Clinical Cancer Research

# Vaccination against Oncoproteins of HPV16 for Noninvasive Vulvar/Vaginal Lesions: Lesion Clearance Is Related to the Strength of the T-Cell Response

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## Abstract

**Purpose:** Therapeutic vaccination with human papillomavirus type 16 (HPV16) E6 and E7 synthetic long peptides (SLP) is effective against HPV16-induced high-grade vulvar and vaginal intraepithelial neoplasia (VIN/VaIN). However, clinical nonresponders displayed weak $CD8^+$ T-cell reactivity. Here, we studied if imiquimod applied at the vaccine site could improve $CD8^+$ T-cell reactivity, clinical efficacy, and safety of HPV16-SLP (ISA101).

**Experimental Design:** A multicenter open-label, randomized controlled trial was conducted in patients with $HPV16^+$ high-grade VIN/VaIN. Patients received ISA101 vaccination with or without application of 5% imiquimod at the vaccine site. The primary objective was the induction of a directly *ex vivo* detectable HPV16-specific $CD8^+$ T-cell response. The secondary objectives were clinical responses (lesion size, histology, and virology) and their relation with the strength of vaccination-induced immune responses.

**Results:** Forty-three patients were assigned to either ISA101 with imiquimod ($n = 21$) or ISA101 only ($n = 22$). Imiquimod did not improve the outcomes of vaccination. However, vaccine-induced clinical responses were observed in 18 of 34 (53%; 95% CI, 35.1–70.2) patients at 3 months and in 15 of 29 (52%; 95% CI, 32.5–70.6) patients, 8 of whom displayed a complete histologic response, at 12 months after the last vaccination. All patients displayed vaccine-induced T-cell responses, which were significantly stronger in patients with complete responses. Importantly, viral clearance occurred in all but one of the patients with complete histologic clearance.

**Conclusions:** This new study confirms that clinical efficacy of ISA101 vaccination is related to the strength of vaccine-induced HPV16-specific T-cell immunity and is an effective therapy for HPV16-induced high-grade VIN/VaIN. *Clin Cancer Res; 22(10); 2342–50. ©2016 AACR.*

*See related commentary by Karaki et al., p. 2317*

## Introduction

Persistent infection of vulvar or vaginal epithelium with HPV16 may cause vulvar or vaginal intraepithelial neoplasia (VIN/VaIN) and progress into cancer (1, 2). $CD4^+$ and $CD8^+$ T-cell immunity to human papillomavirus (HPV) is required to prevent chronic disease (3) but is often weak or not demonstrable in patients with

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

M.I.E. van Poelgeest and M.J.P. Welters contributed equally to this article.

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## Translational Relevance

The spontaneous regression of HPV16$^+$ high-grade vulvar intraepithelial neoplasia (VIN) lesions occurs in less than 1.5% of patients. Recommended treatment is therapeutic excision by surgery, which is associated with high recurrence rates, disfiguration, and psychosexual morbidity. This clinical study demonstrates that vaccination with ISA101 induces objective, partial, or complete histologic regression of the lesion in more than 50% of the patients and, as such, is an effective means to treat VIN that may prevent surgical mutilation in a substantial number of patients. In addition, this study is the first, to our knowledge, that does not use *post hoc* analyses for efficacy but reports on predetermined endpoints showing that the clinical efficacy of the vaccine is related to the strength of vaccine-induced immune responses.

HPV-induced premalignant lesions (4). Consequently, spontaneous regression happens in <1.5% of VIN patients and occurs within the first 10 months following diagnosis (5). As conventional surgery is associated with high recurrence rates and disfigurations, alternative treatments are needed. Recently, we demonstrated that therapeutic vaccination HPV16 E6 and E7 synthetic long peptides (HPV16-SLP) resulted in complete and partial regressions of HPV-induced VIN lesion(s), especially in patients who displayed a strong vaccine-induced HPV16-specific T-cell response (6, 7).

Comprehensive immune monitoring revealed that CD8$^+$ T-cell reactions were weaker than those of CD4$^+$ T cells with this HPV16-SLP vaccine. Furthermore, responses were dominated by E6 oncoprotein–directed reactivity, but this could be prevented by separation of the E6- and E7-SLP (6, 8, 9). As a result, the ISA101 vaccine, comprising two simultaneous injections with a mixture of seven E6-SLP and a mixture of two E6-SLP and four E7-SLP, was developed.

The application of imiquimod on the peptide vaccine injection site enhanced the CD8$^+$ T-cell response in mice (10) and cancer patients (11). To study whether imiquimod could also improve CD8$^+$ T-cell reactivity and clinical benefit in women with HPV16$^+$ high-grade VIN/VaIN, a randomized study was conducted to investigate the immunogenicity, clinical efficacy (lesion size, histology, and virology), and safety of ISA101 with and without imiquimod at the vaccine site.

## Materials and Methods

### Study design and patients

We performed an open-label, randomized phase I/II study at two university hospitals in the Netherlands (Leiden University Medical Centre, Leiden and University Medical Centre Groningen, Groningen). Women of ≥18 years old with histologically confirmed HPV16$^+$ high-grade VIN/VaIN lesions were included. Eligible patients were HIV and HBV seronegative, not pregnant, and without autoimmune or other systemic diseases that could affect the immunocompetence of the patient. Exclusion criteria were diseases or therapies affecting immunocompetence and known hypersensitivity to the treatment components. All patients gave written informed consent before randomization in accordance with the institutional and national guidelines. The study was approved by the national Central Committee on Research Involving Human Subjects (NL21215.000.08) and the local institutional ethical committees.

