Early phase clinical drug development for HPV-induced disorders: novel tools and treatments

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Chapter 5

A RANDOMIZED CONTROLLED PROOF-OF-CONCEPT TRIAL OF DIGOXIN AND FUROSEMIDE IN ADULTS WITH CUTANEOUS WARTS

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Abstract

BACKGROUND Topical Ionic Contra-Viral Therapy (ICVT) comprised of digoxin and furosemide inhibits the potassium influx on which DNA viruses rely for replication. Therefore, ICVT was hypothesised to be a potential novel treatment for cutaneous warts.

OBJECTIVES To assess clinical efficacy, safety and tolerability of ICVT in adults with cutaneous warts. Secondary objective was to gain insight into underlying working mechanism of ICVT.

METHODS Treatment with ICVT was assessed for efficacy, safety and tolerability in a single- centre, randomised, double-blind, placebo-controlled phase 2A trial. Eighty adult subjects with at least 2 cutaneous warts (plantar or common) were randomised to one of 4 treatments: digoxin + furosemide (0.125%), digoxin (0.125%), furosemide (0.125%) or placebo and administered the gel once daily for 42 consecutive days. Pre-defined statistical analysis was performed with a mixed model analysis of covariance.

RESULTS Wart size and HPV load reduction was achieved in all active treatment groups. A statistically significant reduction in wart diameter of all treated warts was shown in the digoxin + furosemide treatment group versus placebo (-3.0mm; 95% CI -4.9 to -1.1mm; p=0.002). There was a statistically significant reduction in HPV load of all treated warts in the digoxin + furosemide group compared to placebo (-94%; 95% CI -100 to -19; p=0.03). With wart size reduction, histologically and immunohistochemically defined viral characteristics disappeared from partial and total responding warts.

CONCLUSIONS This study demonstrates proof-of-concept for the efficacy of topical ICVT in adults with cutaneous warts.

Introduction

Cutaneous warts, or verrucae, are a common benign skin condition with an estimated prevalence of 3-13% in the general population in the Western world.[1] Most people are affected by cutaneous warts, either plantar warts (located on foot soles), or common warts (mostly located on hands or dorsal feet), at some point in their life (Kilkenny and Marks, 1996).[1–4]

Although cutaneous warts are benign and usually resolve spontaneously, they affect both physical and psychosocial discomfort.6 Many patients use a variety of wart-removing products.[5–8] Efficacy rates of common treatments are approximately 39% for cryotherapy, 24% for salicylic acid and 46% for monochloroacetic acid, whereas spontaneous regression rates are around 16%.[7,9–11] As current treatments such as cryotherapy and monochloroacetic acid often have side effects (e.g., pain, erythema and burning sensation) and low efficacy rates, there is a need for therapies with a greater efficacy and minimal side effects.[12–15]

Cutaneous warts are caused by the human papillomavirus (HPV). The great majority (>80%) of verrucae in the general population is related to HPV1, 2, 27 and 57.[16–21]

It is well known that papillomaviruses are dependent of the milieu of the infected host cell for proliferation.[22,23] More specifically, it has been shown that DNA viruses, such as HPV rely on potassium influx (K^+) for replication.[24] The cardiac glycoside digoxin and loop diuretic furosemide both inhibit de K^+ influx by interacting with the cell membrane ion co-transporters Na^+/K^+-ATPase and Na^+-K^+-$2Cl^-$. These two compounds may therefore be valuable for the treatment of HPV-induced diseases, such as cutaneous warts. In 2006, an in vitro study found that the inhibitory effect on DNA replication was most potent when digoxin and furosemide were combined. This new approach with two well-know, established drugs, described as Ionic Contra-Viral Therapy (ICVT), is suggested to be most effective via local application.[25]

A previous phase 1/2 open-label study recently demonstrated safety and efficacy of ICVT in a group of 12 healthy subjects with common warts.[26] The aim of the current proof-of-concept study was to assess clinical efficacy, safety and tolerability of ICVT in adults with cutaneous warts in a single-center, randomised, double-blind, placebo-controlled phase 2A trial. The secondary objective was to gain insight into the underlying working mechanism of ICVT.

