Clinical Research

Cotton swabs for the measurement of NF-κB, IFN-γ, and FOXP3+Treg from lesions of anogenital wart patients

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pISSN: 0853-1773 • eISSN: 2252-8083
https://doi.org/10.13181/mji.oa.247496
Med J Indones. 2024;33:165–72

Received: March 11, 2024
Accepted: June 11, 2024

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ABSTRACT

BACKGROUND Local tissue immunity plays a significant role in anogenital warts' (AGW) pathomechanism and persistence. Assessing biomarkers from lesions instead of serum is recommended to evaluate therapeutic response. Since biopsy is invasive, it is necessary to find less invasive and more comfortable methods. This study aimed to assess the reliability of cotton swabs and tape stripping for evaluating AGW's lesions biomarkers.

METHODS We compared cotton swab *versus* tape stripping method to quantify nuclear factor-κappaB (NF-κB), interferon-gamma (IFN-γ), and FOXP3+regulatory T cell (FOXP3+Treg) from 3 patients with AGW in the preliminary study. The method was selected based on contamination possibility, side effects, and a simpler approach. The main study examined 48 patients with AGW for reliability and reproducibility using the best sampling method from preliminary result and Spearman's Rho analysis, while considering the HIV status and CD4+ counts.

RESULTS Both cotton swabs and tape stripping obtained adequate protein content for biomarkers examination. However, the tape stripping method was causing serum contamination and painful for patients due to the stripping. The total lesion volume in cotton swab method was positively correlated with all patients' NF-κB ($p = 0.001$). IFN-γ had a negative correlation in all reactive HIV patients ($p = 0.012$). FOXP3+Treg and CD4+ counts were negatively correlated with total volume in reactive HIV patients ($p = 0.046$ and 0.017, respectively).

CONCLUSIONS The cotton swab method was reliable in examining NF-κB, IFN-γ, and FOXP3+Treg due to its convenience and lack of serum contamination from AGW lesions, potentially improving patient comfort and practical benefits.

KEYWORDS immunity biomarker, interferon-gamma, NF-κB, regulatory T cell, skin tape

Anogenital warts (AGW) are a common sexually transmitted infections (STIs), with millions of cases occurring each year in both sexes worldwide. They are caused by human papillomavirus (HPV), and approximately 90% of cases are caused by types 6 and 11.[1,2] The Indonesian Sexually Transmitted Infections Study Group detected an increasing trend of AGW cases compared to other STIs in recent decades. Treatment has been challenging owing to the high recurrence rate and prolonged therapy.[3,4] Biomarker screening is necessary to assess disease severity, persistence, recurrence, and response to therapy. Moreover, local immunity is essential in pathomechanism and persistence; thus, collecting samples from AGW lesion tissue to examine immune biomarkers is preferable.[5] However, challenges come from collecting samples, especially in sensitive areas such as genital and perianal regions, as

biopsy procedures can cause injury and discomfort. Therefore, a less invasive, easier to perform, less traumatic, and more comfortable method is required for collecting AGW tissue samples.

Existing studies for immunity biomarkers have collected samples only from biopsies and serums of patients with AGW.[5–7] Research on interferon-gamma (IFN-γ) in AGW has predominantly utilized serum samples from patients[6,7] and FOXP3+regulatory T cell (FOXP3+Treg) from biopsy [5]. The cotton swab and tape stripping sampling technique (stripping adhesive tape glued to lesions sequentially) are minimally invasive and have been widely used in dermatological research but have never been performed on AGW tissue.[8–11] In AGW, the host's immune system becomes less effective because HPV has several evasion mechanisms and strategies, such as minimal expression of viral proteins and the absence of an inflammatory process.[12] The complete HPV life cycle occurs solely within epidermal cells, where virus-laden keratinocytes naturally die and shed following the normal skin keratinization cycle; therefore, no danger signal is present.[13]

The shifting of T helper (Th) responses in HPV infection (Th1 decreasing and Th2 increasing) results in decreased serum IFN-γ.[14] However, Radi et al[6] reported that 90% of patients of AGW had a 13-fold increase in IFN-γ serum level. The "dual face" of the IFN-γ effect plays a controversial role in inflammation and tumor progression, specifically through the downregulation of major histocompatibility complexes and the upregulation of indoleamine 2,3-dioxygenase.[14,15] Thus, it is difficult to draw a definitive conclusion about the exact role of IFN-γ in HPV infection, especially within lesions, as there have been no previous studies on IFN-γ in HPV lesions.