### Randomization and masking

Eligible patients were randomly assigned (1:1) to ISA101 vaccinations with or without imiquimod on the vaccine site by a computer-generated list of random numbers created by an independent contract research organization (Trial Coordination Center, University Medical Center Groningen, Groningen, the Netherlands). As the study had an open-label design, the treatment allocation was not masked from patients or treating clinicians. HPV typing, histologic assessment, and immunomonitoring was blinded with respect to the treatment and clinical outcome of the patients. After all immunologic tests were performed and the data were locked, the immunomonitoring laboratory was unblinded to be able to analyze the immunologic outcomes versus lesion size, histologic, and virologic outcomes.

### Procedure

ISA101 consists of two mixtures of in total 13 SLP (25–35 amino acids in length; overlapping by 10–18 amino acids) covering the entire amino acid sequence of HPV16 E6 and E7 oncoproteins dissolved in dimethylsulfoxide/20 mmol/L PBS/Montanide ISA-51 (Seppic) 20/30/50 v/v/v. One mixture comprised seven E6-SLP injected subcutaneously in the left arm or leg; the other comprised two E6-SLPs and four E7-SLPs injected in the right limb. Each mixture contained a dose of 300 μg per peptide in a volume of 1.4 mL. The injections were given four times, with 3-week intervals. All vaccinations were administered in patient rooms by trained personnel and under supervision of the investigator (or a delegate). For patients assigned to imiquimod, commercially available sachets of 5% imiquimod were first applied on the skin overlaying the vaccination site by the study nurse 1 hour after vaccination; the patient, after appropriate instructions by the study nurse, applied the second dose of ointment at 48 hours. Patients were observed for 1 hour and later, after amendment of the protocol due to acute allergic reactions, for 4 hours following vaccination. After each vaccination, a diary was provided to the patients to help them to record any adverse events (AE) that occurred, which was collected at the subsequent visits to the clinic. Blood samples for safety and immunologic analyses were collected at baseline, just prior to the third vaccination, and 3 weeks after the last vaccination. Clinical efficacy assessments were performed at 3 and 12 months after the last vaccination. All AEs were recorded from baseline until 3 months after last vaccination and only the related (serious) AEs between the subsequent follow-up period (3 and 12 months after the last vaccination).

HPV16-specific T-cell reactivity was determined in peripheral blood mononuclear cells (PBMC) by a set of complementary assays: the lymphocyte stimulation test (LST), ICS, IFNγ-ELISPOT, cytometric bead array (CBA), and regulatory T-cell (Treg) analysis (6, 7). All assays were performed by trained personnel using standard operating procedures with predefined acceptance criteria for a positive and vaccine-induced response. The immunologic data were locked before the laboratory was unblinded to analyze immune responses versus lesion size, histologic, and virologic outcomes (S.H. van der Burg and M.J.P. Welters).

Detailed information on the immunologic assessments is described in the Supplementary Appendix.

Clinical response assessment of the lesions was performed by the same two clinicians (M.I.E. van Poelgeest and H.W. Nijman) of both centers, taking the (sum of) largest diameter in two dimensions, and reviewed together afterwards. Lesions were digitally photographed with a reference grid next to the lesion. A complete response (CR) was defined as a complete disappearance of the lesion and a partial response (PR) when $\geq$50% of the total lesion areas had disappeared. No response (NR) was defined as <50% reduction in lesion size. Biopsies were evaluated histologically according to the national guidelines for gyneco-oncologic pathology by two independent experienced gyneco-oncologic pathologists (H. Hollema and T Bosse) in a blinded fashion. A histologic response was defined as regression of VIN/VaIN to no dysplasia. The presence or absence of HPV16-DNA was determined by HPV16 PCR analysis. AEs [defined by Common Terminology Criteria for Adverse Events (CTCAE), version 3.0], injection site reactions, clinical assessments, and laboratory tests were performed after each vaccination and every 3 months during the 12 months of follow-up. On the basis of a preplanned interim safety evaluation in 10 patients, 5 of whom had been treated with at least one vaccination plus imiquimod, an independent Data and Safety Monitoring Board concluded that the treatment was safe.

### Endpoints

The primary endpoint was comparison of the directly *ex vivo* detectable $CD8^+$ T-cell response to HPV16 E6 and E7 between the treatment arms at 3 weeks after the last ISA101 vaccination. Secondary endpoints were comparison of clinical response as assessed by evaluation of the lesion size, as well as by histology and HPV16-DNA assessments at 3 and 12 months after the last vaccination, with the HPV16-specific immune response as measured by ICS, IFNγ-ELISPOT, LST, and CBA 3 weeks after the second and last vaccination. Finally, these parameters were compared with safety during the study with a focus on administration site reactions.

### Statistical analysis

To detect directly *ex vivo* vaccine-induced $CD8^+$ T-cell responses in at least 60% of the patients with the assumption of 10% at baseline based on our previous study (6) required a sample size of 16 evaluable patients per treatment arm to have 90% power with $P < 0.05$. Therefore, enrolment of at least 32 and maximally 43 patients was planned.

