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Novel Serum Protein Biomarkers for Precancerous Cervical Lesions and Cervical Cancer

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Abstract

Cervical cancer is a health problem worldwide, although it is preventable and curable. Timely detection is crucial for eliminating this disease. Cytology is the official test for cervical cancer screening in most countries. Unfortunately, it has multiple barriers, e.g., its low sensitivity (47-55%) and its invasive nature. There is a need for alternative screening tests that can complement cytology's limitations. Molecular biomarkers can fill this gap. The present study aimed to identify candidate cervical cancer biomarkers in human sera. We selected five human proteins from a previously reported secretome of cervical cancer cell lines as candidate biomarkers. We tested these proteins in a cohort of 212 Mexican women, divided into four clinical groups: control, low and high-grade squamous intraepithelial lesions, and cervical cancer. Immunodetection was done by Western blotting, ELISA, and/or surface plasmon resonance. Four of these five proteins were in higher abundance in sera of precancerous cervical lesions (GAPDH) or cervical cancer (EIF4A1, HNRNPA1, and FDPS) patients ($p < 0.05$). When tested individually, we found that these biomarkers were able to distinguish serum samples from healthy donors from those with cervical disease. Also, a lateral flow assay was developed for detecting FDPS in whole blood, paving the way for detecting these pathologies using rapid tests.

Keywords: Biomarkers, cervical cancer, precancerous cervical lesion, cervical intraepithelial neoplasia, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, cervical cancer screening

1. Introduction

Cervical cancer (CC) is a significant health problem, in 2020 was the fourth most common malignancy among women worldwide. (World Health Organization & International Agency for Research on Cancer, n.d.) In the same year, it was the second most common cancer among Mexican women. (World Health Organization et al., 2021; World Health Organization & International Agency for Research on Cancer, n.d.) CC is preceded by precursor lesions known as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL).(Buckley et al., 1982; Cohen et al., 2019) Both CIN and early stages of CC are usually asymptomatic. (Cohen et al., 2019)

Risk factors for CC include the age of sexual debut, parity, tobacco smoking, folate deficiency, multiple lifetime sexual partners, prolonged use of oral contraceptives, human papilloma virus (HPV) infections, coinfection with other sexually transmitted agents (e.g., herpes simplex virus or *Chlamydia trachomatis*), presence of precursor lesions, and non-attendance to regular cervical screening. (Appleby et al., 2009; Berrington De González & Green, 2007; Bezabih et al., 2015; Bos et al., 2006; Collins et al., 2010; Hwang et al., 2009; International Collaboration of Epidemiological Studies of Cervical Cancer, 2007; Johnson et al., 2019; McGraw & Ferrante, 2014; Mexico's Ministry of Health, 2007; Plummer et al., 2003; Ribeiro et al., 2015; Ruiz et al., 2012; Tekalegn et al., 2022; Vesco et al., 2011)

More than 85% of CC patients are young women, which inflicts a heavy burden on societies, especially in developing countries. (World Health Organization, 2020) For example, in the United States, almost 50% of diagnosed CC cases are women under 35 years old. (Cohen et al., 2019) It is estimated that CC mortality will reach 700,000 deaths by 2030. (World Health Organization, 2020) Although CC is a preventable and curable disease, its elimination requires coordinated action among countries and the development of new technologies for early detection. (World Health Organization, 2020)

Prevention of CC is divided into primary and secondary approaches. (Cohen et al., 2019) Primary prevention is achieved through education and HPV vaccination. (Cohen et al., 2019) Secondary prevention relies on screening methods such as cytology or visual inspection with acetic acid.(Cohen et al., 2019; Ferreccio & Gage, 2003; Wilbur, 2020; L. Zhang et al., 2023) Most countries use cytology as their standard method for CC screening. (GVR, 2021; L. Zhang et al., 2023) HPV detection is performed as a complementary test in many countries, including Mexico. (Cohen et al., 2019; GVR, 2021; Mexico's Ministry of Health, 2007; L. Zhang et al., 2023) Cytology faces multiple barriers, including sociocultural, religious, structural, and methodological, as well as the invasive nature of the test. (Akinlotan et al., 2017; Bravington et al., 2022; Petersen et al., 2022; Shin et al., 2021) Additionally, cytology can show 25% of false-negative results with sensitivity ranging between 47.2% to 55.4%. (Coleman, 2001; Kitchener et al., 2006; Mayrand et al., 2007; Sadat Najib et al., 2020) It is estimated that 64% of women worldwide have never been screened. (Bruni et al., 2022; World Health Organization, 2019) For these reasons, the medical and scientific community continues to develop alternative screening tools. Human biomarkers associated with CC represent one such alternative. (Ruiz Esparza Garrido et al., 2023)

