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The likely possibility of predicting treatment outcomes of cervical lesions using serum FOXP3 and P16INK4A as shown by a cohort study in South Western Uganda

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Abstract

Introduction There is a need to assess the potential of blood-based biomarkers to detect treatment outcomes of cervical lesions. We determined the association between serum P16ink4A and FOXP3 concentrations and treatment outcomes of cervical lesions at a clinic in Southwestern Uganda.

Methods In this prospective cohort study, participants with cytologically and/or histologically confirmed cervical intraepithelial neoplasia (CIN) ($n=90$) and cervical cancer (CC) ($n=90$) were monitored for 12 months. After consent, clinical and demographic data were recorded, blood was collected, and serum P16ink4A and FOXP3 were measured (quantitative ELISA) at baseline and 12 months post-treatment. With multinomial logistic regression, we determined the association between treatment outcomes and serum P16ink4A and FOXP3 concentrations in STATA 17 using P-values of <0.05 as statistically significant.

Results Of the 180 participants initially enrolled, 62 returned for the 12-month follow-up assessment. At this time point, 47 participants presented with cleared lesions, 6 with persistent lesions, and 9 with progressed lesions. All participants exhibiting disease progression ($n=9$) were CC cases, while 82.98% (39/47) of those with cleared lesions had LSIL. For raised (>0.0545 ng/ml), relative to reduced serum FOXP3 (≤ 0.0545 ng/ml), the risk of progression relative to clearance of invasive cervical cancer would increase by 27.82. Also raised (>0.946 ng/ml) relative to reduced (≤ 0.946 ng/ml) serum P16INK4A, the risk of persistence relative to clearance of low grade cervical lesions would increase by 5.16 times, given other variables remain constant.

Conclusion Though results are not statistically significant and imprecise, serum FOXP3 and P16ink4A concentrations are likely associated with persistence and progression of cervical lesions. Their measurement may benefit the prognostic monitoring of cervical lesions.

Keywords Cervical intraepithelial neoplasia, Cervical cancer, P16ink4A, FOXP3, Persistence, Progression, Clearance

1 Introduction

The increased burden of cervical cancer in low-resource settings is largely due to disparities in diagnostics [1]. Worldwide, there were 770,828 estimated incident cervical cancer cases in 2020 [2]. Cervical cancer (CC) is the second most common cancer among women aged 15 to 44 years [2]. While in the US, over 11,500 new cases are diagnosed and about 4,000 women die of this cancer per year [3], rates are much higher in developing countries where it accounts for more than 270,000 deaths annually [5, 6], especially in sub-Saharan Africa [4–6]. Cervical cancer incidence stands at 43/100,000 cancer cases in East Africa [7], and in Uganda, the age-standardized cervical cancer incidence is higher than the global average at 56.2 per 100,000 women [8]. Based on our most recent study in western Uganda, the prevalence of cervical intraepithelial lesions is approximately 6% [9].

Uganda has implemented a test and treat approach towards cervical cancer [10], in which the main screening tests include Pap smear cytology and visual inspection with acetic acid (VIA) [11]. Women who undergo such screening are aged 18 to 65 years, with screening recommended every three years [11]. The Uganda Ministry of Health has started rolling out HPV test as a cervical cancer screening test for HIV-positive women. For women with negative results from VIA or Pap smear cytology, the World Health Organisation (WHO) recommends screening intervals of three to five years. For women with negative HPV-DNA tests, re-screening should be done after five years. Extended intervals beyond five years may be considered following consecutive negative screenings and for older women. The WHO also recommends that women who have undergone treatment for cervical pre-cancerous lesions should receive post-treatment follow-up testing and review within 12 months [12].

In addition to other challenges, diagnosis of cervical lesions remains key, especially in resource-limited countries. Traditional diagnostic techniques such as VIA and Pap tests are discomforting for patients during specimen collection and carry many barriers to their uptake [13–18]. Both tests are based on subjective results and thus bear challenges with respect to uniformity [19]. The Pap test has been reported to have low to moderate sensitivity [20] in screening for cervical neoplasms. The Pap test is said to lead to many repeat tests and many unnecessary colposcopies [21].

Limitations with respect to Pap smear's modest sensitivity (50–75%) [22–24] and specificity (80–90%) [20, 25] have prompted the search for new biomarkers specific not only for the different histologic subtypes of cervical cancer [26] but also for predicting diagnosis and prognosis [27]. Such biomarkers may be used as stand-alone tests or in conjunction with traditional tests to improve prognostic monitoring of cervical lesions [28]. A combination of different blood-based biomarkers, such as proteins, can greatly improve accuracy in detecting cervical cancerous lesions [29] even at the post-treatment level.