Materials and methods

STUDY DESIGN, PARTICIPANTS AND RANDOMIZATION

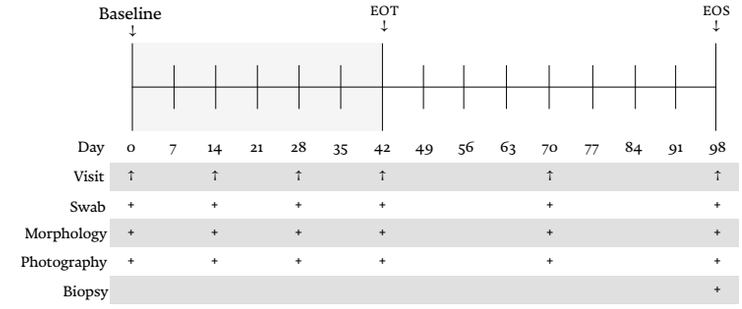
A randomised, double-blind, placebo-controlled, parallel-group, single-center phase 2 trial was conducted. The Declaration of Helsinki was the guiding principle for trial execution, and the study was approved by the independent Medical Ethics Committee "Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek" (Assen, the Netherlands) prior to any procedure. Patients were included if they were (other than the skin condition) healthy, 18 years or older, and had at least 2 (non-subungual, non-genital and non-facial) common or plantar warts with a diameter of minimal 3 mm, diagnosed by a dermatologist and after giving written informed consent. A maximum of 5 warts per subtype were followed during the study. Patients were excluded if they had been exposed to wart-removing products within 30-60 days prior to enrolment, depending on the treatment. For women of childbearing age, effective contraception was required during study execution and at least 90 days afterwards. The study consisted of a screening phase (weeks -4 to 0), a treatment phase (weeks 0 to 6) and a follow-up phase (weeks 6 to 14), as shown in Figure 1.

Subjects were randomised 1:1:1:1 in blocks of four to receive one of the four treatment regimens: digoxin + furosemide (0.125%, w/w) digoxin (0.125% w/w), furosemide (0.125% w/w) or vehicle, which served as placebo with an identical appearance. Randomization was predefined and performed in SAS by an independent statistician and subject numbers were sequentially allocated by chronological enrollment. Subjects, study personnel and investigators were blinded for allocated treatment throughout the study. At baseline, all warts were numbered by a blinded independent clinical staff member; for common warts starting from 1 with a maximum of 5 and for plantar warts starting from 6 with a maximum of 10. Wart number 1 or 6 was selected as untreated wart (N=80) and the other warts were selected as treated warts. Of the treated warts 1 wart per subject was selected as primary wart (biopsy wart, N=80) using a randomly generated number in SAS drawn by an independent statistician.

STUDY SITE

The study was conducted from December 2014 to August 2015 at the Center for Human Drug Research, Leiden, The Netherlands.

Figure 1. Study design. The treatment phase entailed 42 days with study visits at day 0, 14, 28 and 42. The follow-up phase lasted for 56 days with study visits at day 70 and 98. The treatment period was 42 days with patient visits at day 0, 14, 28 and 42. At all visits the following assessments were performed of all warts: wart size measurement, wart morphology, photography, swab. At day 98, a biopsy was performed of the primary and untreated wart.



EOT = End of treatment; EOS = End of study.

STUDY PROCEDURES

The primary objective was to investigate clinical efficacy of ICVT by analyzing wart size reduction and viral load in primary warts in the four treatment groups. Wart size reduction was assessed in diameter and height (mm) by a digital vernier caliper (0-150 mm, Aerospace). Wart clearance (defined as 100% reduction) was assessed by a dermatological sub-investigator. Viral load was measured with use of skin swabs.[26] In addition, 2 biopsies of the primary wart and the untreated reference wart were taken at the end of study (EOS). The HSL-PCR/MPG assay (LMNX kit HSL-PCR, Labo Bio-medical Products) enables the simultaneous identification of 23 warts-associated HPV types from the alpha (HPV2, 3, 7, 10, 27, 28, 29, 40, 43, 57, 77, 91 and 94), gamma (HPV4, 48, 50, 60, 65, 88 and 95), mu (HPV1 and 63) and nu-genus (HPV41).[16,27] Viral load was determined for all swabs and biopsy samples of primary warts that were positive for HPV1, 2, 27 or 57 by qPCR.

The secondary objective was to gain insight into the underlying working mechanism of ICVT. Therefore, wart morphology was assessed to confirm or reject the hypothesis that wart size reduction can be predicted by morphological aspects of all warts in this study. Standardized photographs of the primary wart were taken and wart morphology was assessed using the CWARTS diagnostic tool.[28,29] Complete responders were defined as showing a reduction of 100% in size, partial responders a reduction between 25% and 100% in

size and non-responders less than 25% reduction of wart size at EOS compared to baseline. A subset of 20 warts of subjects was chosen based on response (complete, partial or non-responders) for a histopathology and immunohistochemistry (IHC) analysis, in order to confirm or reject the hypothesis that wart size reduction can be predicted by viral characteristics, Ki-67 (cell proliferation) and HPV E4 (marker of a productive infection) patterns. Viral characteristics (histopathology), Ki-67 (clone MIB-1; Dako/Agilent Technologies) and HPV E4 patterns (SILgrade-E4-1 kit containing XR-E4-1 monoclonal antibody, Labo Bio-medical Products) were assesses by two blinded reviewers and without prior knowledge of responder or HPV status. All analyses were independently performed by two reviewers except for the Ki67 analysis that was discussed during microscopy.

