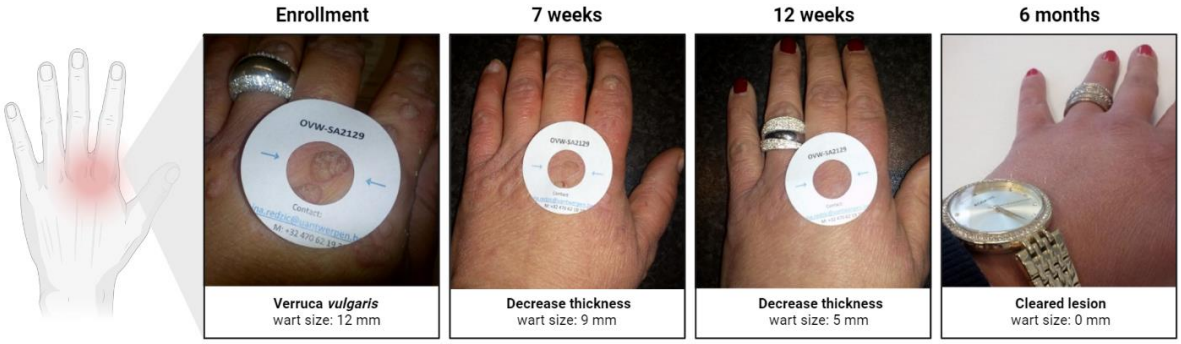


Figure 1. HPV type-specific prevalence in cutaneous warts with 95% Confidence Intervals: **(A)** cutaneous HPV types and **(B)** mucosal HPV types.

6.3 Case Reports

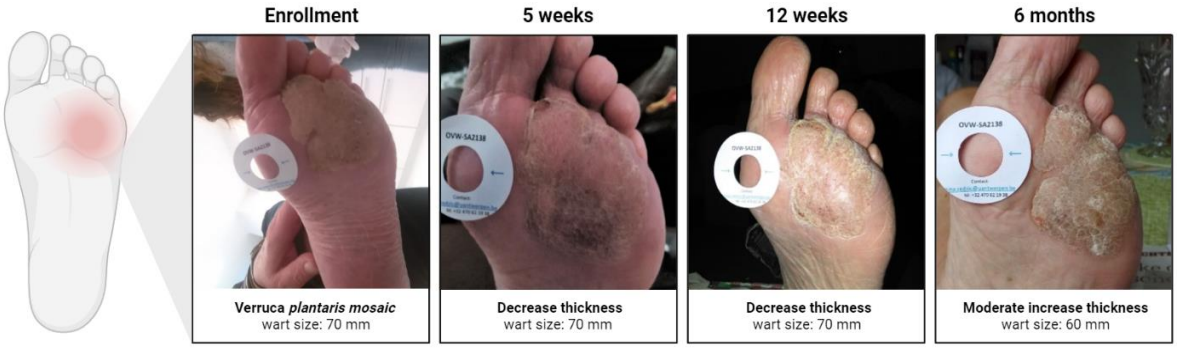
6.3.1 OVW-SA2129



A 42-year-old woman presented with 49 individual warts on both hands. All warts were verruca vulgaris with an average wart size of 7 mm. The index wart size was 12 mm. The warts have been present for already more than 5 years and were resistant to numerous both topical (i.e. over-the-counter Wartner Wart & Verruca pen with trichloroacetic acid, 40% SA ointment and cryotherapy) as well as systemic (i.e. cimetidine and zinc) treatments. The patient worked in a retail store, where she had daily contact with customers and due to the visible location and abundant number of lesions, was ashamed of her condition. The patient performed daily taping of all of her warts to keep them obscured, a very cumbersome and time-consuming task with a serious impact on her quality of life. The patient initiated the daily application of study treatment with weekly spray at the time of enrollment, and after 7 weeks of treatment, visible changes in wart appearance were observed i.e. a decrease in thickness of lesions was evident. After 12 weeks the lesions were almost smooth, but the skin lines were not completely restored yet. Treatment was stopped as instructed by protocol and at 6 months after enrolment skin lines were completely healed with no remaining lesions on both hands. The patient was part of group 2 (subjects OVW-SA2099-190), the group that received two batches i.e. Lot no. 18C07 and 19A21 (for more details see Chapter 5.2.2 Product Stability). After unblinding, it became apparent that the patient received the study drug treatment i.e. AV2-SA. Considering the low SA concentration (6,79 – 3.38%) in the batches received and rather extensive patient treatment history with SA solutions to 7 times more concentrated than the one received