In untreated AGW, FOXP3+Treg cells are observed in high numbers in HPV 6 and 11 lesions and are suspected to have immunosuppressive functions and correlate with the size of AGW lesions, allowing AGW's progression and recurrence.[5] Viruses can manipulate and modulate the signaling pathway generated by nuclear factor-κappaB (NF-κB), blocking apoptosis and various viral elimination processes and providing negative feedback to the virus's E1 (early gene) to mediate HPV replication.[16] However, no studies have examined NF-κB from AGW lesions. Thus, this study aimed to determine the correlation between immunological biomarkers and total lesion volume of AGW in different HIV statuses. Nonetheless, a preliminary study is needed to determine the most comfortable and reliable method between tape stripping and cotton swabs to collect specimens for immunological biomarkers measurement, namely NF-κB, IFN-γ, and FOXP3+Treg.

METHODS

The study included three patients for the preliminary study and 48 patients in the main study. The preliminary study qualitatively assessed three patients to determine which technique would be adequate, entail fewer side effects, reduce the risk of systemic (blood and serum) contamination, offer a simpler approach, and enhance comfort for collecting lesion material using cotton swabs or tape stripping. All lesion samples obtained using tape stripping and cotton swabs were analyzed in duplicate for the NF-κB, IFN-γ, and FOXP3+Treg concentrations. The study was conducted in the Dermatology and Venereology ward of Cipto Mangunkusumo Hospital between July and November 2022. The protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No: KET-686/UN2.F1/ETIK/PPM.00.02/2022) and all patients signed an informed consent. This trial was registered under protocol ID NCT06281353 in ClinicalTrials.gov.

Selection criteria

The inclusion criteria included patients with AGW lesions measuring 5–50 mm in diameter, who had not received any AGW therapy in the past 2 weeks, who were willing to participate, and who had signed an informed consent form. Three AGW lesions per patient were selected from a consistent location and displaying identical clinical forms and sizes, for sampling using cotton swabs and tape stripping. The total volume of each lesion (calculated as length × width × height) was measured in mm^3 using skin calipers. Tissue samples from different lesions on the same patients were collected via cotton swabs and tape stripping for the enzyme-linked immunosorbent assay (ELISA) examination of NF-κB, IFN-γ, and FOXP3+Treg.

Sample size calculation

The sample size for the main study was calculated to obtain a more comprehensive assessment of the biomarker's reliability and reproducibility, using the formula of the correlative study to assess the strength

measurement of the R coefficient with an estimation of R = 0.4 between the total lesion volume and biomarker values, taking into account the HIV status of the patients (using the 4th generation HIV ELISA test) and CD4+ counts. The correlation coefficient (r) measures the strength and direction of the linear relationship between two variables, and in this context, Spearman's correlation coefficient was utilized. This calculation resulted in a minimum of 47 participants.

Sample collection

Samples were obtained by skin stripping and cotton swabs, as shown in Figure 1. All samples were sent to the laboratory for <2 hours. Samples were weighed, converted to homogenate, and frozen at –80°C.

Skin stripping

The tape was applied to the dry surface lesion with standard pressure (225 g/cm^2) for 10 sec and then quickly stripped using tweezers.[8] This process was repeated up to 20 times on the same lesion. No blood was allowed on the tape, or the procedure had to be repeated on another lesion with a new sterile tape.

Cotton swab

The AGW lesion area was cleaned using a wet gauze with 0.9% NaCl for 5 min. Then, the selected lesion was swabbed slowly on a rotated axis using a sterile cotton swab (M or S size; Onemed®, Indonesia) dipped in 0.9% NaCl 2–5 times under moderate pressure until all sides of the cotton swab touched the lesion for at least 20 swabs. No blood was allowed on the swab; if so, the procedure was repeated on another lesion using a new sterile cotton swab. The swab was then placed in a sample cup marked with the patient's data and sampling date, cut at the base of the tip, sealed, and placed in a transfer container containing ice packs.