Safety and tolerability were monitored by tracking of adverse events (AEs), performing physical examination, measuring vital signs, 12-lead electrocardiograms, and laboratory tests (i.e. hematology, chemistry, coagulation, and urinalysis) and by systemic therapeutic drug monitoring for systemic exposure of digoxin at multiple time points throughout the study. Treatment adherence was measured by monitoring all daily dose administrations via a validated mobile e-diary application. After application of the gel, trial subjects took a photo of all warts with use of the mobile e-diary.

STATISTICS

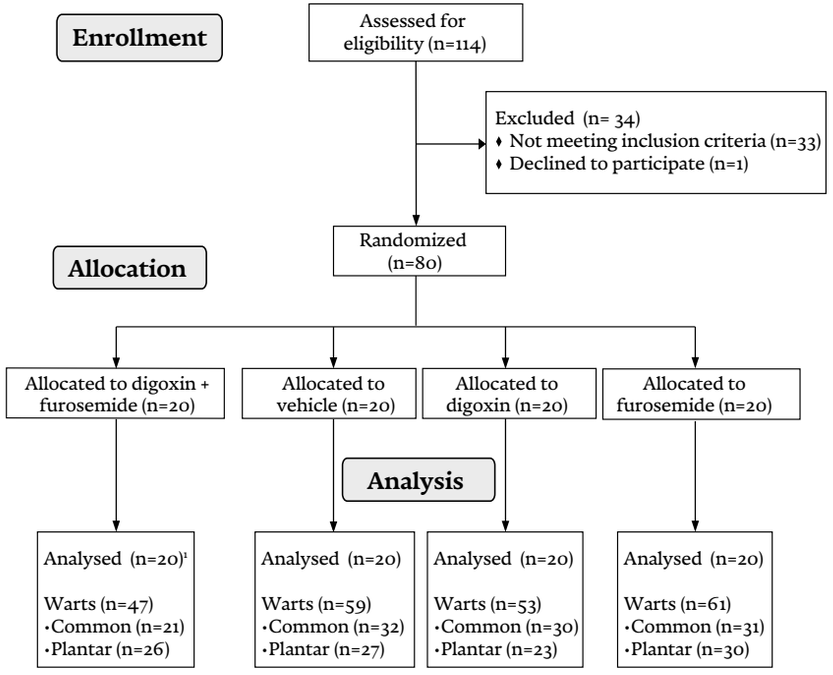
A sample size of 20 patients per treatment group was estimated based on the analysis of primary warts to provide >90% power to demonstrate superiority of digoxin and/or furosemide over placebo with a difference in means of 31.6mm^3, assuming that the common standard deviation is 30, using a two group t-test with a 0.05 two-sided significance level.[26] All efficacy/pharmacodynamic endpoints were analyzed in the intention to treat population, with a mixed model using treatment, time and treatment by time as fixed factors and subject as random factor. The pre-defined primary analyses to investigate clinical efficacy of ICVT were performed for primary warts only. For pre-defined secondary analyses to gain insight into underlying working mechanism of ICVT was based on all treated warts, and within subject was added as random factor to the model. All statistical tests were two-tailed with α-level of 0.05. A two-sided Fisher's exact and a two-sided Wilcoxon exact rank test were used to analyze wart clearance. Correlation between qPCR in swab samples and biopsies was investigated using a linear regression model with subject as random factor.

Results

PATIENTS

Hundred-and-fourteen (114) otherwise healthy subjects with cutaneous warts were screened of whom 81 (71%) were enrolled in the trial; 1 withdrew before randomization (see Fig. 1 and Fig. 2). All subjects (N=80) completed the study and there were no treatment discontinuations or early withdrawals. Baseline demographic and disease characteristics were comparable in all four treatment groups (see Table 1).

Figure 2. Flowchart of the study of all subjects and warts. One-hundred and fourteen (114) otherwise healthy subjects with cutaneous warts were screened of whom 81 (71%) were enrolled in the trial; 1 withdrew before randomization. Of the 80 remaining subjects, 20 were randomly assigned to one of four treatment groups: digoxin + furosemide, digoxin, furosemide or placebo, all to be locally applicated in gels. All subjects (N=80) completed the study and there were no treatment discontinuations or early withdrawals.



1: In the digoxin+furosemide group the pharmacodynamics measurements of the primary wart of 1 subject were excluded.

Table 1. Patient characteristics.