during the study, it can be concluded that the treatment effect observed in this patient is attributable to AV2 treatment and that this patient benefited significantly from the AV2-SA combination therapy.

6.3.2 OVW-SA2129



A 66-year-old man presented with 4 individual warts on both feet. All warts were verruca plantaris mosaic with an average wart size of 40 mm and an astonishing index wart size of 70 mm and thickness of 15 mm. The warts were present for more than 3 years and were resistant to numerous very extensive topical treatments simultaneously applied. The patient's treatment schedule consisted of biweekly visits to the dermatology practice where cryotherapy was administered, followed by the excision of as much tissue as possible and the application of Cantharone (0.7% cantharidin, a natural toxin that possesses both blistering and keratolytic effects). After treatment by the dermatologist, the patient continued treatment at home by applying daily over-the-counter 17% SA solution and taking a disinfecting foot bath. The patient had already employed this treatment approach for more than 3 months without any visible effect. Not only did the patient had to endure a rather intensive treatment on a frequent basis, but he also experienced pain while walking and could not travel long distances by foot resulting in an impaired quality of life. The patient initiated daily application of study treatment with weekly spray as described in study protocol at the time of enrollment and after only 5 weeks of treatment already visible treatment effects were observed i.e. the thickness of the index wart decreased considerably. At 12 weeks of treatment, the thickness of the index wart was even less while

the size still stayed constant. Although the index wart was not completely cleared, the patient had to end treatment per study protocol. 6 months after enrolment the index wart again visibly gained in thickness. The patient was part of group 2 (subjects OVW-SA2099-190), the group that received two batches i.e. Lot no. 18C07 and 19A21 (for more details see Chapter 5.2.2 Product Stability). Unblinding revealed that the patient was given the study drug treatment i.e. AV2-SA. Considering the low SA concentration (6,79 – 3.38%) in the study drug batches applied and rather intensive patient treatment history where a combination of aggressive treatments was applied without any effect, it can be concluded that the treatment effect observed in this patient can be attributed to AV2.

Chapter 7 Concluding Remarks

7.1 Summary and General Discussion

Cutaneous warts are common skin lesions caused by infection with the human papillomavirus (HPV). Although benign in nature they can often be painful and cause impaired quality of life due to social stigma or inhibition of the natural function. For this reason, many patients do seek treatment, but these treatments often remain unsatisfactory and cause unwanted adverse events. Currently, a reliable approach to deciding on optimal treatment is not available. Previous studies suggest that HPV type can influence response to treatment and natural course in cutaneous warts. For this purpose, the pertinence of HPV genotyping as a triage method for the clinical management of cutaneous warts is explored.

Chapter I provides a comprehensive introduction to HPVs, their genomic structure and organization, phylogenetic and topical classification, and infectious life cycle as well as a summary of available molecular HPV detection methods. In a subsequent section, an overview of the cutaneous clinical manifestations where HPV has a potential impact is presented, with an in-depth focus on cutaneous warts. Together these two sections provide a broad basis for the thesis outline and specific objectives, describing the current gaps in the knowledge surrounding HPV and cutaneous warts and the potential use of HPV genotyping in clinical management of these lesions.