Biomarkers examination

Total protein content

Total protein content was measured using a colorimetric Bradford protein assay method (Protein Assay Dye Reagent Concentrate No. 500-0006; Bio-Rad®, USA) and with 0.2–0.9 mg/ml bovine serum albumin as the protein standard solution. The sample was put into a different test tube, added with 5 ml of diluted dye reagent and vortex, and incubated at room temperature (5 min–1 hour). The absorbance was measured at 595 nm.[17]

NF-κB immunoassay

Semiquantitative measurement of NF-κB p65 was used for the preliminary study, using SimpleStep ELISA Kit (ab176648; Abcam®, UK). A 50 µl control sample was put into appropriate wells; subsequently, 50 µl of the antibody cocktail was added to all wells and incubated for 60 min under room temperature. The samples were aspirated and washed thrice with 350 µl 1× wash buffer. Next, each well was supplemented

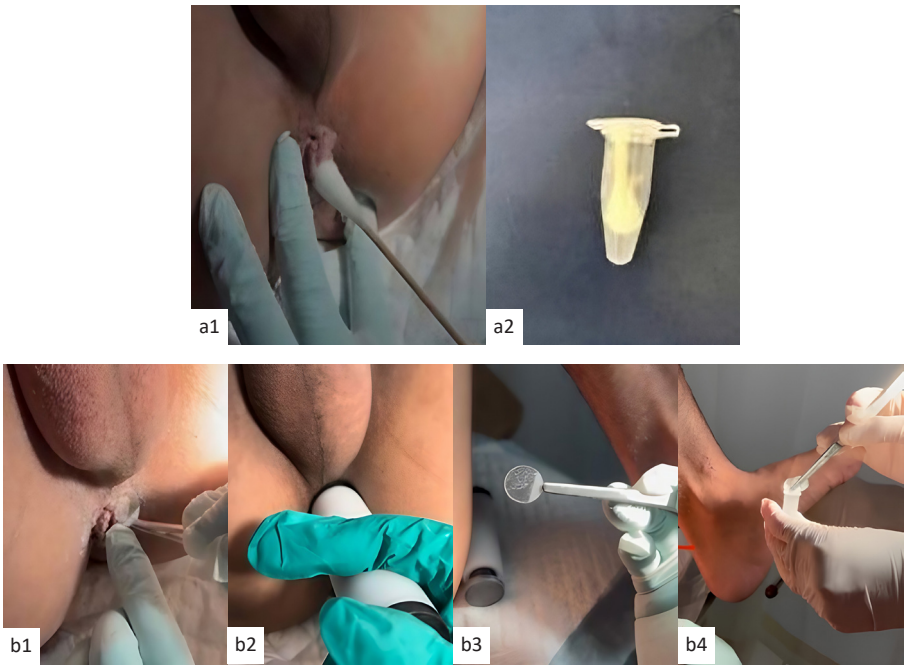

Figure 1. Sampling techniques using cotton swab and tape stripping. The anogenital wart (AGW) lesion was swabbed using the sterile cotton swab tip (a1) and the swab tip was inserted into the sample cup (a2) and delivered immediately to the laboratory. The surface of AGW was placed with the tape (b1), then the applicator was used to press the tape (b2), next the tape stripping was removed using tweezer (b3) and inserted into the sample cup (b4)

with 100 µl tetramethylbenzidine (TMB) substrate and incubated for 15 min. A stop solution was added, and the optical density (OD) of the plate was read at a wavelength of 450 nm.[18]

Quantitative measurement was used in the main study to assess NF-κB levels using Human NF-κB-p65 ELISA Kit (E-EL-H1388; Elabscience®, USA), where 100 µl of the standard solution was added to various wells and incubated for 90 min at 37°C. Subsequently, the solution was replaced with 100 µl of biotinylated detection Ab and further incubated at 37°C for 60 min before the plate was washed and aspirated 3 times. A conjugate solution was then added and incubated for 30 min at 37°C. Afterward, the plates were washed and aspirated 5 times, and 90 µl of substrate reagent was incubated for 15 min at 37°C. Finally, 50 µl of stop solution was added, and the plate was read at a 450 nm wavelength.[19]