In this study, we evaluated five human proteins known to be secreted by CC cell lines. We found that four of these proteins are associated with precancerous cervical lesions or CC: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and eukaryotic initiation factor 4A1 (EIF4A1), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), and farnesyl pyrophosphate synthase (FDPS). Although these proteins have been reported to participate in different types of cancer, including CC, this is the first time they have been detected as circulating biomarkers in human sera. To the best of our knowledge, there is currently no screening test for precancer or CC based on human circulating protein biomarkers.

2. Materials and Methods

2.1 Patients and Venous Blood Sera

The IMSS' (Instituto Mexicano del Seguro Social) National Ethics Committee approved the present study (R-2019-785-070), and all samples were collected after obtaining informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Whole venous blood was collected from a total of 283 women, seventy-one women were excluded due to insufficient sample volume, hemolysis, lipemia, loss to follow-up and/or incomplete documentation. A total of 212 women were assessed and divided into four clinical groups: control (CTR; n=56, 7% HPV positive, average age 30 years), low-grade squamous intraepithelial lesion (LSIL; n=67, 99% HPV positive, average age 35 years), high-grade squamous intraepithelial lesion patients (HSIL; n=68, 94% HPV positive, average age 42 years), and cervical cancer (CC; n=21, 100% HPV positive, average age 50 years).

Group assignment was confirmed through cytology, colposcopy, and/or histopathology results (Supplementary Table S1). Cytology tests were performed on all participants, and those with abnormal results underwent colposcopy and histopathologic studies, in accordance with the Mexican official clinical guidelines.(Mexico's Ministry of Health, 2007) HPV status was determined by IMSS staff, with some cases confirmed by a certified third-party laboratory.

Whole blood samples were centrifuged for 15 min at 1000 rpm at room temperature (RT) to obtain sera. Sera were stored at -70 °C until use.

2.2 Selection of Candidate Cervical Cancer Biomarkers

Secreted proteins from CC cell lines HeLa and SiHa were obtained from Checa-Rojas et al. (Checa-Rojas et al., 2018) Initially, 266 secreted proteins identified in both CC cell lines were preselected. GSTM3 and GSTP1 were also included due to their role in tumor progression, bringing the total to 268 proteins. (Checa-Rojas et al., 2018)

Two criteria were used to filter the initial list. The first criterion discarded proteins if their names were related to terms such as *tubulin*, *actin*, *keratin*, *albumin*, *collagen*, *histone*, *microtubule*, *membrane*, *cytoskeleton*, *centromere*, *telomere*, *ribosome*, *DNA polymerase*, and *putative*/*probable*/*hypothetical*. The second criterion removed proteins based on their subcellular location (reported by UniProt). Exclusions were made if the proteins were located solely

in a membrane, in two or more membranes, secreted, part of vesicles, endoplasmic reticulum, chromosome, cell projections, or uncorroborated location (not supported by at least one reference). (Bateman et al., 2023) GSTM3 was retained for its previously stated importance in tumor progression. (Checa-Rojas et al., 2018)

This filtering process resulted in a final list of 52 candidate biomarkers. Then, a list of different commercial antibodies for the 52 target proteins were obtained and quoted with various Mexican distributors. Due to availability, delivery times, project management, and COVID-19 restrictions, we were able to acquire antibodies for only five protein targets: GSTM3, EIF4A1, HNRNPA1, GAPDH, and FDPS (Supplementary Table S2).

2.3 Dot Blotting

Primary and secondary (horseradish peroxidase-conjugated) antibodies were purchased from various manufacturers. Polyvinyl difluoride membranes were activated with absolute methanol for 30 seconds and washed three times with distilled water at RT. The membranes were then incubated with 1x Western blotting transfer buffer for 20 min and air dried for 30 seconds. Samples (2 µL each; 1 µg of total protein) were manually spotted onto the membranes.

After fully air-drying, the membranes were blocked with 1x Tris-buffered saline with Tween20 (TBST) added with 5% nonfat milk for 1 hour at RT and washed three times for 5 minutes each with 1x TBST buffer at RT. The membranes were then immersed in absolute methanol for 5 seconds, air-dried for 10 minutes, and incubated with primary antibodies (1:500) for 1 hour at RT. Following primary anti-body incubation, the membranes were washed three times in 1x TBST for 5 minutes each, immersed in absolute methanol for 5 seconds, and air-dried for 10 minutes at RT.