In **Chapter 2**, a step-by-step description of the development and optimization of a patient-friendly, non-invasive sampling technique for HPV-related skin disorders is provided, in combination with automated DNA extraction. Due to its noninvasive character, the novel sampling technique is especially applicable to children and can be easily implemented in clinical routine.

Chapter 3 describes a low-cost, high-throughput, ultra-low volume qPCR assay capable of detecting the most prevalent HPV types in cutaneous warts i.e. the wart-associated HPV genotyping assay. Due to its unique design, the assay can be used as a general screening assay as well as a genotyping assay capable of identifying 12 distinct HPV genotypes,

resulting not only in lower screening costs but also providing an option for faster and more simplified testing results.

The complete workflow, i.e. the combination of the novel skin sampling technique with the highly automated HPV qPCR assay, is easily implementable in large high throughput clinical trials as described in **Chapter 4**. The OVW-SA001 clinical trial was designed to assess the efficacy of a novel anti-viral drug i.e. AV2, in combination with salicylic acid as treatment for cutaneous warts. It was a single-center randomized controlled trial with two treatment arms comprising a total of 269 participants. Each participant either received SA as a control treatment or AV2 in combination with SA as a study treatment. Subjects were instructed to apply treatment daily for 12 weeks and provide weekly pictures via a messaging application (WhatsApp) as well as fill out several online follow-up questionnaires. Full details of the OVW-SA001 clinical trial protocol are provided including study design, objectives, recruitment strategy, eligibility criteria, power analysis, treatment interventions as well as data management and statistical analysis.

Unfortunately, distinct claims about treatment efficacy of the AV2-SA treatment could not be made due to several complications during the conduct of the trial (as described in **Chapter 5**). Nevertheless, some patients did benefit from the treatment provided during the study, with a total clearance rate of 46/237 (19%) and 89/237 (38%) of patients experiencing reduction in wart size, contributing to an overall positive effect of treatment in 135/237 of patients (57%). Despite the trial issues, AV2-SA seems to be a promising novel therapy against cutaneous warts as demonstrated by specific case reports where certain individuals did experience a vast improvement in their quality of life (cf. **Chapter 6**). However, the formulation of the treatment is not yet optimal, and future formulation studies should focus on ease of use (e.g. addition of collodion) and optimization of SA stability in AV2.

A thorough epidemiological description of the study population is provided, which is to date the largest study population with cutaneous warts described in Belgium. Furthermore, we were also able to demonstrate the utility of the novel workflow in the OVW-SA001 trial

with 97% (261/269) of initial study samples being valid and 98% (263/269) of study participants being positive for at least one of the HPV types included in the wart-associated HPV genotyping qPCR assay. The study population was highly enriched with persistent warts considering that 98% (n=263) of the sampled skin lesions were older than six months and 92% (n=247) had undergone previous treatment. The most prominent wart type was the mosaic verruca plantaris (42%, n=113). The most prevalent HPV types were cutaneous HPV types 27 (73%, n=195), 57 (63%, n=169), and 2 (42%, n=113). Only 2% (5/263) of HPV-positive warts were negative for HPV 2, 27 or 57. The highest median viral loads were observed with HPV27 and 57 (i.e. 6.29E+04 and 7.47E+01 viral copies per cell respectively), suggesting that high viral loads are associated with the highest infectious potential due to copious viral shredding in the environment. A multivariate analysis was performed and significant associations between wart persistence and certain wart types, the number of warts, and HPV genotypes were identified. Based on these findings, persistent warts were more likely to: (1) be verruca vulgaris, verruca plantaris simple, or mosaic, (2) to manifest as multiple warts, (3) and to be negative for HPV type 2 or 4.

These characteristics can be useful in the clinical setting for future risk stratification when considering treatment triage and patient management. Patient triage will ensure that optimal treatment is provided from the start, increasing the chances of a positive outcome and improving the time to lesion clearance. An additional advantage of rapid clearance is that the transmission of the infection in the general population is then inhibited as well.