P16ink4A is a cell cycle regulator protein. Its amount and role are tightly regulated in normal cells [30, 31] and it inhibits cyclin-dependent kinases 4 and 6 through phosphorylation of the retinoblastoma (Rb) protein [31]. During the transformation phase of the HPV infection, the viral oncogene E7 binds the Rb protein and results in increased P16ink4A levels [32]. Therefore, P16ink4A has been considered to serve not only as a surrogate marker for persistent high-risk (Hr) HPV infection [33, 34] but also as a sensitive and specific marker for cervical dysplasia, calling for its usage in screening and

diagnosis [35–39]. Indeed immunohistochemical expression of P16ink4A has been shown to be related to the degree of histological dysplasia and malignancy [40]. Reduced expression of P16ink4A is also associated with improved disease-free survival and overall survival, thus indicating its potential as a valuable prognostic marker [41].

Forkhead/winged-helix transcription factor box P3 (FOXP3) is a regulator for regulatory T cells (T reg) development and function, and belongs to the Forkhead protein family of transcription regulators. It is expressed in regulatory T cells [42] and drives their potential to block an immune response [43–48]. There is no demonstratable FOXP3 expression in normal cervical tissue. However, there is a significant increase in FOXP3 expression from CIN1 all through to cervical cancer [42]. This expression has been associated with not only the development, progression and prognosis of cancers including cervical cancer [42, 49–54], but it is also significantly and positively related to P16ink4A expression [42].

Hence, FOXP3 and P16ink4A have been widely and successfully applied in grading of cervical lesions using immunohistochemical methods [33, 34, 55–57] though with several challenges including longer turn-around-time and poor reproducibility [58]. It is against such a background that we studied serum concentrations of proteins like FOXP3 and P16ink4A for the likely possibility of predicting treatment outcomes of cervical cancer and cervical intraepithelial neoplasia.

2 Materials and methods

2.1 Study design

This study employed a prospective cohort study design in which we followed up the cases from our earlier case-control studies [59, 60]. In the previous studies, we purposively sampled women who sought cervical cancer care at the cervical cancer clinic of Mbarara Regional Referral Hospital (MRRH) from April 2022 to June 2023. The cases were those women who had a positive VIA and were confirmed (Pap smear cytology or Histology) to have any cervical lesion of any grade (prior to treatment) and were followed up as guided by the routine standard of care. The controls were those women who were confirmed negative for intraepithelial lesion or malignancy and were not followed up in this current study. This prospective cohort took twelve months.

2.2 Study setting

We conducted this prospective study at the cervical cancer clinic and the histopathology laboratory at Mbarara Regional Referral Hospital (MRRH) and the pathology department of Mbarara University of Science and Technology (MUST), respectively. MUST and MRRH are located in Mbarara City, southwestern Uganda and are both government institutions. MRRH runs specialised clinics including the cervical cancer clinic and it serves all districts of western Uganda as well as neighbouring countries including Tanzania, Rwanda and Democratic Republic of Congo. The clinic is run by a gyneoncologist, medical residents, nurses, and midwives. At MRRH, the screening program employs a "screen and treat" approach (immediate treatment after positive screening), in which the main screening tests include Pap smear cytology or VIA [11] for women aged 20 to 65 years and < 50 years respectively, depending on clinical observations and availability, at a screening interval of 3 years [11]. The Ministry of Health has started rolling out HPV tests as a cervical cancer screening test for HIV-positive women.

The confirmatory tests for cervical intraepithelial lesions, including PAP smears or histology, are performed in the pathology department of the hospital/university. The colposcopic examination is done following a positive screening test, and it helps to verify the presence of lesions and determine the degree and type of lesions, either CIN or cancer [12, 61, 62]. During colposcopy, biopsies can be collected directly from any area that appears abnormal [12]. Also from colposcopy, it can be determined whether to use cryotherapy (for low grade precancerous lesions) or LEEP (for larger or high grade precancerous lesions) as treatment strategies [63] or surgery, radiotherapy and chemotherapy (for CC) mostly offered at UCI [11]. Uganda has also opted for the neoadjuvant chemotherapy (NACT) based on the resource stratified clinical practice guideline from the American Society of Clinical Oncology (ASCO) [64]. Early cervical cancer stages are being managed following the modified NACT protocol, after a multidisciplinary discussion on a case by case basis, while late stages are managed with high doses of vaginal brachytherapy with or without chemotherapy [11]. Women who undergo treatment for cervical pre-cancerous lesions receive post-treatment follow-up testing and review at 6 months and again at 12 months while those women who undergo treatment for cervical cancer receive post-treatment follow-up testing at 12 months.

2.3 Sampling procedure

We followed up with cases from previous studies [59, 60], which had been selected through purposive sampling method. These participants were selected from among all women who sought cervical cancer services at the cervical cancer clinic of MRRH during the time of the study.

2.4 Sample size determination

In this prospective cohort study, we followed up with a total of 180 cases (90 CIN cases and 90 CC cases), as calculated and used previously [59].

2.5 Inclusion and exclusion criteria

This study included all women who reported to the cervical cancer clinic at MRRH during the time of the study and consented to participate. However, any woman who was too ill to consent was excluded. We excluded all those women who had already been started on treatment, those with insufficient data in their patient charts, as well as those with other secondary malignancies already diagnosed. We also planned to exclude or stop following up any woman that, at any time, developed any other malignancy.