The primary analysis of the study data was done after the database was locked. All analyses were by intention to treat (ITT) and when indicated for the per protocol (PP) population. The ITT population consisted of all patients who were compliant to all inclusion criteria, received at least one vaccination, and at least one post-vaccination endpoint assessment for clinical and immunologic outcomes. The PP population consisted of all patients who received at least three vaccinations, had no major deviations of the time between vaccinations (<5 weeks), had endpoint assessments performed in accordance with the study protocol, and had received no additional treatment. The safety population consisted of all patients who received at least one vaccination. A statistical analysis plan was written before statistical analysis of the primary and secondary immunologic endpoints was performed (see Supplementary Appendix). Briefly, for the primary endpoint, the number of patients displaying a direct *ex vivo* detectable HPV16-specific $CD8^+$ T-cell response or not after at least three vaccinations in each treatment arm was compared using the Fisher exact test. Patients not selected for *ex vivo* analysis because of nonresponsiveness in the screening (ICS after expansion) were included as nonresponders. The secondary immunologic endpoints (proliferation, cytokine production, and IFNγ-ELISPOT) were shown to correlate with clinical efficacy in our previous VIN study (6). The strength of the HPV16-specific immune response defined as the median-specific spot count (IFNγ-ELISPOT), median stimulation index (SI in LST), or median amount of cytokine production (pg/mL in CBA) among different defined patient groups was tested by the nonparametric Mann–Whitney test. For each assay the patients were grouped according to either the treatment arm or clinical outcome after treatment: changes in lesion size (NR/PR or CR), histology (VIN present or absent in biopsy), and virology (HPV16-DNA present or absent in biopsy). In addition, patients were grouped based on the best overall response defined as a CR of the lesion and/or a histologically cleared lesion at 12 months. $P < 0.05$ was considered significant. The relation between the immunologic reactivity to one and the other peptide pools was analyzed by the linear mixed model using SPSS Statistics (version 20).

AEs were interpreted and graded according to CTCAE (version 3.0), with a focus on vaccination site reactions, 3 weeks after each vaccination and at 3 and 12 months after the last vaccination. AEs were grouped into categories for analyses. For the clinical efficacy and safety parameters, differences between treatment groups for quantitative variables were evaluated using a Student *t* test for normally distributed variables or a Mann–Whitney test for skewed distributed variables. For qualitative parameters, overall differences were evaluated using Fisher exact test (dichotomous response) or $\chi^2$ test (exact when indicated). The statistical analysis and reporting was done using SAS for Windows version 9.3. This trial is registered in the Dutch Trial Registry (http://www.trialregister.nl; number NTR1526).

## Results

### Patient characteristics

Between October 14, 2008, and September 8, 2011, 43 patients were randomized into the study (Supplementary Fig. S1). Four patients did not receive treatment and were excluded. Thirty-three (85%) patients completed the entire study. Thirty-four patients were included in the ITT analysis for clinical efficacy and 31 patients for immunologic responses. Baseline demographic and clinical characteristics were balanced between treatment groups (Table 1). Reasons for exclusion from outcome analyses are listed in Supplementary Table S1.

### A high number of objective clinical responses after vaccination

Table 2 shows the clinical results at 3 and 12 months after the last vaccination for the ITT population. No differences were observed between the two treatment arms (Table 3). Three months after the last vaccination, 53% (18/34; 95% CI, 35.1–70.2) of the patients displayed an objective regression in lesion size (CR or PR), 7 patients (21%; 95% CI, 0.1–0.4) displayed a full histologic regression, and 6 patients (18%; 95% CI, 0.1–0.4) successfully cleared HPV16. Twelve months after the last vaccination, 22 of 29 patients (75%; 95% CI, 0.6–0.9) displayed CR or PR. Seven of these patients (24%; 95% CI, 0.1–0.4) had received additional treatment to vaccination. Fifteen of 29 patients (52%;

**Table 1.** Baseline demographic and clinical characteristics of the study groups

| | ISA101 + Imiquimod (*N* = 17) | ISA101 (*N* = 22) |
|---|---|---|
| Age (years) | | |
| Mean (SD) | 41.6 (10.2) | 43.4 (12.0) |
| Range | 23.5–58.7 | 29.8–71.8 |
| Ethnic origin | | |
| Caucasian | 17 (100%) | 21 (95.5%) |
| Asian | — | — |
| Black | — | — |
| Other | — | 1 (4.5%) |
| Smoking status | | |
| None | 2 (11.8%) | 3 (13.6%) |
| Past | 4 (23.5%) | 3 (13.6%) |
| Current | 11 (64.7%) | 16 (72.7%) |
| VIN or VaIN lesion | | |
| VIN | 16 (94.1%) | 18 (81.8%) |
| VaIN | 0 | 3 (7.7%) |
| VIN and VaIN | 1 (5.9%) | 1 (5.1%) |
| Lesion grade at baseline | | |
| No dysplasia | 1 (5.9%) | 0 |
| Grade II | 5 (29.4%) | 5 (22.7%) |
| Grade III | 11 (64.7%) | 17 (77.3%) |
| Prior therapy | 11 (64.7%) | 16 (72.7%) |
| Laser therapy | 7 (63.7%) | 13 (81.3%) |
| Local excision | 7 (63.7%) | 10 (62.5%) |
| Imiquimod | 5 (45.5%) | 7 (43.8%) |
| Other | 1 (9.1%) | 3 (18.8%) |

NOTE: Data are mean (SD) or number of patients.

95% CI, 32.5–70.6) had an objective reduction in lesion size without additional treatment, 7 of whom displayed a CR and 8 a PR. A full histologic response was seen in 8 of 23 (35%; 95% CI, 0.2–0.6) and 7 of 22 (32%; 95% CI, 0.1–0.5) patients. The full histologic response coincided with clearance of HPV16.