7.2 Future Outlook

As already outlined above the newly developed workflow is readily implementable in routine clinical practice and patient triage based on the newly discovered epidemiological profile of persistent warts can directly be applied in patient management as well.

Furthermore seeing that we provide a step-by-step development and optimization of this sampling and analysis workflow, the same guidelines can be applied in further research regarding other HPV-related cutaneous diseases.

Considering that the current trial comprised a study population enriched with persistent warts, a low-cost but potentially very valuable study would be to assess the HPV types in warts of patients visiting the general practice for other reasons than wart treatment. If the study is performed in the same region as the current trial i.e. Antwerp province, the two study populations could be directly compared and additional claims about the epidemiological profile of a non-persistent wart could be made, further optimizing and filling the gaps in current knowledge about wart triage with a low-cost study budget.

A more direct approach would be a large clinical trial comparing three principal treatment groups i.e. cryotherapy, salicylic acid, and a wait-and-see group. The study sample size should be sufficiently powered to ensure that each cutaneous HPV type is adequately represented in each treatment group. This study design would unravel the role of HPV genotyping in treatment efficacy and would validate the true added value of HPV as a triage method for the treatment of cutaneous warts. In addition, the study would not only identify certain epidemiological profiles of warts for which a specific treatment is more effective but also ones that have a favorable natural course and should not be treated at all, therefore saving not only economic resources but also preventing patients from experiencing unnecessary painful side effects of treatment.

In addition, future research should definitely also focus on high-risk immunocompromised individuals such as organ transplant patients, who are at increased risk of developing large numbers of persistent warts and where cutaneous warts have a serious impact on quality of life. Studies have suggested that these patients exhibit similar HPV-type specific distribution as the general population, making the current wart-associated HPV genotyping assay easily implementable in these studies as well (Köhler et al., 2009; Rubben et al., 1993; Surentheran et al., 1998). Furthermore, based on this data, future vaccines could be developed against the most prevalent cutaneous HPV types and used in prevention strategies in immunocompromised patients (Bouwes Bavinck et al., 2007).

Regarding AV2, our data suggest a beneficial effect in the treatment of cutaneous warts in certain subjects. The efficacy of AV2 as a treatment of HPV-related cervical lesions has been

investigated in two previous studies (Martinez et al., 2017; Mutombo et al., 2019). In a small-scale, phase 2 randomized controlled trial, Martinez et al., demonstrated that AV2 treatment resulted in a statistically significant reduction of more than 50% of the original lesion size for 21/28 (75%) of patients that received study treatment in comparison to 0/22 (0%) of patients in the placebo group, 2 months after study enrollment ($p<0.001$; Martinez et al., 2017). In another, larger study conducted by Mutombo et al., lesion size reduction was observed 2 months after treatment in 127/142 (89%) of patients in the AV2 group compared to 120/131 (92) of patients in the placebo group (Mutombo et al., 2019). The lesion size reduction rates between the two groups were not statistically significant ($p> 0.1$) and the authors did not succeed in reproducing the positive effect of AV2 described by Martinez et al. (Martinez et al., 2017). The authors highlighted certain limitations in the study design and concluded that further evaluation of the effects of AV2 is warranted considering not only different diagnostic methods, treatment regimens, and mode of application as well as different comparator arms. This is in line with our findings emphasizing the need for additional studies to focus on optimizing the formulation of AV2-SA combination therapy in treatment of cutaneous warts. When proven to be an effective wart treatment, AV2 could also be assessed as a treatment of other more detrimental cutaneous as well as mucosal HPV-related diseases such as non-melanoma skin cancer, anogenital and oropharyngeal cancer.