2.6 Data collection

Upon arrival at the clinic, participants were assessed for eligibility to participate in the study, and written informed consent was sought. After the provision of written informed consent, participants were taken through a description of the study and its objectives. Pap smears were then collected, and VIA and other tests were performed thereafter for confirmation of lesions.

2.7 Demographic and clinical data collection

At baseline, (case-control study) [59], after routine care at the clinic, potential participants were explained to and taken through the consent process, filling out the

questionnaire and blood specimen collection. A validated questionnaire was used to collect the quantitative data from respondents, which was supplemented with more information from patient files or charts if needed. Recordable data on the enrolment form included patient's demographics, including country, district, age, and other risk factors to cervical intraepithelial lesions. We also captured the VIA, Pap test, and colposcopy results together with histology results, if necessary, stating the exact grade of lesion and the treatment modality offered. For all cytology and histology specimens, examination and grading of lesions followed the Bethesda grading system (2014). Cytological grading of cervical lesions included LSIL, ASCUS, ASCH, and HSIL while histological grading of cervical lesions included CIN1, CIN2, CIN3, and CIS. We also captured the clinical stage for CC cases at baseline and at 12 months post-treatment.

Qualified and experienced research assistants collected blood specimens from every participant, using the venipuncture technique, and these blood specimens were used to determine levels of all the biomarkers independently. Blood specimens were collected from participants at baseline and at 12 months after the initial visit.

At follow up, we ascertained treatment outcomes following the standard of care. Clearance was ascertained by complete elimination or resolution of precancerous lesions, or cancerous cells following treatment at follow-up screenings, with tests such as VIA or Pap smears [65, 66]. Persistence was ascertained by the continued presence of lesions after treatment, indicating that the treatment did not fully eliminate the disease [67]. Progression was ascertained by the advancement of abnormal cell changes in the cervix from a lower-grade state (such as low-grade squamous intraepithelial lesions (LSIL) or cervical intraepithelial neoplasia (CIN 1) to a more severe state (such as high-grade squamous intraepithelial lesions (HSIL) or CIN 2/3 and potentially to invasive cervical cancer, or from a lower clinical stage to a higher clinical stage (such as from stage 1 to stage 2, 3 or 4), often indicating that the cancer is growing or spreading despite treatment [65, 66].

2.8 Blood specimen handling

Centrifugation, separation, and storage of samples was performed by a Medical Laboratory scientist in the MUST Clinical and Research Laboratory. For P16ink4A and FOXP3, the collected blood specimens were left to stand upright at room temperature to ensure complete clotting and clot retraction. Then, the samples were centrifuged at 1500 g for 15 min at 4 °C to separate the serum. Serum was then picked with an automatic pipette, and transferred into new sterile cryovial tubes, leaving behind the cells and the buffy coat. The cryovial tubes with their contents were capped, and immediately frozen at -80°c until analysis.

2.9 Measurement of serum FOXP3 and P16ink4A concentrations

We used P16ink4A (Human) ELISA Kit and FOXP3 (Human) ELISA Kit, all from Elab sciences, following manufacturer's instructions. These blood samples were stored in MUST Clinical and Research Laboratory at -80 °C and then analysed at the Makerere University College of Veterinary Medicine, Centre for Biosecurity and Global Health. We adopted the biomarker concentrations categories as described previously [59, 60]. These categories were generated using Phil Clayton's cutpt, which establishes a cut-off

point for each biomarker on the ROC curve that is closest to the point with ideal diagnostic values for sensitivity.

2.10 Statistical analysis

Data was collected by the principal investigator with the help of research assistants at baseline and at 12 months post-treatment. It was then entered into an Excel spreadsheet (Microsoft Office Professional Plus 2013, version 15.0.4675.1003, Microsoft Inc, USA) and then imported into STATA 17 (Stata Corp LLC, College Station, Texas, United States) software. Descriptive statistics were employed to describe the population using frequencies, means ± standard deviations (SDs), and median values for continuous variables. We also employed chi-square tests, frequencies, and proportions to describe categorical variables.

Serum levels of each biomarker were determined at baseline and at the end of the follow-up period, and compared using the analysis of variance (ANOVA) test, for all the categories of lesions and treatment outcomes, taking p-values of < 0.05 as significant. We used multinomial logistic regression to determine the association between treatment outcomes at 12 months and serum P16ink4A and FOXP3 concentrations. Data was presented as relative risk ratios with 95% confidence intervals, and P-values of < 0.05 were taken to be statistically significant.

3 Results

3.1 Socio-demographic characteristics of study participants

We followed and monitored a total of 180 participants from women who sought services at the cervical cancer clinic at MRRH, and these included 90 cases of CC and 90 cases of CIN. The mean age among the CC cases (51.13, +/-13.01) was significantly higher than that of CIN cases (35.06, +/-7.71) ($p < 0.001$).