Characteristics	Digoxin+ Furosemide (N= 20)1	Digoxin (N= 20)	Furosemide (N= 20)	Placebo (N= 20)	Total (N=80)
Mean age in years (SD)	23.8 (±7.9)	30 (±13.5)	23.5 (±5.5)	26.1 (±12.7)	25.8 (±10.6)
SEX – NO. (%)					
Male	6 (30)	11 (55)	7 (35)	7 (35)	31 (39)
Female	14 (70)	9 (45)	13 (65)	13 (65)	49 (61)
Mean time since diagnosis in years	5.3	7.6	6.9	4.9	6.2
Total amount of warts – no.	47	53	61	59	220
Mean number of warts per subject – no.	2.4	2.7	3.1	3	2.8
Subjects with common warts – no. (%)	9 (45)	10 (50)	10 (50)	10 (50)	39 (49)
Amount of common warts – no. (%)	21 (45)	30 (57)	31 (51)	32 (54)	114 (52)
Treated common warts – no. (%)	12 (57)	19 (63)	21 (68)	21 (66)	73
Subjects with plantar warts – no. (%)	11 (55)	9 (45)	10 (50)	9 (45)	39 (49)
Amount of plantar warts – no. (%)	26 (55)	23 (43)	30 (49)	27 (46)	106
Treated plantar warts – no. (%)	15 (58)	13 (57)	20 (67)	17 (63)	65
Subjects with both common and plantar warts – no(%)	0 (0)	1 (5)	0 (0)	1 (5)	2 (3)
Size of warts – mean diameter (mm)	6.6	6.4	6.4	6.5	6.5
Size of primary wart – mean diameter (mm)	6.02	6.56	6.47	6.45	6.38
HPV TYPE PRIMARY WART					
HPV1	0	0	0	0	0
HPV2	5	4	3	6	18
HPV27	6	10	10	3	29
HPV57	6	2	3	6	17
Other[2]	2	4	4	5	15
Any previous treatment – no. (%)	16 (80)	17 (85)	14 (70)	15 (75)	62 (78)
Cryotherapy – no. (%)	12 (60)	16 (80)	12 (60)	14 (70)	54 (68)
Cimetidine – no. (%)	0 (0)	1 (5)	0 (0)	0 (0)	1 (1)
Electrocoagulation – no. (%)	0 (0)	1 (5)	0 (0)	0 (0)	1 (1)
Fluorouracil – no. (%)	0 (0)	1 (5)	0 (0)	0 (0)	1 (1)
Monochloor, Salicylic and trichloric acid – no. (%)	7 (35)	8 (40)	9 (45)	6 (30)	30 (38)
Surgery – no. (%)	1 (5)	2 (10)	0 (0)	0 (0)	3 (4)

1: In the digoxin + furosemide group the pharmacodynamics measurements of the primary wart of one subject were excluded / 2: Other = HPV3, HPV4and HPV10.

TREATMENT ADHERENCE

Seventy-eight (78) of the 80 subjects (97.5%) applied the gel once daily for more than 35 consecutive days and only sporadically subjects did not comply to the daily treatment regimen. Most subjects applied a dose within the range of 5-30 mg per wart per day. However, the mean amount of study medication applied per wart per day was highly variable (range: 2.9-118 mg).

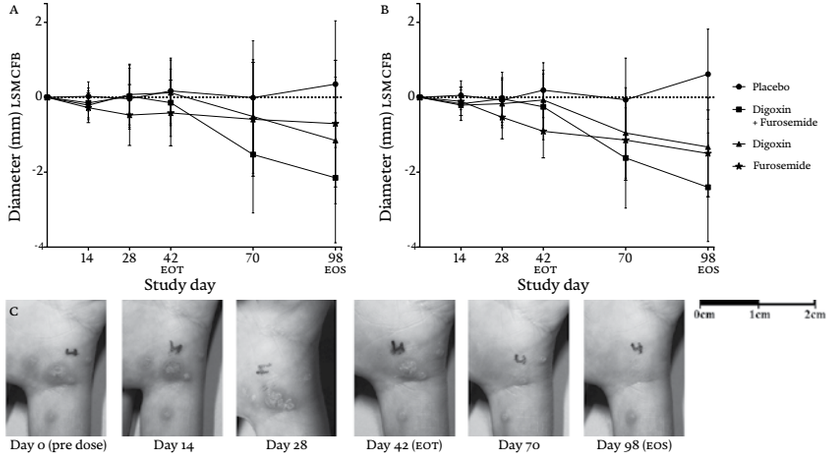
WART SIZE REDUCTION

Figure 3A shows a reduction in primary wart diameter measured by caliper from baseline to end of study (EOS) in all active treatment groups. A statistically significant effect ($p<0.05$) was found in the digoxin + furosemide group versus placebo (-2.5mm; 95% CI -4.9 to -0.1; p=0.04), while the two other treatment groups (digoxin vs placebo and furosemide vs placebo) showed no statistically significant effects (-1.5mm; 95% CI -3.9 to 0.9; p=0.21, and -1.1mm; 95% CI -3.4 to 1.3; p=0.38, respectively). Changes in diameter were most pronounced after end of treatment (EOT), as shown in Figure 3A. In the analysis of all treated warts (N=139) a statistically significant wart size reduction measured by caliper was observed between each active treatment group and placebo (digoxin + furosemide versus placebo; -3.0mm; 95% CI -4.9 to -1.1; p=0.002, digoxin vs placebo -1.9mm; 95% CI -3.7 to -0.2; p=0.03, furosemide versus placebo -2.1mm; 95% CI -3.8 to -0.4; p=0.01) as is shown in Figure 3B.