In the past HPV has been a difficult virus to address using antiviral therapy, seeing that the pharmaceutical industry has historically preferred enzymes encoded by the viral genomes as antiviral drug targets and HPV only encodes a single enzyme i.e. E1 helicase (Fradet-Turcotte & Archambault, 2007). Nowadays in addition to inhibitors that target E1 helicase activity, other antivirals exist capable of hindering E1-E2 protein interaction and E1/E2 binding to DNA (Liu et al., 2019). Other non-HPV-specific approaches are the so-called host-dependent viral inhibitors such as DNA-polymerase inhibitors. Seeing that these therapeutics are less specific for HPV-infected cells and also affect normal cell functions, they are more likely to exhibit mechanism-based toxicity (Fradet-Turcotte & Archambault, 2007; Liu et al., 2019). A major drawback when considering HPV-antiviral therapy is that

antivirals are indeed capable of inhibiting HPV proliferation but are unable to eradicate the infection in case of viral integration in the host genome. Once the integration has occurred E6 and E7 oncogenes should be explored as main targets of anti-viral therapy given their overexpression due to disruption of the E2 open reading frame (Fradet-Turcotte & Archambault, 2007).

Considering the current dynamic landscape in antiviral therapy, with numerous promising products under investigation, in the mid-term future it can be foreseen that these products will find a prominent place in the clinic and offer a strong alternative in the treatment of viral-induced disease.

Samenvatting

Cutane wratten zijn veel voorkomende huidaandoeningen veroorzaakt door infectie met het humaan papillomavirus (HPV). Alhoewel ze in principe goedaardig zijn, kunnen ze vaak pijnlijk zijn en een verminderde levenskwaliteit veroorzaken door sociaal stigma of belemmering van de natuurlijke functie. Bijgevolg gaan meeste patiënten toch op zoek naar een behandeling, maar deze behandelingen zijn vaak onsuccesvol en veroorzaken ongewenste bijwerkingen. Op dit moment is er nog geen betrouwbare methode beschikbaar voor het bepalen van een optimale behandeling. Eerdere studies suggereren dat het HPV-type de respons op behandeling en het natuurlijke beloop bij cutane wratten kan beïnvloeden. Derhalve wordt de waarde van HPV-genotypering als een triagemethode voor klinisch management van cutane wratten onderzocht.

Hoofdstuk I geeft een uitgebreide inleiding in HPV's, hun genomische structuur en organisatie, fylogenetische en topische classificatie, infectieuze levenscyclus, alsook een overzicht van beschikbare moleculaire HPV-detectietechnieken. In een bijkomend gedeelte wordt een overzicht gegeven van de cutane klinische manifestaties waar HPV een potentiële impact heeft, met een diepgaande focus op cutane wratten. Samen vormen deze twee gedeeltes een brede basis voor de opzet van het proefschrift en de specifieke doelstellingen, waarbij de huidige hiaten in de kennis rond HPV en cutane wratten en het potentiële gebruik van HPV-genotypering in het klinische management van deze laesies worden beschreven.

In **Hoofdstuk 2** wordt een stapsgewijze beschrijving gegeven van de ontwikkeling en optimalisatie van een patiëntvriendelijke, niet-invasieve staalafname techniek voor HPV-gerelateerde huidaandoeningen, in combinatie met geautomatiseerde DNA-extractie. Dankzij het niet-invasieve karakter is de ontwikkelde techniek eveneens toepasbaar bij kinderen en kan deze eenvoudig in de klinische routine worden geïmplementeerd.

Hoofdstuk 3 beschrijft een kostenefficiënte PCR-test met een hoge verwerkingscapaciteit, die gebruik maat van ultralage volumes en waarmee de meest voorkomende HPV-typen in cutane wratten kunnen worden gedetecteerd, i.e. de wrat-geassocieerde HPV-genotyperingstest. Dankzij het unieke ontwerp kan het assay gebruikt worden zowel als een algemene screeningstest en als een genotyperingstest die 12 verschillende HPV-genotypen kan identificeren, wat niet alleen resulteert in lagere screeningskosten, maar ook een mogelijkheid biedt voor snellere en eenvoudigere testresultaten.