IFN-γ immunoassay

Using the IFN-γ Human ELISA Kit (ab100538; Abcam®), standard solutions, reagents, and samples were put into different wells, covered, and incubated for 2.5 hours under room temperature. The solution was discarded, and the cells were washed 4 times using a 1× wash solution. A total of 100 µl of the previously prepared biotin antibody was added and incubated for 1 hour at room temperature. Subsequently, 100 µl of streptavidin solution was added and incubated for 45 min. The solution was then discarded, and 100 µl of TMB One-Step Development Solution was added and incubated for 30 min. Afterward, 50 µl of stop solution was added, and readings were measured at a wavelength of 450 nm.[20]

FOXP3+Treg immunoassay

After preparing the Human FOXP3 ELISA Kit (ab285290; Abcam®), the plate was washed twice with 1× wash solution. Standard solution (100 µl) was added to different wells, followed by 0.1 ml of biotin detection antibody solution. This mixture was incubated and then discarded, and the wells were washed 3 times with 1× wash solution. Subsequently, each well was filled with 350 µl of wash buffer, then aspirated, and incubated for 30 min at 37°C. Afterward, the solution was discarded, and the wells were washed 5 times with 1× wash solution. In each well, 90 µl of TMB substrate was added, covered, and incubated in the dark (15–30 min, 37°C). At the end of the incubation, blue shadows should be visible in the first three to four wells. Then, 50 µl of stop solution was added to each well, and the result was read at 450 nm within 20 min.[21]

Statistical analysis

The correlation between total lesion volume, NF-κB concentration, IFN-γ, FOXP3+Treg, and CD4+ counts in lesions of patients with AGW was analyzed using Spearman's Rho correlation coefficients.[22] A subgroup analysis of the correlations was performed based on HIV status and CD4+ counts of the patients with AGW.

Table 1. AGW location, total volume, biomarker examination, and side effects from the three patients in the preliminary study

Characteristics	Patient 1	Patient 2	Patient 3
Total volume (mm^3)	4,557	1,196.75	875.3
Total protein (ng/ml)			
Tape stripping	21.03	34.46	15.1
Cotton swab	102.57	272.02	105.6
NF-κB concentration (10^{-3} pg/ng protein)			
Tape stripping	3.20	1.94	4.80
Cotton swab	0.71	0.20	0.70
IFN-γ concentration (10^{-1} pg/ng protein)			
Tape stripping	5.17	3.28	7.32
Cotton swab	1.15	0.41	1.12
FOXP3+Treg concentration (10^{-3} ng/ng protein)			
Tape stripping	6.51	2.90	10.30
Cotton swab	2.00	0.30	0.90
Side effect			
Tape stripping			
Pain	Y	Y	Y
Bleeding	Y	N	Y
Erosion	Y	Y	Y
Uncomfortable	Y	Y	Y
Cotton swab			
Pain	N	N	N
Bleeding	N	N	N
Erosion	N	N	N
Uncomfortable	N	N	N

AGW=anogenital wart; FOXP3+Treg=FOXP3+regulatory T cell; IFN-γ=interferon-gamma; N=no; NF-κB=nuclear factor-κappaB; Y=yes
All patients were male and had AGW in perianal area

RESULTS

The characteristics on three adult male patients with AGW in the preliminary study are shown in Table 1. Tape stripping was repeated 20 times on the same lesion; however, this method induced pain, erosion, and excoriation, making the samples unreliable due to blood contamination. The procedure was only performed until 10 strips before placing them in a cup and cryobox with ice packs. Repeated washes and enhanced OD are needed due to the low total protein extraction from tape strips and some falling below detectable levels.

Table 2. Patient's characteristics and biomarker's concentrations of the AGW lesions by cotton swab technique from the main study

Variables	n (%) (N = 48)
Male sex	39 (81)
Age (years), median (min–max)	27.5 (19–52)
HIV status	
Reactive	32 (67)
CD4+ <200 cells/µl	8 (25)
CD4+ ≥200 cells/µl	24 (75)
Nonreactive	16 (33)
Total lesion volume (mm^3), median (min–max)	712.56 (51.00–14369.26)
FOXP3+Treg concentration (10^{-3} ng/ng protein), median (min–max)	1.17 (0.004–200.00)
IFN-γ concentration (10^{-1} pg/ng protein), median (min–max)	63.16 (0.12–1782.00)
NF-κB concentration (10^{-3} pg/ng protein), median (min–max)	9.95 (0.10–85.25)

AGW=anogenital wart; FOXP3+Treg=FOXP3+regulatory T cell; IFN-γ=interferon-gamma; NF-κB=nuclear factor-κappaB