Most of the CC cases (79%, 71/90) and 57% (51/90) of CIN cases were residents of districts of Southwestern Uganda other than Mbarara and central Uganda ($p < 0.001$). The majority of CC participants (57%, 51/90) and 60% (54/90) of the CIN participants were married, while 4% and 22% of the CC and CIN, respectively, were single and this distribution in marital statuses was statistically significant ($p < 0.001$).

The highest attained level of education among our study participants was generally primary level and below, with 53%(48/90) of CC cases having attained a maximum of preprimary and 52%(46/90) of CIN cases having attained a primary level of education ($p < 0.001$). The proportion of study participants living with HIV was generally high, with 76%(68/90) of CC cases and 52%(47/90) of CIN cases reporting so, and this distribution across study groups was statistically significant ($p < 0.002$). More than half of CC participants (63%, 57/90) and 44% (38/90) of CIN participants were not using contraceptives. Among those that reported usage of contraceptives, 57.6% (19/33) of the CC and 77.6% (38/49) of CIN participants used hormonal methods ($p < 0.009$).

The mean FOXP3 baseline concentration (0.13 +/- 0.15) among CC participants was significantly higher than that of CIN participants (0.06 +/- 0.02) ($p < 0.001$). Similarly, the baseline P16INKK4A mean concentration (1.45 +/- 1.11) among CC participants was significantly higher than that of CIN participants (1.09 +/- 0.66) ($p < 0.01$), as shown in Table 1.

Table 1 Socio-Ddemographic haracteristics of study participants

Variable	Category	Cancer	CIN	Test	*p*-value
		N **= 90**	***N*** **= 90**		
Age		51.13(13.01)	35.06(7.71)	Ind. t test	< 0.001*
Age group	20–29	2 (2%)	22 (24%)	Fisher's exact	< 0.001*
	30–39	18 (20%)	38 (42%)		
	40–49	21 (23%)	29 (32%)		
	50–59	20 (22%)	1 (1%)		
	60-max	29 (32%)	0 (0%)		
Region of residence	Central	4 (4%)	2 (2%)	Fisher's exact	< 0.001*
	Other districts	71 (79%)	51 (57%)		
	Mbarara	15 (17%)	37 (41%)		
History of high BP	No	71 (79%)	67 (75%)	Fisher's exact	0.6
	Yes	19 (21%)	23 (25%)		
History of Diabetes	No	70 (78%)	78 (87%)	Chi-square	0.12
	Yes	20 (22%)	12 (13%)		
Marital status	Single	4 (4%)	20 (22%)	Fisher's exact	< 0.001*
	Married	51 (57%)	54 (60%)		
	Divorced	28 (31%)	16 (18%)		
	Widowed	7 (8%)	0 (0%)		
Highest level of education	Never studied	32 (36%)	5 (6%)	Fisher's exact	< 0.001*
	Preprimary	48 (53%)	3 (3%)		
	Primary	10 (11%)	46 (52%)		
	Secondary	0 (0%)	22 (25%)		
	Tertiary	0 (0%)	6 (7%)		
	University	0 (0%)	7 (8%)		
HIV status	Negative	22 (24%)	42 (47%)	Fisher's exact	0.002*
	Positive	68 (76%)	47 (52%)		
	Unknown	0 (0%)	1 (1%)		
Smoking	No	90 (100%)	85 (94%)	Fisher's exact	0.059
	Yes	0 (0%)	5 (6%)		
Contraceptive use	No	57 (63%)	38 (44%)	Chi-square	0.009*
	Yes	33 (37%)	49 (56%)		
Type of contraceptive ($n = 82$)	IUD	14 (42.4%)	8(16.3%)	Fisher's exact	0.009*
	Hormonal	19 (57.6%)	38 (77.6%)		
	Condom	0 (0%)	3 (6.1%)		
Presenting complaint	None	52 (58%)	61 (68%)	Fisher's exact	0.011*
	Abnormal discharge	6 (7%)	4 (4%)		
	Others	8 (9%)	11 (12%)		
	Cervicitis	14 (16%)	14 (16%)		
	Painful micturition	10 (11%)	0 (0%)		
Age at sexual debut (years)		18.96(2.87)	18.79(2.77)	Ind. t test	0.69
Baseline FOXP3 concentration (ng/ml)		0.13(0.15)	0.06(0.02)	Ind. t test	< 0.001*
Baseline P16INK4A concentration (ng/ml)		1.45(1.11)	1.09(0.66)	Ind. t test	0.01*

FOXP3- Forkhead-Box-Protein P3; *-statistically significant ($p \leq 0.05$); Continuous variables, Age, Baseline FOXP3 concentration, Baseline P16INK4A concentration and Age at sexual debut are presented as mean (standard deviation)

3.2 Treatment outcomes for cervical lesions at 12 months post-treatment of study participants at Mbarara regional referral hospital

Of the 180 at baseline, 62 study participants completed the post-treatment follow-up and monitoring at 12 months, giving a loss to follow-up rate of 65.6%. Of those who returned for 12 months post-treatment follow-up, 47 participants had cleared lesions,

6 had persistent lesions, and 9 participants had progressed lesions. Most of the participants with cleared cervical lesions, 82.98% (39/47), initially had LSIL. However, all those participants who had persistent lesions also initially had LSIL 100% (6/6). A total of 9 participants had progressed either from lower CC clinical stage to a higher stage or from precancerous lesions to CC. This distribution in the 12 months post-treatment follow-up was statistically significant ($p < 0.001$).