### The use of imiquimod as adjuvant has no effect on the vaccine-induced HPV16-specific T-cell response

The goal of the study was to assess if the application of imiquimod on the vaccination site increased the HPV16-specific $CD8^+$ T-cell response. Only 4 of the 31 patients in the ITT population failed to show a $CD8^+$ T-cell response to the 24 peptide pools tested by ICS (Supplementary Fig. S2 and Fig. 1A). On average, the patients recognized 6 peptide pools (range 1–14). The use of imiquimod neither increased the number of peptide pools, to which $CD8^+$ T cells reacted as measured by ICS, nor significantly increased the response rate in the direct *ex vivo* $CD8^+$ T-cell IFNγ-ELISPOT assay (Supplementary Fig. S3 and Fig. 1B). The same holds true for the PP population (Supplementary Fig. S4). The HPV16-specific immune response was also measured by our standard 4-day IFNγ-ELISPOT, proliferation, and cytokine production assays (6). A vaccine-induced increase in HPV16-specific T-cell reactivity was observed in all patients after 2 and 3 to 4 vaccinations (Fig. 2), while the reactivity to a mix of recall antigens remained the same over all time points (Supplementary Fig. S5). The median frequency of HPV-specific T cells was high, with about 1 specific cell per 1,000 PBMC exceeding the responses observed against a mix of recall antigens. However, the increase in HPV-specific T cells was similar in both treatment arms. Thus, ISA101 induced a strong and broad HPV-specific T-cell response in all patients, but the concomitant administration of imiquimod on the vaccination site did not affect ISA101-induced immunity. Hence, all further analyses on HPV-specific immunity versus clinical responses included patients from both treatment arms.

### Predefined immune correlates of efficacy correlate with clinical outcome

We previously reported that the strength of the HPV16-specific immune response as measured by the 4-day IFNγ-ELISPOT, proliferation, and cytokine production assays correlated with clinical efficacy of vaccination (6). To validate these functional assays as immune correlates for clinical outcome, we divided the ITT patients based on clinical responses by changes in lesion size, histology, or virology. At 3 months follow-up, the group of complete clinical responders, by either of these criteria, showed a stronger HPV16-specific reactivity after vaccination in the IFNγ-ELISPOT assay (Fig. 3) in comparison with patients without such a complete clinical response. Also HPV16-specific proliferation and TNFα production was stronger after 3 to 4 vaccinations in the group of patients with a CR based on either of these three clinical measurements. Production of HPV16-specific IFNγ, IL5, and IL10 was stronger in the group of patients with a complete regression compared with noncomplete regressors (Fig. 3). After 12 months of follow-up, all immune response assays showed a stronger vaccine-induced immune response in patients with virus clearance and better overall clinical responses (Fig. 3 and Supplementary Fig. S6). All immune reactions, except IFNγ production, were stronger in patients with a histologic response, and all immune reactions, except proliferation, were stronger in the group of patients with a complete regression of the lesion. When the patients of the PP population were grouped according to outcome, differences in specific spot counts (IFNγ-ELISPOT) and both TNFα and IL5 production were comparable with those for the ITT patient population (Supplementary Fig. S7). The linear mixed model analyses revealed that responsiveness to one peptide pool correlated to a response to multiple peptide pools, suggesting a better overall fitness of the immune system of the patient to respond to vaccination (Supplementary Table S2). A *post hoc* analysis showed no correlation between the *ex vivo* detectable HPV16-specific $CD8^+$ T-cell response and clinical outcome (data not shown). In addition, no correlations were found between previously reported baseline lesion size or the percentage of HPV16-specific Tregs after vaccination (6) and clinical outcome (data not shown).

### AEs

There were two study stops during the study. Because of a sudden death after a second ISA101 vaccination, the study was put on hold on August 26, 2009, and restarted on October 8, 2009, after it became clear that the patient had died of a myocardial infarction, which was considered unlikely to be related to vaccination. On June 21, 2010, the study was put on hold because a patient developed a type I allergic reaction within two hours after vaccination, classified as a Suspected Unexpected Serious Adverse Event. The study was resumed after a protocol amendment with respect to an increased observation time after vaccination was approved by the IEC (October 28, 2010).

All vaccinated patients displayed treatment-related local and systemic AEs (Supplementary Table S3), similar to our previous study (7). Injection site reactions were long lasting, with swellings still present after 12 months (Supplementary Table S4). Treatment-related severe AEs (4% of total) are shown in Supplementary Table S5 and did not differ between the two treatment arms. As mentioned above, one patient died due to a myocardial infarction unlikely related to vaccination and 10 other patients experienced 16 serious AEs, 6 of which were treatment related