Next, the membranes were incubated with secondary antibodies (1:20,000) for 1 hour at RT and washed five times with 1x TBST. Finally, the membranes were air-dried at RT, and the specific signal was visualized by chemiluminescence. Primary and secondary antibodies were purchased from various manufacturers.

2.4 Western Blotting

The primary and secondary antibodies used in dot blotting were also employed in Western blotting. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes using a semidry system (Bio-Rad Trans-Blot SD semi-dry transfer cell). The blots were blocked with 1x TBST with 5% nonfat milk for 15 minutes at RT, washed three times in 1x TBST for 10 minutes at RT, and incubated with primary antibodies (1:10,000) at 4 °C overnight.

After primary antibody incubation, the membranes were washed three times in 1x TBST for 10 minutes at RT and incubated with secondary antibodies (1:20,000) for 2 hours at RT. The membranes were then washed three times with 1x TBST for 10 minutes at RT before incubation in the developing solution. Data band intensities were obtained using an iBright imaging system (Thermo Fisher) and processed with the included software.

2.5 Enzyme-Linked Immunosorbent Assay

Commercially available enzyme-linked immunosorbent assay (ELISA) kits for detecting GAPDH were purchased from different manufacturers. ELISA was performed according to manufacturers' instructions.

2.6 Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were performed using a Biacore T200 instrument (GE Healthcare). The primary monoclonal antibody anti-FDPS (rabbit) and FDPS recombinant protein (His tag) were used, along with the following reagents: sensor chip CM5 series S, an amine-coupling kit [1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC)], N-hydroxysuccinimide (NHS), 1 M ethanolamine-HCl (pH 8.5)]; 50 mM NaOH, glycine (pH 2.0), acetate buffer (pH 4.0), NSB reducer and Hanks balanced saline (HBS)-EP+ buffer (pH 7.4) (0.1 M HEPES, 1.5 M NaCl, 0.03 M EDTA, 0.5% [vol/vol] surfactant P20) (GE Healthcare, North Richland Hills, TX, USA). Human ultrafiltrated pooled male serum (BioChemed Services catalog number 751NS-MP-SUF; Winchester, VA, USA) was used as a reference serum.

To determine the number of binding–regeneration cycles before analyte measurements were affected, the binding capacity of FDPS was monitored by tracking the signal of the 1-µg/mL standard solution over multiple cycles.

The anti-FDPS antibody was immobilized on the CM5 sensor chip using the amine-coupling kit. The chip was activated with a 1:1 mixture of EDC to sulfo-NHS, injected over the surface at a flow rate of 10 µL/min for 500 seconds, to obtain densities of approximately 14,000 resonance units (RU), and the unresected groups were blocked with ethanolamine. Biacore HBS-EP (10 mmol/L [pH 7.4]) was used as the running buffer. A solution of 15 µL of antibody in 96 µL of 10 mM sodium acetate (pH 4.0) was used; lanes 1 and 3 were left blank intentionally.

The standard curve was prepared by passing different concentrations of purified human recombinant protein at different concentrations: 0.1, 0.5, 1, 5, 10, 30, 60 and 100 µg/mL. All serum samples were diluted 1:5 in NSB and HBS EP+ buffers, and simple injections were performed with a flow rate of 10 µL/min, a contact time of 180 seconds, and dissociation time of 15 seconds. After every analyte injection, the surface was regenerated with 10 mM glycine–HCl (pH 2.0), injected for 30 seconds at a flow rate of 30 µL/min. All samples were injected in triplicate, and measurements were performed at 25 °C.

The concentration of human FDPS protein in serum samples was estimated from the standard curve. Calibration curves were prepared by spiking human recombinant protein into pooled normal serum at concentrations of 0.1, 0.5, 1, 5, 10, 30, 60, and 100 µg/mL. The standard curve served as reference for measuring FDPS concentration. Using this calibration curve, the concentrations of controls and unknown samples were determined. Controls were prepared by spiking human recombinant protein into pooled normal sera at a concentration of 10 µg/mL to determine the precision and accuracy of FDPS measurement.

All binding sensorgrams were collected, processed, and analyzed using Biacore T200 Evaluation Software 2.0 (GE Healthcare). To monitor nonspecific binding, lanes 1 and 3 were left blank, i.e., the protein was not immobilized on these lanes. Additionally, blank serum was periodically injected across all rows to check for nonspecific binding to the anti-FDPS antibody immobilized to the surface.