For age distribution, more than a third (44%, 4/9) of all participants with progressed lesions were aged between 50 and 59 years. Also, 33% (3/9) of participants with progressed lesions were aged 60 years and above. Among participants whose lesions persisted, 33% (2/6) were aged between 40 and 49 years. The same proportion was observed for those aged between 30 and 39 years.

The study found that 64% (30/47) of participants with cleared lesions and 78% (7/9) of those with progressed lesions were married. However, 50% (3/6) of those with persistent lesions were single ($p = 0.18$). The majority of participants with cleared lesions (60%, 28/47), persistent lesions (50%, 3/6), and progressed lesions (56%, 5/9) were living with HIV though not statistically significant ($p = 0.91$), as shown in Table 2.

3.3 Distribution of follow-up serum Foxp3 and p16ink4a concentrations across treatment outcomes of cervical lesions of study participants at Mbarara regional referral hospital

The 12 months follow-up mean serum FOXP3 concentration was found to be significantly ($p < 0.001$) high among participants with progressed lesions (0.13 ng/ml +/-0.09) compared to mean concentrations among those with persistent (0.05ng/ml +/-0.01) and cleared lesions (0.05ng/ml +/-0.02). Similarly, the follow-up mean serum p16INK4A concentration was significantly ($p = 0.006$) high (1.66ng/ml +/-0.99) among participants with progressed lesions compared with mean concentrations among participants with cleared lesions (0.98ng/ml +/-0.47) and persistent lesions (1.12ng/ml +/-0.35). We observe that the follow-up mean serum p16INK4A and FOXP3 concentrations were almost the same across cleared and persistent categories of treatment outcomes.

Considering the categorization of serum concentrations, raised FOXP3 was observed in 78% (7/9) of participants with progressed lesions. In the same regard, 72% (34/47) of participants with cleared lesions and 67% (4/6) of participants with persistent lesions had reduced FOXP3 concentrations. Furthermore, raised p16INK4A was observed in 67% (6/9) of participants with progressed lesions. Also, 40% (19/47) of participants with cleared lesions, 67% (4/6) of participants with persistent lesions had a raised p16INK4A as shown in and Table 3.

3.4 Association between follow up serum FOXP3 concentrations and treatment outcomes of cervical lesions among study participants at Mbarara regional referral hospital

For raised follow-up serum FOXP3 concentrations relative to reduced follow-up serum FOXP3, the relative risk for persistence relative to clearance of low grade cervical lesions would be expected to increase by a factor of 1.34 given the other variables in the model are held constant, though not statistically significant ($p = 0.81$) and imprecise. Also, for raised follow-up serum FOXP3 concentrations relative to reduced follow-up serum FOXP3, the relative risk for progression relative to clearance of high grade cervical lesions and ICC would be expected to increase by a factor of 27.82 given the other

Table 2 Treatment outcomes for cervical lesions at 12 months' post-treatment of study participants

Variable	Category	Cleared	Persisted	Progressed	Test	*p*-value
		***N*=47**	***N*=6**	***N*=9**		
Initial cervical Lesions	ASCUS	1 (%)	0 (%)	0 (%)	Fisher's exact	< 0.001*
	CANCER	2 (%)	0 (%)	9 (%)		
	HSIL	5 (%)	0 (%)	0 (%)		
	LSIL	39 (%)	6 (%)	0 (%)		
Age group	20–29	12 (26%)	2 (33%)	0 (0%)	Fisher's exact	< 0.001*
	30–39	19 (40%)	2 (33%)	0 (0%)		
	40–49	14 (30%)	2 (33%)	2 (22%)		
	50–59	1 (2%)	0 (0%)	4 (44%)		
	60-max	1 (2%)	0 (0%)	3 (33%)		
Residence	Central	4 (9%)	2 (33%)	2 (22%)	Fisher's exact	0.27
	Other districts	42 (89%)	4 (67%)	7 (78%)		
	Mbarara	1 (2%)	0 (0%)	0 (0%)		
History of high BP	No	37 (79%)	4 (67%)	8 (89%)	Fisher's exact	0.66
	Yes	10 (21%)	2 (33%)	1 (11%)		
History of Diabetes	No	39 (83%)	5 (83%)	8 (89%)	Chi-square	0.91
	Yes	8 (17%)	1 (17%)	1 (11%)		
Marital status	Single	8 (17%)	3 (50%)	0 (0%)	Fisher's exact	0.18
	Married	30 (64%)	2 (33%)	7 (78%)		
	Divorced	9 (19%)	1 (17%)	2 (22%)		
Education level	Never studied	4 (9%)	0 (0%)	4 (44%)	Fisher's exact	0.027*
	Preprimary	0 (0%)	1 (17%)	0 (0%)		
	Primary	22 (47%)	2 (33%)	5 (56%)		
	Secondary	10 (21%)	3 (50%)	0 (0%)		
	Tertiary	6 (13%)	0 (0%)	0 (0%)		
	University	5 (11%)	0 (0%)	0 (0%)		
HIV status	Negative	19 (40%)	3 (50%)	4 (44%)	Fisher's exact	0.91
	Positive	28 (60%)	3 (50%)	5 (56%)		
Smoking	No	45 (96%)	5 (83%)	9 (100%)	Fisher's exact	0.31
	Yes	2 (4%)	1 (17%)	0 (0%)		
Contraceptive use	No	18 (40%)	3 (50%)	7 (78%)	Chi-square	0.11
	Yes	27 (60%)	3 (50%)	2 (22%)		
Type of contraceptive	IUD	6 (20%)	0 (0%)	7 (78%)	Fisher's exact	0.007*
	Hormonal	23 (77%)	3 (100%)	2 (22%)		
	Condom	1 (3%)	0 (0%)	0 (0%)		
Initial presenting complaint	None	1 (2%)	0 (0%)	0 (0%)	Fisher's exact	0.53
	Abnormal discharge	7 (17%)	0 (0%)	0 (0%)		
	Others	2 (5%)	1 (17%)	0 (0%)		
	Cervicitis	32 (76%)	5 (83%)	9 (100%)		
Age at sexual debut (years)		18.94(2.59)	19.17(2.71)	17.45(2.29)	ANOVA	0.26