**Table 2.** Clinical results at 3 and 12 months after the last vaccination - ITT population

| | | | | | At 3 months | | | | At 12 months | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient ID | Baseline histology | Treatment group ISA101 | Number of vaccines | Baseline lesion Size ($cm^2$) | Size ($cm^2$) | Lesion response | Histology | HPV16 | Size ($cm^2$) | Lesion response | Histology | HPV16 |
| 1 | VIN3 | + imiq. | 4 | 15.5 | 7 | PR | VIN3 | + | . | . | . | . |
| 2 | VIN3 | | 4 | 16 | 0 | CR[a] | No dysplasia | − | 0 | CR[a] | No dysplasia | − |
| 3 | VIN2 | | 4 | 3 | 0 | CR | No dysplasia | − | 0 | CR | . | . |
| 6 | VIN3 | | 4 | 8 | 3 | PR | VIN2 | + | 1.5 | PR | . | . |
| 9 | VIN3 | + imiq. | 4 | 3.5 | 1 | PR | VIN3 | + | 0 | CR | No dysplasia | − |
| 10 | VIN2 | | 4 | 4 | 1.5 | PR | VIN2 | + | 0.8 | PR | VIN2 | + |
| 11 | VIN3 | | 4 | 25.3 | 14.8 | NR | VIN3 | + | 0.02 | PR[b] | . | . |
| 12 | VIN2 | + imiq. | 4 | 5 | 0.2 | PR | No dysplasia | − | 0.3 | PR | VIN3 | + |
| 14 | VIN3 | + imiq. | 4 | 0.3 | 0.1 | PR | VIN3 | + | 0 | CR | No dysplasia | − |
| 15 | VIN2 | + imiq. | 4 | 2 | 1.2 | NR | VIN1 | + | 0.5 | PR | VIN1 | + |
| 17 | ValN3 | | 4 | 2 | 1.5 | NR | ValN3 | + | . | .[c, d] | ValN3[d] | +[d] |
| 18 | ValN3 | | 4 | 2 | 2 | NR | ValN3 | + | . | .[c] | Dysplasia | + |
| 19 | VIN3 | | 4 | 4.5 | 3 | NR | VIN3 | + | 6 | NR | VIN3 | + |
| 20 | VIN2 | | 4 | 2 | 0.7 | PR | VIN3 | + | . | . | VIN3 | + |
| 21 | VIN3 | | 4 | 6.5 | 6.5 | NR | Carcinoma[e] | + | . | . | . | . |
| 22 | VIN3 | + imiq. | 4 | 2 | 1.7 | NR | VIN2 | + | 1.5 | NR | VIN3 | + |
| 24 | VIN3 | | 4 | 6.2 | 33 | NR | VIN3 | + | 10.5 | NR[c] | . | . |
| 26 | VIN2 | | 2 | 1 | 4 | NR | VIN3 | + | 0.3 | PR[c] | VIN3 | . |
| 28 | VIN3 | | 4 | 9.5 | 5.6 | NR | VIN3 | + | 9 | NR | VIN3 | + |
| 29 | VIN2 | + imiq. | 4 | 4.2 | 1 | PR | VIN3 | + | 0.2 | PR | . | . |
| 30 | VIN3 | | 3 | 10.3 | 0.5 | PR | No dysplasia | − | 0.4 | PR | No dysplasia | − |
| 31 | VIN3 | | 2 | 1.4 | 0 | CR[a] | No dysplasia | + | 0 | CR[a] | No dysplasia | − |
| 32 | VIN3 | + imiq. | 2 | 1.5 | 0 | CR | No dysplasia | . | 0 | CR | . | . |
| | ValN | | | 2.3 | 0 | CR | No dysplasia[f] | −[f] | 0 | CR | . | . |
| 51 | VIN2 | | 4 | 19 | 20 | NR | VIN2 | + | 0 | CR[g] | . | . |
| 52 | VIN3 | | 4 | 120 | 83 | NR | VIN3 | + | 121 | NR | VIN3 | + |
| 53 | VIN3 | + imiq. | 4 | 42 | 42 | NR | VIN3 | + | 17 | PR | VIN3 | + |
| 55 | VIN3 | + imiq. | 4 | 104 | 84 | NR | VIN3 | + | 33 | PR | VIN3 | + |
| 57 | VIN3 | | 4 | 3 | 4 | NR | VIN3 | + | 4 | NR | VIN3 | + |
| 58 | VIN3 | + imiq. | 4 | 44 | 2 | PR | No dysplasia | − | 0 | CR | . | . |
| 59 | VIN3 | | 4 | 8 | 6 | NR | VIN3 | + | 26 | NR | VIN3 | + |
| 60 | VIN3 | + imiq. | 4 | 30 | 0.4 | PR | Microinvasive carcinoma | + | 0 | CR[g] | . | . |
| 61 | VIN3 | + imiq. | 4 | 12 | 4 | PR | VIN2 | + | 0 | CR | . | . |
| 62 | ValN3 | | 4 | 3 | 1.5 | PR | ValN2 | + | 0 | CR[h] | . | . |
| 63 | VIN3 | | 3 | 3.2 | 1.5 | PR | VIN3 | + | 0 | CR[a] | No dysplasia | + |

NOTE: Lesion response: CR was defined as a complete disappearance of the lesion; PR was defined as a disappearance of at least 50% of the total lesion area; NR was defined as a disappearance of less than 50% of the total lesion area or as an increase in extent of the lesion. In case no lesion was visible at 3 months follow-up, no biopsy was taken at 12 months unless VIN/ValN lesions had recurred. Dots mean not available. Patients 32 and 52 had HPV16+ VIN and ValN lesions at baseline.

Abbreviation: imiq., imiquimod.

[a]Imiquimod during follow up.

[b]Laser treatment and local excision.

[c]Laser treatment.

[d]No clinical lesion assessment available. Histology and HPV typing results based on excision specimen vaginal top performed six weeks after 3 month follow-up visit.

[e]Patient 21 underwent wide local excision and sentinel node biopsy for stage 1 vulvar carcinoma.

[f]Vaginal smear showed no dysplasia, HPV16 negative. Not enough material for HPV typing on vulvar biopsy was available.

[g]Patient 51 and 60 were surgically treated for microinvasive carcinoma 9 months and 6 months after the last vaccination, respectively.

[h]Patient developed microinvasive vagina carcinoma second year vaccination.