Table 3. Analysis of correlations between lesion's total volume and immunological biomarkers from AGW patients with regards of their HIV status

Total lesion volume	IFN-γ	NF-κB	FOXP3+Treg	CD4+
All patients (N = 48)				
r	−0.362	0.420	−0.207	-
p	**0.006**	**0.001**	0.079	-
Nonreactive HIV patients (n = 16)				
r	−0.291	0.526	−0.132	-
p	0.137	**0.018**	0.313	-
Reactive HIV patients (n = 32)				
r	−0.398	0.347	−0.303	−0.377
p	**0.012**	**0.026**	**0.046**	**0.017**

AGW=anogenital wart; FOXP3+Treg=FOXP3+regulatory T cell; IFN-γ=interferon-gamma; NF-κB=nuclear factor-κappaB
Spearman's Rho test were used for these correlation analyses

Table 2 showed that males were among 81% of participants. In terms of HIV status, 67% were reactive, 75% of them with CD4+ counts above 200 cells/µl. Clinical measurements revealed an initial visit lesion with a total volume median of 712.56 mm^3. Ratios of various biomarkers were also assessed: FOXP3+Treg (median of 1.17 10^{-3} ng/ng protein), IFN-γ (median of 63.16 10^{-1} pg/ng protein), and NF-κB (median of 9.95 10^{-3} pg/ng protein).

Table 3 showed that total lesion volume exhibited a strong, positive, statistically significant correlation with NF-κB ($p<0.001$), a moderate negative correlation with IFN-γ ($p = 0.006$), and a weak, statistically insignificant negative correlation with FOXP3+Treg, as illustrated in Table 3.

Infection, inflammation, and immune status significantly affect various aspects of AGW. Accordingly, we assessed the levels of NF-κB, IFN-γ, FOXP3+Treg, and CD4+ counts based on HIV status (Table 3). Patients with nonreactive HIV demonstrated that the initial total lesion volume was strongly positively correlated with NF-κB ($p = 0.018$) and exhibited weak, statistically insignificant negative correlations with IFN-γ and very weak negative correlations with FOXP3+Treg. Conversely, patients with reactive HIV displayed statistically significant moderate negative correlations between

initial total lesion volume and CD4+ counts, IFN-γ, and FOXP3+Treg ($p<0.05$). In these patients, NF-κB was moderately positively correlated with total lesion volume ($p = 0.026$).

DISCUSSION

No studies have examined the biomarkers of AGW lesions using noninvasive methods. However, some of the methods have already been reported for disorders, including tape-stripping for atopic dermatitis,[9,11] and cotton swabs for diabetic ulcers[10]. Thus, this study is the first study examining several immunological biomarkers using noninvasive sampling method for AGW lesions.

A preliminary study showed that both methods could successfully and appropriately collect sufficient protein. The fragile AGW surface contributed to clumps or fragments when collected using cotton swabs; thus, the protein concentration was 4–8 times higher. Repeated stripping was required after lifting the visible thin tissue layer, especially when contaminated with serum or blood due to skin erosion and excoriation. In laboratory assessments, the total protein content from tape stripping often falls below the detectable levels. Therefore, the cotton swab method has emerged as a reliable approach for laboratory analysis.

Furthermore, we observed that the amount of NF-κB, IFN-γ, and FOXP3+Treg in the preliminary study was higher in the cotton swab method than in the tape stripping samples, considering the concentration calculation based on total protein. This phenomenon arose from potential serum contamination during the tape stripping process despite excluding blood-contaminated samples. Upon calculation, it was observed that the concentrations of NF-κB, IFN-γ, and FOXP3+Treg in cotton swab samples were lower than in tape stripping, which is attributable to the higher total protein content in the former. Moreover, we observed the same fluctuation trends between the two techniques, indicating that both are reliable sampling methods for AGW lesions.