IUD-Intrauterine Device; BP-Blood Pressure; Continuous variable Age at sexual debut is presented as mean (standard deviation); *-statistically significant ($p \leq 0.05$)

Table 3 Distribution of follow-up serum Foxp3 and p16ink4a concentrations across treatment outcomes of cervical lesions of study participants

Variable	Category	Cleared	Persisted	Progressed	Test	*p*-value
		N = 47	*N* = 6	*N* = 9		
Follow UP FOXP3 (ng/ml)		0.05(0.02)	0.05 (0.01)	0.13 (0.09)	ANOVA	< 0.001*
Follow UP P16INK4A (ng/ml)		0.98(0.47)	1.12(0.35)	1.66(0.99)	ANOVA	0.006*
Follow UP FOXP3 Categories	Reduced Foxp3	34 (72%)	4 (67%)	2 (22%)	Fisher's exact	0.017*
	Raised Foxp3	13 (28%)	2 (33%)	7 (78%)		
Follow UP P16INK4A Categories	Reduced p16	28 (60%)	2 (33%)	3 (33%)	Fisher's exact	0.26
	Raised p16	19 (40%)	4 (67%)	6 (67%)		

Continuous variables FOLLOW UP FOXP3 (ng/ml) and FOLLOW UP P16 (ng/ml) are presented as mean (standard deviation); *-statistically significand ($p \leq 0.05$)

Table 4 Association between follow up serum FOXP3 concentrations and treatment outcomes of cervical lesions among study participants

Variable	Relative risk	*P* Value	95% CI
Persisted lesion (LSIL)	1.34	0.81	0.12–15.18
Progressed lesion (HSIL + CC)	27.82	0.10	0.52-1483.94

CI-Confidence Interval; All data was presented after controlling for age, region of residence, family planning usage and type, HIV status, highest education attained, marital status, smoking, history of blood pressure and history of diabetes

Table 5 Association between follow-serum P16INK4A concentrations and treatment outcomes of cervical lesions among study participants

Variable	Relative risk	*P* Value	95% CI
Persisted lesion (LSIL)	5.16	0.22	0.37–71.91
Progressed lesion (HSIL + ICC))	0.44	0.63	0.02–12.25

CI-Confidence Interval; All data was presented after controlling for age, region of residence, family planning usage and type, HIV status, highest education attained, marital status, smoking, history of blood pressure and history of diabetes

variables in the model are held constant, though not statistically significant ($p = 0.10$) and imprecise as shown in Table 4.

3.5 Association between follow-up serum P16INK4A concentrations and treatment outcomes of cervical lesions among study participants

For raised follow-up serum P16INK4A concentrations relative to reduced follow-up serum P16INK4A, the relative risk for persistence relative to clearance of low grade cervical lesions would be expected to increase by a factor of 5.16 given the other variables in the model are held constant, though not statistically significant ($p = 0.22$) and imprecise. For raised follow-up serum P16INK4A concentrations relative to reduced follow-up serum P16INK4A, the relative risk for progression relative to clearance of high grade cervical lesions and ICC would be expected to reduce by a factor of 0.44 given the other variables in the model are held constant, though not statistically significant ($p = 0.63$) and imprecise as shown in Table 5.