**Table 3.** Summary of clinical results at 3 and 12 months after ISA101 vaccination with and without imiquimod - ITT population

| | ISA101 + Imiquimod ($N = 13$) | ISA101 ($N = 21$) | Total ($N = 34$) |
|---|---|---|---|
| **3 Months after last vaccination** | | | |
| Lesion response | | | |
| CR | 1 (8%) | 3 (14%)[a] | 4 (12%) |
| PR | 8 (61%)[b] | 6 (29%) | 14 (41%) |
| NR | 4 (31%) | 12 (57%) | 16 (47%) |
| Total | 13 (100%) | 21 (100%) | 34 (100%) |
| Histologic response | | | |
| Grade II/III to no dysplasia | 3 (23%) | 4 (19%) | 7 (21%) |
| No response | 9 (69%) | 16 (76%) | 25 (73%) |
| Grade II/III to carcinoma | 1 (8%)[c] | 1 (5%) | 2 (6%) |
| Total | 13 (100%) | 21 (100%) | 34 (100%) |
| Presence of HPV16 | | | |
| Negative | 3 (23%)[d] | 3 (14%) | 6 (18%) |
| Positive | 10 (77%) | 18 (86%) | 28 (82%) |
| Total | 13 (100%) | 21 (100%) | 34 (100%) |
| **12 Months after last vaccination** | | | |
| Lesion response | | | |
| CR | 6 (50%)[b] | 6 (35%)[a,c] | 12 (41%)[b,a,c] |
| PR | 5 (42%) | 5 (29%)[e,f] | 10 (34%)[e,f] |
| NR | 1 (8%) | 6 (35%)[f] | 7 (24%)[f] |
| Total | 12 (100%) | 17 (100%) | 29 (100%) |
| Missing | 1 | 4[f] | 5 |
| Histologic response | | | |
| Grade II/III to no dysplasia | 3 (38%) | 5 (33%) | 8 (35%)[g] |
| No response | 5 (62%) | 10 (67%) | 15 (65%) |
| Grade II/III to carcinoma | 0 | 0[h] | 0 |
| Total | 8 (100%) | 15 (100%) | 23 (100%) |
| Missing | 5 | 6 | 11 |
| Presence of HPV16 | | | |
| Negative | 3 (38%) | 4 (29%) | 7 (32%)[g] |
| Positive | 5 (62%) | 10 (71%) | 15 (68%) |
| Total | 8 (100%) | 14 (100%) | 22 (100%) |
| Missing | 5 | 7 | 12 |

NOTE: 11 patients had other treatment during the study, one patient in the imiquimod group and 10 patients in the no imiquimod group. Of these 11 patients, five patients were classified as CR and three patients as PR.

[a]Three patients (2, 31, and 63) used topical imiquimod.

[b]One patient (60) in the vaccination + imiquimod group underwent surgical treatment for microinvasive carcinoma.

[c]Two patients (21 and 51) were surgically treated for invasive vulvar cancer 3 and 9 months after the last vaccination, respectively.

[d]One patient (32) had both VIN and VaIN lesions. HPV test result based on vaginal smear.

[e]One patient (11) had laser treatment and surgical excision.

[f]Four patients (17, 18, 24, and 26) had laser treatment. Patients 24 and 26 had a PR, from patients 17 and 18 no lesion assessment available at 12 months.

[g]No biopsy was done at 12 months after last vaccination in case no lesion was visible at 3 months after last vaccination. In that case, the 3 months outcome was taken at 12 months. This was applicable for two patients.

[h]One patient (51) was diagnosed with vulvar carcinoma 9 months after the last vaccination and had surgical treatment.

(Supplementary Table S6). Seven patients developed an injection site ulcer, 2 of whom required special treatment (Supplementary Fig. S8). Vaccine-related acute allergic reactions occurred in 18% of the patients in each treatment arm and were likely related to the peptides (Supplementary Fig. S9). No clinically significant abnormalities in laboratory values and vital signs were observed during the study (data not shown).

## Discussion

This is the first randomized trial to evaluate the clinical and immunologic effects of the therapeutic HPV16 vaccine ISA101 and the immune modifier imiquimod applied to the skin overlaying the vaccination site. Our study shows that vaccination with ISA101 is an effective treatment for high-grade VIN/VaIN lesions and sustains the notion that clinical efficacy of HPV16-SLP vaccines is related to the strength of the vaccine-induced T-cell response, also when patients received additional treatment. The application of imiquimod to the vaccine site neither altered the safety profile of ISA101 nor the immunologic and clinical outcomes. Failure of imiquimod to improve the immunologic reactivity may be due to the superior immunogenicity of long peptide vaccines over minimal peptide vaccines (12), as the immune responses to minimal peptides was enhanced by imiquimod (13).

In this study, 84% of the patients displayed a vaccine-induced HPV16-specific IFNγ-associated $CD4^+$ and $CD8^+$ T-cell response. On the basis of our previous study (6, 7), the results of three different immune assays were predicted to correlate with clinical outcome. Indeed, the measured strength of the HPV16-specific T-cell response by IFNγ-ELISPOT, proliferation, and cytokine