WART CLEARANCE

At the EOS, primary warts (N=80) showed comparable clearance rates in all active treatment groups, i.e. 3/19 (16%) in the digoxin + furosemide group, 3/20 (15%) in the digoxin group and 3/20 (15%) in the furosemide group. In contrast, no clearance was observed in the placebo treated group (N=20). A two-sided Fisher's exact test revealed no statistically significant differences when active treatment groups were compared to the placebo group. Table 2 shows comparable clearance rates in all treated warts in the 3 active treatment groups. Supplemental data (Table A) shows the rates of clearance observed in treated common warts (24-27%) and treated plantar warts (8-15%) at EOS. When including all warts with a reduction of ≥90% diameter, the highest response rate was seen in common warts treated with digoxin + furosemide (N=5) at EOS with a response rate of 45%. In Figure 3C an example of a photography assessment of a treated wart in the digoxin + furosemide group is shown.

Figure 3. Change from baseline (CFB) least squares mean (LSM) of diameter of primary warts (A) and all treated warts (B) and photography assessments of a common wart of subject #6 (digoxin+furosemide) (C). (A) Analysis of the primary endpoint for the intention-to-treat population (N=79) was performed using a mixed model with treatment, time and treatment by time as fixed factors and subject as random factor. All statistical tests were two-tailed with α-level of 0.05. Results showed a statistically significant reduction of wart size in the digoxin+furosemide group compared to placebo (-2.5mm; 95% CI -4.9 to -0.1; p=0.04). Single treatment groups (digoxin vs placebo and furosemide versus placebo) showed no statistically significant effects (-1.5mm; 95% CI -3.9 to 0.9; p=0.21, and -1.1mm; 95% CI -3.4 to 1.3; p=0.38, respectively). Changes in diameter were most pronounced after EOT, as shown in Figure 3A. (B) In the analysis of all treated warts (N=139) a statistically significant wart size reduction was observed between each active treatment group and placebo (digoxin+furosemide versus placebo; -3.0mm; 95% CI -4.9 to -1.1; p=0.002, digoxin vs placebo -1.9mm; 95% CI -3.7 to -0.2; p=0.03, furosemide versus placebo -2.1mm; 95% CI -3.8 to -0.4; p=0.01). (C) A photography assessment of a treated wart in the digoxin+furosemide group. *(see inside back-cover for image c in color)*



EOT= end of treatment; EOS= end of study.

Table 2. Clearance of all warts per subject at end of study.

Characteristics	Digoxin+ Furosemide (N= 191)	Digoxin (N= 20)	Furosemide (N= 20)	Placebo (N= 20)
Wart clearance[2] (p-value treatment vs placebo)	0.11	0.23	0.11	-0 (0)
All warts[3] – no. (%)	2 (11)	2 (10)	2 (10)	0 (0)
At least 1 wart, but not all warts[4] – no. (%)	1 (5)	1 (5)	2 (10)	20 (100)
No clearance – no. (%)	16 (84)	17 (85)	16 (80)	

1: In the digoxin+furosemide group the pharmacodynamics measurements of the primary wart of one subject were excluded / 2: Clearance defined as 100% reduction / 3: All warts of the subject were cleared / 4: At least one wart, but not all warts, of the subject was cleared

VIRAL LOAD

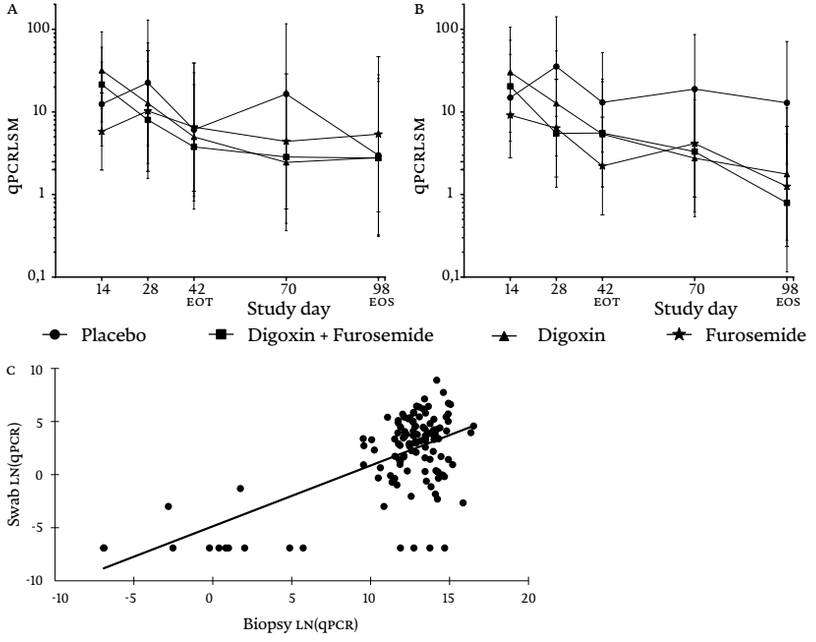
At baseline, 200 of the 219 (91%) warts (one missing sample) were positive for DNA from the 23 tested HPV types. HPV27 was most prevalent (38%) followed by HPV57 (26%) and HPV2 (24%). Of the 219 warts, 186 (85%) were positive for one of the HPV types for which viral load testing was available (i.e., HPV1, 2, 27, 57). No statistical differences were found when comparing the HPV load of primary warts (N=79) in swabs from baseline to EOS in the treatment groups with the placebo group (digoxin + furosemide -8%; 95% CI -96 to 1952; p=0.96, digoxin -6.3%; 95% CI -96 to 2086; p=0.97 and furosemide 80%; 95% CI -92 to 3966; p=0.71), as is shown in Figure 4A. However, when comparing the viral load change of HPV from baseline to EOS in the swabs of all treated warts (N=139), there was a statistically significant reduction of viral load, only in the digoxin + furosemide group versus placebo (-94%; 95% CI -100 to -19; p=0.03) (see Fig. 4B). In biopsies, no statistically significant differences in HPV load were seen in the treatment groups versus placebo. There was a significant correlation (p<0.0001) between viral load in swabs and biopsies at the EOS (Fig. 4C). We observed a significant correlation (p=0.001) between wart size reduction and reduction in HPV load (data not shown).