4 Discussion

Here, we reported a likely association between raised serum concentrations of FOXP3 and P16ink4A and the progression of cervical lesions. This shows the potential usage of serum FOXP3 and P16ink4A serum concentrations in predicting the treatment outcomes of cervical cancer and cervical intraepithelial neoplasia in our study population. To the best of our knowledge, this is the first study to predict treatment outcomes of

cervical lesions using blood-based biomarkers. In this study, baseline serum concentrations of FOXP3 and P16ink4A following initial diagnosis and 12 months post-treatment for cervical cancer and cervical intraepithelial neoplasia were measured.

We observed that most of the cervical cancer participants with progressed lesions were aged 50 years and above, were married, living with HIV, using IUDs, less educated, and presented with cervicitis. It has been demonstrated earlier that HIV positivity and being married likely increase rates of HPV infection and probably persistence [68–70]. Also, we take note of the fact that the majority of women in Uganda are engaged in polygamous relationships [71], which likely increases their chances of HPV infection and hence persistence. However, this population had earlier been described to have a mean CD4 count of 493.392 cells per μl and a viral load of.

4719.833 copies per μl [9]. The fact that cervicitis was a common complaint among those with progressed lesions highlights the importance of STIs in the development of cervical lesions as well as treatment outcomes. In this population, we earlier demonstrated a high prevalence of Chlamydia [72], one of the most important STIs in cervical cancer, which could be responsible for cervicitis. A low level of education, as shown in this study, is strongly linked with low socio-economic status as well as reduced knowledge of cervical cancer [73] and they could be responsible for the progression of cervical lesions.

In this study, we demonstrated the likely importance of FOXP3 and P16ink4A in predicting treatment outcomes of cervical lesions. FOXP3 and P16ink4A genes display co-expression, while FOXP3 significantly regulates P16ink4A expression [74, 75]. FOXP3 is a member of the forkhead/winged-helix family and a transcription factor specifically expressed on Tregs. It plays a crucial roles in the development and functions of Treg cells [76, 77]. Recent studies revealed that FOXP3 expression is not only specifically linked to Tregs naturally generated in the thymus but also in the cytoplasm and/or nucleus of cancer cells, and it promotes tumor progression and, consequently, poor disease outcomes characterized by metastases [78, 79]. P16ink4A, a cyclin-dependent kinase inhibitor 2 A, is a tumor suppressor protein that downregulates cell multiplications and plays important roles in cell cycle arrest in malignancies, and hence loss of this tumor suppressor biomolecule in malignancies has been associated with tumor progression [80].

The baseline and follow-up distribution of serum FOXP3 and P16ink4A across treatment outcomes were significantly different among our study participant groups. This finding was in line with our earlier observations in this population which reported the likely beneficial use of circulating P16ink4A in the detection of cervical lesions [74]. FOXP3 expression was significantly higher ($p < 0.001$) among participants with progressed lesions 0.13(0.09) than those with persistent lesions 0.05(0.01) as well as those with cleared lesions 0.05(0.05).

Similarly, serum P16ink4A concentrations across treatment outcomes were significantly high ($p = 0.009$) among participants with progressed lesions 1.66(0.99) compared to those with persisted 1.12(0.35) and cleared lesions 0.98(0.47). This demonstrates the extent of gene expression as it has been shown to be highest in CC cases followed by CIN II-III and CIN I [81]. It is worth noting that all participants with progressed lesions were from the cervical cancer group. These findings uphold the fact that FOXP3 and P16ink4A expressions are upregulated in cervical cancers [74, 82, 83].

Although not statistically significant (p = 0.10), we observed that in the cases of raised follow-up serum FOXP3 concentrations relative to reduced follow-up serum FOXP3, the relative risk for progression relative to clearance of cervical lesions would be expected to increase by a factor of 27.82. This demonstrates that elevated FOXP3 expression would likely result in the progression of cervical cancer. This is in line with the roles of FOXP3. FOXP3 elevation has been demonstrated as a mechanism of immune evasion by tumor cells as it downregulates chronic inflammation in the tumor microenvironments thereby promoting tumorigenesis [48, 84, 85].

The prognostic role of FOXP3 has also been shown in other cancers. For instance, Lee et al. studied the prognostic impact of FOXP3 expression in triple-negative breast cancer and reported that FOXP3-positive Treg cells had stronger prognostic significance than FOXP3-negative Treg cells and concluded that FOXP3-positive Tregs are an independent prognostic factor for overall survival (hazard ratio 2.4) and progression-free survival with hazard ratios of 2.0 [86].

Similar observations were recorded for the association between follow-up serum P16ink4A concentrations and treatment outcomes. For raised follow-up serum P16ink4A concentrations relative to reduced follow-up serum P16ink4A, the relative risk of persistence relative to clearance of cervical lesions was expected to increase by 5.16, although this observation was not statistically significant (p = 0.22). Conversely, in cases of raised follow-up serum P16ink4A concentrations relative to reduced follow-up serum P16ink4A, the relative risk for progression relative to clearance of cervical lesions was expected to reduce by a factor of 0.44. This justifies the upregulation of P16ink4A in cancer progressions and its downregulation in cleared cervical cancer cases. P16ink4A has been reported to be a major antitumor biomolecule (a potent inhibitor) that arrests cytokinesis at the G1 or S stage of the cell cycle [74, 87]. Raised serum FOXP3 and P16ink4A concentrations are thus associated with cervical cancer progression. However, they are not only specific to cervical cancers as they have been reported as prognostic indicators in several other cancers like breast cancers, colorectal cancers, and squamous cell carcinomas [88–90].