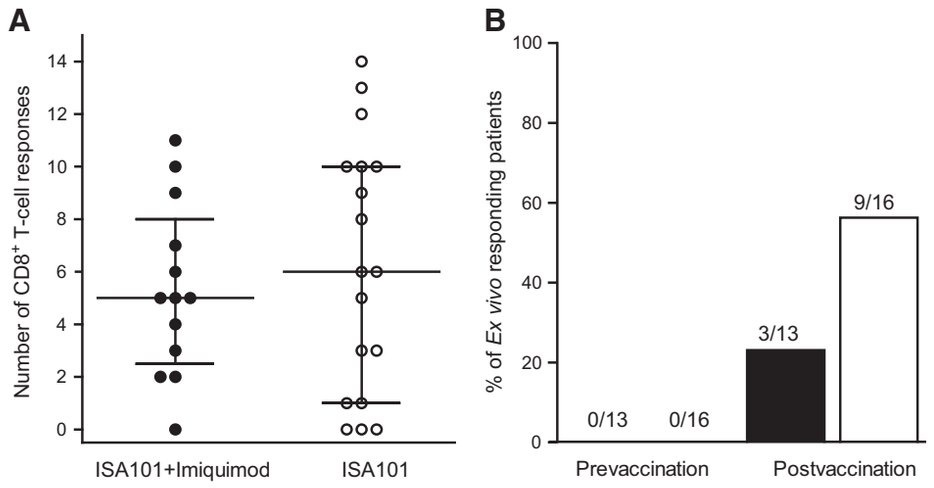


**Figure 1.**
HPV16-specific $CD8^+$ T-cell reactivity of the ITT population. A, number of positive peptide pools (of a total of 24 peptide pools) recognized by $CD8^+$ T cells after culture per patient as determined by intracellular IFNγ staining. No difference was observed ($P = 0.30$; Fisher exact test) among patients in the two treatment arms. B, percentage of patients displaying an *ex vivo* detectable HPV16-specific $CD8^+$ T-cell response as determined by IFNγ-ELISPOT assay. A nonsignificant difference was observed ($P = 0.06$; Fisher exact test) among patients in the two treatment arms. Patients are grouped according to their treatment. Black bars: ISA101 vaccination with imiquimod. White bars: ISA101 vaccination.

production at 3 and 12 months correlated with clinical responses as defined by complete disappearance of the lesions, histology, and the clearance of HPV16 from full histologic responders. This consistent finding validates the use of these immune assays for therapeutic HPV16 vaccine development.

Although in our earlier study, the presence of HPV16 virus was only assessed at 3 months after the last vaccination, this parameter was now assessed at both 3 and 12 months after the last vaccine dose. HPV-DNA was again no longer demonstrable by PCR in biopsies of patients with a full histologic response (no dysplasia) in 6 of 7 at 3 months and in 7 of 8 at 12 months. The absence of the virus in the tissue of patients with no remaining dysplasia 12 months after vaccination is likely to indicate a full cure.

Ten patients in the ISA101 group and one patient in the ISA101 plus imiquimod group received additional treatment of the lesion, mostly between 3 and 12 months after the last vaccination. Overall, 75% of the patients displayed a clinical response at 12 months follow-up. Eight patients displayed a complete histologic regression at this time, 3 of whom had also received imiquimod treatment on the lesion during the study. Although it cannot be excluded that in these patients, imiquimod may have caused the complete histologic regression (14), we have reported earlier that the clinical efficacy of imiquimod was associated with preexisting

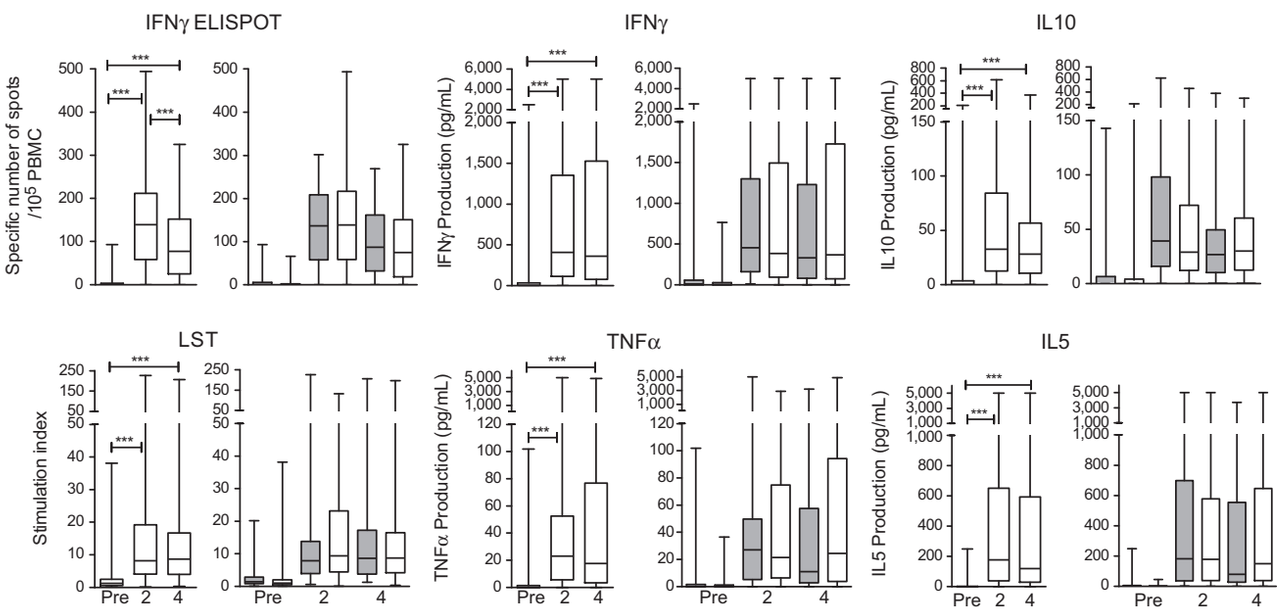


**Figure 2.**
Immune response of ITT population according to treatment. Plotted are box-whiskers, in which the median, interquartile range, as well as the minimum and maximum values, are depicted. Left are the measured values for the assays shown for all patients prior to vaccination (Pre), after two vaccinations (2) and after four vaccinations (4). Of note, one patient received three instead of four vaccinations; hence the results of this time point were used. In the plots to the right for each immune assay, the patients are grouped according to the treatment: ISA101 + imiquimod is indicated by gray bars; ISA101 by white bars. Paired *t* test analysis was performed to determine significant differences between the post-vaccination sample and pre-vaccination sample. ***, $P < 0.0001$. No differences were observed between the two treatment arms (Mann–Whitney).