RESPONSE ANALYSES

In case of wart elevation, a significantly decreased wart diameter was observed at EOS after 6 weeks of treatment with the combination treatment digoxin + furosemide compared to placebo (-5.2 mm; 95% CI -8.6 to -1.8; p=0.003). The morphologic aspects callus and smooth/rough wart did not show any difference in prediction of wart size reduction (see Table 3).
In Table 4, a summary of the responder analysis is given, based on 9 responder warts (i.e., 3 complete responders, 6 partial responders) and 11 non-responders. The individual data is available in Supplemental Data: Table B. H&E staining showed changes characteristic of viral infection in biopsies from non-responder warts in contrast to the biopsies from complete and partial responder warts. In the IHC of the non-responders the Ki-67 was positive suprabasal (scattering) in all biopsies, comparing to a basal Ki-67 pattern in all complete responders, i.e. 100% and 5 out of 6 partial responders, i.e. 83.3% (Table 4). Staining of the HPV E4 protein, indicative of a productive HPV infection, was positive in all non-responders and related to a high HPV load in EOS biopsies and swabs (see Table 4). Concordantly, in all complete and partial responders

the E4 staining was negative. The mean viral load in biopsies and swabs at EOS was lower in the complete and partial responders compared to the non-responders. In Figure 5 examples of the H&E staining of a classical verruca vulgaris and plana are illustrated, showing typical viral characteristics.

Figure 4. HPV viral load in swabs depicted as percentage change from baseline (CFB) least squares mean (LSM) of primary wart (A) and all treated warts (B) and correlation of HPV viral load in swabs versus biopsy at end of study (C). (A) Analysis of primary warts (N=79) was performed using a mixed model with treatment, time and treatment by time as fixed factors and subject as random factor. All statistical tests were two-tailed with α-level of 0.05. No statistical differences were found when comparing the HPV load of primary warts (N=79) in swabs from baseline to EOS in the treatment groups with the placebo group (digoxin + furosemide -8%; 95% CI -96 to 1952; p=0.96, digoxin -6.3%; 95% CI -96 to 2086; p=0.97 and furosemide 80%; 95% CI -92 to 3966; p=0.71). (B) Viral load change of HPV from baseline to EOS in the swabs of all treated warts (N=139) was a statistically significant only in the digoxin + furosemide group versus placebo (-94%; 95% CI -100 to -19; p=0.03). (C) Correlation between qPCR in swab samples and biopsies was investigated using a linear regression model with subject as random factor. There was a significant correlation (p<0.0001) between viral load in swabs and biopsies at the EOS. The line depicts the linear correlation: Viral load swab = -4.8+0.56*viral load biopsy.



EOT= end of treatment; EOS= end of study.

SAFETY

No treatment related study discontinuations occurred. The AE profile was comparable in all treatment groups. Nasopharyngitis, headache and influenza-like illness were the most frequently occurring mild and self-limiting treatment-emergent AEs (see Supplemental Data: Table C). No clinically relevant changes in vital signs and laboratory assessments were observed. Digoxin values measured for therapeutic drug monitoring were all below the Limit of Quantification (LoQ, 300pg/mL).

Table 3. Wart morphology in relation with wart size in digoxin + furosemide treatment group.

	Wart diameter (mm)		
	Difference[1]	**95% CI[2]**	**P-value**
Callus: Present (N=42) / Absent (N=37)	-1.71	-5.12 to 1.70	0.3203
Capillary thrombosis: Present (N=45) / Absent (N=34)	-2.51	-6.15 to 1.13	0.1730
Level Elevation: (N=44) / Flat (N=35)	-5.21	-8.60 to -1.82	0.0031
Aspect Smooth: (N=17) / Rough (N=62)	-1.86	-5.85 to 2.13	0.3357

1: Difference of the mean diameter as measured by caliper / 2: CI=confidence interval

Table 4. Response analyses per responder group. HE + IHC staining biopsies and viral load in biopsies and swabs.