In this population, we take note of other potential factors that could likely be associated with treatment outcomes. From our earlier observations, we reported that lipids and insulin resistance markers are associated with poor treatment outcomes in this same population [91]. Other factors that could have led to poor treatment outcomes include obesity, a condition that is not only prevalent in this population but also associated with cervical lesions [92].

We strongly take note of the preliminary exploratory nature of this study and hence more work needs to be done. All serum samples used in this study were stored at recommended temperatures and then run as a batch. We believe this storage time may have led to some minimal reductions in the concentrations of FOXP3 and P16ink4A. We also report that we experienced a very high loss of follow-up rate, especially among participants in the cancer group, and this could have lowered the power of our study to some degree. However, this study had been designed with an adequate sample size to take care of loss to follow-up, though not to the observed extent. With the small sample size involved in the analysis of treatment outcomes, the regression estimates are unstable, underpowered and hence may not conclusively be relied on. In the presentation of our data, we put emphasis on the overall diagnosis rather than specific histological grading.

Being preliminary in nature, we did not provide exact classifications based on CIN grades for precancerous lesions and clinical staging for CC cases since some categories would have very few observations, which would hinder meaningful statistical analysis. Therefore, this study did not show differences between CIN I and CIN II/III, and early/late stage, which would have been clinically impactful. In the same regard, we did not capture the exact hormonal contraceptives being used by the study participants. We also take note of the fact that the clinical stage of cervical cancer or grades of CIN may have had individual contributions to treatment outcomes, which is not catered for in this study.

5 Conclusion

This study provides preliminary findings that serum concentrations of FOXP3 and P16ink4A are likely associated with the persistence of cervical intraepithelial neoplasia and the progression of cervical cancer. Quantitative measurement of circulating P16ink4A may also be beneficial in monitoring the prognosis of cervical cancer based on objectivity in its measurement and the relatively low cost compared to current testing methods. More population-based studies are recommended to refine the prognostic value of these biomarkers further. Larger prospective studies are also recommended to further understand the biological mechanisms behind FOXP3 and P16ink4A and the clearance, persistence, or progression of cervical lesions.

Abbreviations

ASCUS	Atypical Squamous Cells of Undetermined Significance
CIN	Cervical Intraepithelial neoplasia
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HrHPV	High Risk Human Papilloma Virus
HSIL	High Grade Squamous Intraepithelial Lesion
IUD	Intrauterine device
LEEP	Loop Electrosurgical Excision Procedure
LSIL	Low Grade Squamous Intraepithelial Lesion
MRRH	Mbarara Regional Referral Hospital
MUST	Mbarara University of Science and Technology
PAP	Papanicolaou
SD	Standard deviation
STATA	Statistical Software for Data Science
VIA	Visual Inspection with acetic acid

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Author contributions

The corresponding author, FS, conceived the idea and developed the first draft of the manuscript. Co-authors TCR, DT, NN and JN supervised the whole project, from data collection to analysis and reviewed the manuscript. HL and CMC reviewed and provided overall guidance in the entire write-up and approved the final version prior to submission. JNN participated in data analysis. All authors reviewed and approved the final version of the manuscript. All authors are accountable for all aspects of this manuscript.

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Data availability

Data which was based on to generate this article is available from the corresponding author, but only upon meaningful request.

Declarations

Ethics approval and consent to participate

This study was cleared by the Mbarara University of Science and Technology Research Ethics Committee (MUST-REC) (MUST-2022-612) and Uganda National Council for Science and Technology (UNCST) (HS2722ES). The Hospital Director, Mbarara Regional Referral Hospital, also provided clearance before the commencement of data collection. All participants in this cohort study received the standard package of care at the cervical cancer clinic. The fact that this study involved human research participants, we ensured that all procedures adhered to the declaration of Helsinki. With the help of research assistants, we sought written informed consent from every participant before taking part in the study. Participants also consented to have their blood samples stored and files or chats reviewed, by research assistants, for more clinical and demographic information. We used study numbers, not names, for all collected data and blood specimens. We delinked all participants' identifiable information during data analysis. A separate record was kept in the clinic to help capture patient's telephone numbers that were used during follow up. All participant interaction with the research team took place in a private and comfortable room, free from other disturbances and only accessible to one participant at a time. All filled/completed documents were filed before calling in the next participant. Participants received their VIA results on the spot. Pap smear results were ready in not more than a week's time after sample collection. The results of biomarkers were ready after one month from the time of sample collection. All results were delivered to participants by the nurse at the clinic. In cases where results returned with abnormal results, participants were linked to relevant departments for appropriate management.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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