Further, the total lesion volume of all patients in this study exhibited a moderate negative correlation with IFN-γ, suggesting that the latter had a protective effect and confirming the theory that increased IFN-γ is generally associated with a more effective immune response of the host.[23] However, no study has examined the IFN-γ levels from the lesions tissue of AGW. This contrasts with other research on patients with AGW serum, which differs from the findings of the AGW lesion tissue in our study. Radi et al[6] observed that IFN-γ serum levels were increased in 90% of patients with AGW, and Le Poole et al[7] showed that patients with AGW serum exhibited a 13-fold increase in IFN-γ. No information was available regarding the immunological status of the patients in their study. These findings suggest that IFN-γ exerts an immunosuppressive effect, contradicting our study, which demonstrated that the larger the total lesion volume, the lower the concentration of IFN-γ. These contradictory results stem from the dual role of IFN-γ.[14,15] Despite the theoretical association of increased IFN-γ with a more effective immune response, recent data indicate that a decline in serum IFN-γ corresponds with the transition from a Th1 to a Th2 response in AGW[24] and suggests that IFN-γ contributes to immunosuppression.[7]

This study observed that all patients, including nonreactive and reactive HIV, showed a statistically significant positive correlation between total lesion volume and NF-κB, which varied from moderate to strong correlation. This finding indicated that higher concentrations of NF-κB were associated with increased total lesion volume. Furthermore, this observation supports the theory that HPV exploits NF-κB by manipulating signaling pathways. The E1 and E2 proteins of HPV induce the activation of NF-κB, which typically restricts viral genome replication; however, the E6 and E7 proteins of HPV stimulate NF-κB activation. Contrarily, they mediate negative feedback on E1 and E2, thereby enhancing HPV replication and increasing viral copy numbers.[16] Thus, the increased NF-κB concentration would correspond to the increase in viral replication and total lesion volume.

This study revealed that total lesion volume was statistically significant only for FOXP3+Tregs in patients with reactive HIV, demonstrating a moderate negative correlation. This result indicates that lower concentrations of FOXP3+Tregs in AGW lesions was correlated with increased total lesion volume, particularly in patients with reactive HIV, suggesting an immunoprotective role for FOXP3+Tregs. While statistically insignificant, a negative correlation was observed in both all patients and those with nonreactive HIV. Contrarily, Cao et al[5] reported that higher accumulations of FOXP3+Tregs in the lesions of patients with AGW were associated with the

enlargement of single lesion biopsies but not total volume, suggesting an immunosuppressive effect. However, their study conducted on a small sample with varying AGW sizes and did not assess immune status.

Infection, inflammation, and immune status significantly affect various aspects of AGW. The levels of NF-κB, IFN, Treg, and CD4+ counts differ across immune statuses, impacting the incidence and severity of AGW. Our analysis revealed a statistically significant, moderate negative correlation between CD4+ counts and initial total lesion volume in the 32 reactive HIV cases. We observed that as CD4+ counts decreased, the initial total lesion volume of AGW tended to increase. This observation is consistent with previous studies, which suggest that HIV infection and immunodeficiency alter the relationship between HPV infection and the prevalence of AGW.[6,25] It is well-known that the number of Langerhans cells, CD4+, macrophages, neutrophils, and natural killer cells are reduced in HIV-associated immunodeficiency, leading to alterations in local immunity and modulation of HPV infection at the tissue level.[26] Low CD4+ counts (≤200 cells/µl) are predictors associated with an increased risk of AGW.[27] However, there is currently no existing research that explicitly addresses the correlation between CD4+ counts and total lesion volume.

Furthermore, our study revealed a statistically significant moderate negative correlation between the total lesion volume of patients with reactive HIV and IFN-γ, supporting the theory that IFN-γ has a protective effect, with increased IFN-γ associated with a more effective host immune response.[23] In contrast, among patients with nonreactive HIV, there was a weak and very weak negative correlation between total lesion volume and IFN-γ and FOXP3+Treg, respectively; however, these findings were statistically insignificant, which might be attributable to the small sample size (only 16 patients).

This study investigated the immunology of AGW sourced from lesions using a noninvasive approach not previously used and assessed the reliability and reproducibility of the cotton swab method. This innovative method may offer new insights into the immunological aspects of AGW. Additionally, this study encompassed a larger sample size compared to prior studies, thereby enhancing the credibility of the results. However, a limitation of this research is the absence of serum biomarker analysis to correlate with lesion biomarkers. In conclusion, the cotton swab technique is a favorable and reliable method for the immunoassay examination of AGW lesions, particularly for NF-κB, IFN-γ, and FOXP3+Treg analysis.

Conflict of Interest

The authors affirm no conflict of interest in this study.

Acknowledgment

None.

Funding Sources

None.

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