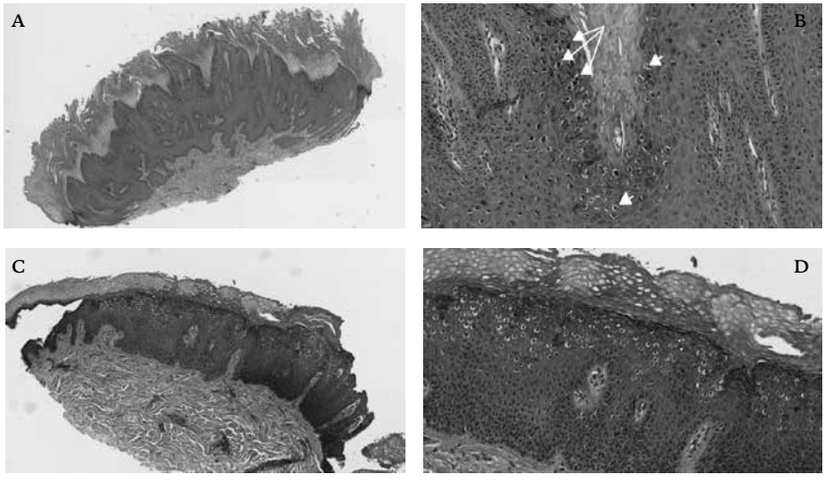
Responder	Swab baseline		Biopsy EOS		Swab EOS	
	HPV positive (n)	Mean log10 copies/PCR	Change in viral characteristics (n)	Positive test for E4, Ki-67& HPV (n)	Mean log10 copies/PCR	HPV positive (n)	Mean log10 copies/PCR
Complete (N=3)	TND[1] (1/3) HPV3 (1/3) HPV57 (1/3)	5.6[2]	0/3	E4 (0/3) Ki-67 (3/3)[3] HPV2 (1/3)	2.9[5]	HPV (0/3)	0[5]
Partial (N=6)	HPV2 (2/6) HPV27 (2/6) HPV57 (2/6)	4.7	0/6	E4 (0/6) Ki-67 (5/6)[3] (1/6)[4] HPV2 (1/6) HPV27 (1/6) HPV57 (3/6)	3.8[5]	HPV2 (2/6) HPV57 (2/6)	1.1[5,6]
Non (N=11)	HPV2 (3/11) HPV27 (4/11) HPV57 (4/11)	4.9	11/11	E4 (11/11) Ki-67 (11/11)[4] HPV2 (4/11) HPV27 (4/11) HPV57 (3/11)	8.8	HPV27 (4/11) HPV2 (4/11) HPV57 (3/11)	4.3

1: Target not detected (TND) for the 23 HPV types included in the broad spectrum genotyping assay / 2: Samples of subject without HPV DNA detected and HPV3 at baseline are not further tested for viral load and therefore not included in the mean / 3: Basal staining, restricted to the basal layer / 4: Scattered staining 5: Samples not tested are considered as zero / 6: Samples where the target is not detected are considered as zero / EOS = end of study

Discussion

This study demonstrates clear and statistically significant pharmacodynamic effects of topical ICVT on common and plantar warts with a favorable safety profile. Both lesion reduction and clearance rates indicate pharmacological activity and demonstrate proof-of-concept of ICVT in adults with cutaneous warts. Effects of ICVT was slightly more pronounced in patients with common warts. This is in accordance with previous studies wherein evident differences between response to treatment of common and plantar warts were reported.7, 30 The increased treatment resistance of plantar warts was previously described and seems to be mainly due to callus formation resulting in a decrease in cutaneous permeability of a drug.[28]

Figure 5. Histological representative cases of classical cutaneous viral warts. (A) Verruca vulgaris H&E low power view (50x) with architectural characteristic inturning of the elongated rete ridges, epidermal hyperplasia, papillomatosis, hypergranulosis, hyperkeratosis and columns of parakeratosis. (B) Verruca vulgaris H&E, detail view (200x): note koilocytes (arrowhead) and coarse granula (arrows) mostly in top layers (stratum granulosum). (C) H&E low power view (50x) of verruca plana with epidermal hyperplasia, hypergranulosis, hyperkeratosis, koilocytes in middle and upper layers. (D) verruca plana H&E, detail view (100x) note the absence of papillomatosis, parakeratosis and coarse granula. *(see inside cover for image in color)*



EFFICACY RATES THERAPIES

Efficacy rates of the most common used treatments are estimated to be around 39% for cryotherapy, 24% for salicylic acid and 46% for monochloroacetic acid. In the current study, ICVT efficacy rates were estimated to be comparable to the efficacy rates reported in literature, i.e., around 45% in common warts. However, it should be noted that the current trial consisted of subjects with treatment resistant warts that had been present for a long time (mean time of onset 4.9 – 7.6 years in the treatment groups). It can therefore be anticipated that ICVT might have shown higher efficacy rates in subjects with more recently developed warts.