De volledige workflow, bestaande uit de combinatie van de nieuwe staalafname techniek met de geautomatiseerde HPV PCR-test, kan gemakkelijk worden geïmplementeerd in omvangrijke klinische onderzoeken, zoals beschreven in **Hoofdstuk 4**. Het klinische onderzoek OVW-SA001 werd ontworpen om de werkzaamheid van een nieuw antiviraal geneesmiddel, namelijk AV2, in combinatie met salicylzuur (SA) te beoordelen als behandeling voor cutane wratten. Het betrof een gerandomiseerde, gecontroleerde, monocentrische studie met twee behandelingsarmen en 269 deelnemers in totaal. Elke deelnemer kreeg ofwel SA als controlebehandeling, ofwel AV2 in combinatie met SA als studiebehandeling. Proefpersonen werden verzocht om gedurende 12 weken dagelijks de behandeling toe te passen, wekelijks foto's te voorzien via een berichtenapplicatie (WhatsApp) en om online follow-upvragenlijsten in te vullen. Volledige details van het klinische onderzoeksprotocol worden beschreven, inclusief onderzoeksopzet, doelstellingen, wervingsstrategie, geschiktheidscriteria, berekening van de steekproefgrootte, behandelingsinterventies evenals gegevensbeheer en statistische analyse.

Spijtig genoeg konden er geen duidelijke uitspraken worden gedaan over de werkzaamheid van de behandeling met AV2-SA vanwege enkele complicaties tijdens de uitvoering van het onderzoek (zoals beschreven in **Hoofdstuk 5**). Desondanks hadden bepaalde patiënten baat bij deelname aan het onderzoek, met een totaal klaringspercentage van 46/237 (19%) en 89/237 (38%) patiënten die een vermindering van de wratgrootte ondervonden, wat bijdroeg aan een algemeen positief effect van de behandeling bij 135/237 patiënten (57%).

Ondanks de complicaties schijnt AV2-SA een veelbelovende nieuwe therapie tegen cutane wratten te zijn, zoals blijkt uit specifieke casusrapporten waarbij specifieke patiënten een enorme verbetering van hun levenskwaliteit ondervonden (zie **Hoofdstuk 6**). De formulering van de behandeling was echter nog niet optimaal en toekomstige studies zouden zich moeten richten op gebruiksgemak (bijv. toevoeging van collodium) en optimalisatie van de SA-stabiliteit in AV2. Verder wordt ook een grondige epidemiologische beschrijving van de studiepopulatie voorzien, wat tot op heden de grootste studiepopulatie met cutane wratten in België betreft. Een multivariabele statische analyse onthulde significante associaties tussen wrat persistentie en specifieke wrattypes, de hoeveelheid wratten en bepaalde HPV-genotypen.

Hoofdstuk 7 voorziet een samenvatting en een algemene discussie van de bevindingen van het proefschrift, evenals een vooruitblik en toekomstige onderzoeksmogelijkheden op dit domein. De praktische implementatie van de ontwikkelde workflow in de klinische praktijk wordt benadrukt, evenals de toepasbaarheid van de opgestelde richtlijnen in onderzoek naar andere HPV-gerelateerde huidaandoeningen. Toekomstig onderzoek moet zich richten op hoog-risico immuungecompromitteerde personen, waar cutane wratten ernstige gevolgen kunnen hebben en de ontwikkeling van vaccins tegen prevalente cutane HPV-typen voor deze bevolkingsgroep van uiterst belang is. In het algemeen wordt benadrukt dat de verworven epidemiologische inzichten gebruikt moeten worden om behandelstrategieën te optimaliseren, onnodige behandelingen te voorkomen en de therapeutische opties voor verschillende HPV-gerelateerde aandoeningen te exploiteren.

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- Rikki Rogers