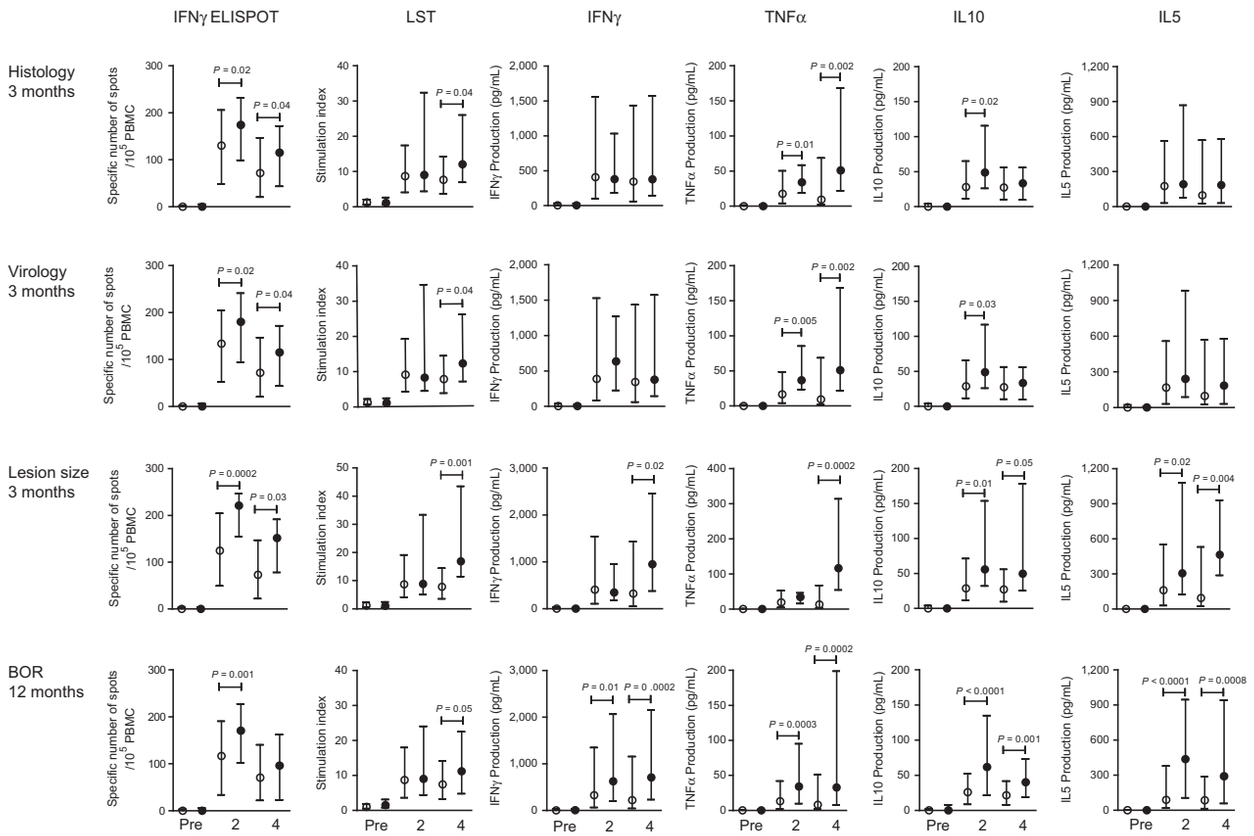


**Figure 3.**

Strength of the HPV16-specific immunity versus clinical measurements. The strength of the immune response prior to vaccination (Pre), after two vaccinations (2), and after four vaccinations (4) is depicted when ITT patients were grouped according to presence (open circle) or absence (closed circle) of VIN lesion (histology), HPV16 DNA (virology), a non-CR (NR/PR; open circles), or CR (closed circles) at 3 months after the last vaccination. In addition, at 12 months the best overall response (BOR) is displayed; closed circles represent a histologic cleared lesion and/or a clinical CR of the lesion. Open circles are all other outcomes.

HPV16-specific IFNγ-producing T cells, which in some patients are likely to have been induced by ISA101 vaccination (15). Importantly, half of the patients had an objective reduction in lesion size without additional treatment. Notably, vaccination may prevent surgical mutilation in more than a quarter of the patients, a percentage which might be increased when cotreatment with imiquimod on the lesion itself is given.

ISA101 vaccination was accompanied by a number of AEs, including allergic reactions. This was also observed after peptide vaccination in renal cell cancer patients (16). In addition, some patients experienced persisting swelling and ulceration of the skin at the injection site, similar to what is found for the tetanus vaccine, hepatitis B, and BCG vaccine (17, 18) and other vaccines with Montanide (19–21). Further optimization of clinical response rates and/or reduction of side effects may be achieved by using alternative adjuvants replacing Montanide, careful dose–response studies, and by combination of vaccination with imiquimod on the lesion.

In two independent studies we have now shown that more than half of patients with high-grade HPV16$^+$ VIN respond clinically to HPV16-SLP vaccination and that the clinical responders display a stronger vaccine-induced HPV16-specific immune response. Individual variation between patients in immune and clinical response may be due to the individual differences in immune microenvironment (22, 23). The selection of patients based on specific microenvironmental factors may increase clinical responsiveness in subsequent trials.

## Disclosure of Potential Conflicts of Interest

C.J.M. Melief reports receiving commercial research grants from and has ownership interest (including patents) in ISA Pharmaceuticals. S.H. van der Burg reports receiving commercial research support from and is a consultant/advisory board member for ISA Pharmaceuticals and is listed as a co-inventor on a patent, which is owned by The Leiden University Medical Center and licensed to ISA Pharmaceuticals on the use of long peptides as a vaccine platform. No potential conflicts of interest were disclosed by the other authors.

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