DELAYED RESPONSE TO ICVT

Interestingly, wart size reduction in diameter and clearance both occurred predominantly after EOT. One explanation might be that ICVT interferes with the HPV life cycle 22, which firstly results in a reduction of HPV load and thereafter reduction in wart size. It looks like the disappearance of signs of HPV infection precedes the actual vanishing of the wart. This is supported by the fact that E4 staining, indicative of a productive infection, in the response analysis showed that partially cleared warts were in viral regression, showing less E4 signals and less papillary patterns. Another explanation could be reservoir forming of ICVT in the hyperkeratotic layer that slowly releases the drug into the lesion and thereby resulting in a delayed and prolonged response. Studies with a longer follow-up period and without the biopsy intervention at EOS have to be considered to better understand effectiveness of ICVT in both mono active and dual active form.

SYSTEMIC EFFECTS OF ICVT

Warts without application of the research gel in the active treatment groups reduced in size, in contrast to the placebo group, which suggests that this reduction was not due to spontaneous regression. The observed clearance might be explained by distant effects of the gel, i.e., increased activation of the immune system might have led to activity in untreated distant warts. Cardiac glycosides such as digoxin are known to influence the immune response at multiple levels 31, thus digoxin in the formulation might be held responsible for this. This distant clearance concept is also known from

another topical compound, imiquimod. Psoriasis patients treated with imiquimod can locally develop total body psoriasis exacerbations during treatment based on distant skin immune system activation by imiquimod.[32–34]

HPV DISTRIBUTION AND LOAD BY SWABS SUITABLE BIOMARKERS FOR CUTANEOUS WARTS

The distribution of HPV types in warts in this study was similar to the distribution found in common and plantar warts in literature 21 except for HPV1. This can logically been explained by the study sample, containing adults, whereas HPV1 infections are more prevalent among children with warts present for less than 6 months.[21]

Skin swabs have been frequently used to determine HPV status of subjects in a research setting, but not yet in relation to antiviral treatment monitoring.[35,36] Wart swabs are ideal for sampling in order to determine viral load, as the golden standard HPV status determination (biopsy) has several disadvantages such as the burden for the patient, the practical difficulty of taking multiple biopsies from a single small lesion, as well as the potential study bias caused by the curative effect of taking a biopsy.[37] The current study showed that viral load determined in swabs correlated with viral load determined from biopsies of the same wart. These data confirm the correlation previously reported by van der Kolk et al, but now in a larger sample set warranting the continued use of this marker in clinical studies.[26]

RESPONSE ANALYSES

Outcomes from microscopical and IHC analyses of the biopsies at EOS correspond with those from the viral load analysis: biopsies and swabs of the complete and partial responders have a lower viral load or are HPV negative which corresponds with loss of changes in the epithelium characteristic of viral infection, absence of E4 staining and a basal Ki-67 staining, whereas the non-responders had high viral loads in swab and biopsies. H&E staining of the biopsies showed signs of changes related to viral infection, E4 staining and a scattered Ki-67 staining. The HPV E4 protein disrupts the keratin filamentous network and inhibits formation of the cornified envelope. Detection of E4 is indicative of a productive viral infection.[22,38] Ki-67 is a biomarker for cell proliferation and in normal epithelium the Ki-67 signals are restricted to the basal layer. By reactive change, the Ki-67 positivity is also observed in the other layers of the epithelium (scattered staining).[39] From this, we can conclude that there is a clear correlation between the histopathological diagnoses, presence of E4 and Ki-67 pattern and HPV load.

Morphologic aspects of the warts could be useful to predict wart size reduction, based on the results of the current study. In clinical practice this might be helpful to have insight into the morphological characteristics when deciding about the most effective and personalised treatment.

SAFETY

Current options for therapy all have high rates of side effects including pain and irritation at the application site, blistering and scarring.[7,13] Such local irritations were not observed in the current trial.

In conclusion, our findings clearly show proof-of-concept of topical ICVT for cutaneous warts with the most pronounced effects of digoxin and furosemide when combined in a formulation for common warts. A treatment period of 42 days was well tolerated and led to significant wart size reduction and occasionally clearance. As hypothesised, wart size reduction was related to HPV load reduction measured by qPCR in swab, proving that this swab method can be a valuable, non-invasive disease biomarker for drug development in cutaneous warts. As clinical outcomes, such as clearance of lesion sites often require long-term treatment and follow-up, we indicate the found efficacy in the current study as proof-of-concept of ICVT in cutaneous warts. Further investigations to evaluate total clearance and recurrence rates after a longer treatment and follow-up period are recommended.

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