2.7 Lateral Flow Assay

Lateral flow assays (LFA) are valuable for detecting target molecules in over-the-counter devices. Control antibodies (anti-IgG), anti-FDPS, and FDPS recombinant protein were purchased from various suppliers. Different combinations of capture and detection antibodies (anti-FDPS) were evaluated. Detection antibodies were coupled to cellulose nanobeads. FDPS LFA strips were prepared by applying 0.5 µL of capture antibody per strip to the test line and 0.5 µL of control antibody per strip to the control line on pre-cut test strips, which were then dried in an oven at 40 °C for 1 hour.

Lyophilized recombinant FDPS was reconstituted to 100 µg/mL and then serially diluted to reach concentrations ranging from 10,000–10 ng/mL. Each conjugate was diluted in borate, casein, and Tween buffer, applied onto the strips and allowed to develop for 10 min at RT before reading. For serum sample testing, each conjugate was diluted to 0.2% solids using borate, casein, and Tween buffer. Cellulose nanobead conjugates and serum samples were pipetted into a glass test tube, with half-strips of LFA placed in glass tubes so that the membrane's bottom was submerged in the testing solution, allowing the conjugate-serum mixture to wick up by the membrane for 10 minutes at RT. An LFA reader recorded line intensities for each strip.

For whole blood sample testing, strips were prepared by applying a sample pad conjugate with cellulose nanobeads and rabbit monoclonal antibody. The test line was stripped at a rate of 1 µL using an Ahlstrom 8964 conjugation pad with rabbit polyclonal antibody, while the control line was sprayed under the same conditions with anti-rabbit antibody. The membrane was dried in an oven at 40 °C for 1 hour, assembled with the backing card, sample, and wick membranes, and cut into 5-mm strips with an electric cutter. The strips were placed in a DCN^{DX} cassette. A control venous blood sample mixed with sera from various clinical groups was pipetted into the cassette's sample well and introduced into the LFA reader after 10 min. All LFA experiments were conducted in collaboration with DCN^{DX} (Carlsbad, California, USA).

2.8 Graphs and Statistical Analysis

Graphs and hypothesis tests were performed using R (version 4.3.1; "Beagle Scouts").(R Core Team, 2023) Kruskal-Wallis, Wilcoxon, and pairwise Wilcoxon tests were employed to compare groups. Receiver operating characteristic (ROC) curve analysis was conducted using the ROCR package, specifically on groups showing significant differences (Supplementary Figures S1-S4). (Sing et al., 2005)

3. Results

Women attending public gynecological services in Mexico City were enrolled after obtaining written informed consent. Initially, 283 women were recruited, but 71 of them were excluded due to reasons such as insufficient sample volume, hemolysis, lipemia, being lost to follow-up or incomplete documentation. Thus, 212 women were ultimately assessed. Among them, four distinct clinical groups were identified: control (CTR, n=56), low-grade squamous intraepithelial lesion (LSIL, n=67), high-grade squamous intraepithelial lesion (HSIL, n=68), and cervical cancer (CC, n=21).

Five candidate biomarkers for cervical cancer were randomly selected from a list of 52 proteins secreted by HeLa and SiHa cell lines obtained from Checa-Rojas et al, 2018. Selection criteria can be consulted on Materials and

Methods (section 2.2), and Supplementary Table S2. Due to limited sera volumes, not all the candidate biomarkers could be tested in every sample.

3.1 Immunodetection of Candidate Biomarkers for Cervical Cancer

3.1.1 Dot Blotting Results

Dot blot experiments were conducted for three candidate biomarkers - GSTM3, EIF4A1, and FDPS – to compare spot intensities across the four clinical groups. Signal intensities for GSTM3 were too low to be reliably detected, prompting its exclusion from further testing. For EIF4A1 and FDPS, significant differences were observed among different pairs of clinical groups (Kruskal-Wallis test, $p < 0.05$). Pairwise Wilcoxon tests were subsequently performed to identify the significant pairs (Table 1).

Table 1. Pairs of clinical groups showing significant differences (pairwise Wilcoxon test, $p < 0.05$) in dot blotting

Candidate CC biomarker	Clinical groups
EIF4A1	CTR vs. CC
FDPS	CTR vs. HSIL
	CTR vs. CC
	LSIL vs. HSIL
	LSIL vs. CC

Abbreviations. CTR, control; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CC, cervical cancer; vs, versus.

3.1.2 Western Blotting Results

We selected sera from control and CC groups for Western blot analysis using EIF4A1 and HNRNPA1 antibodies (Figures 1 and 2). This decision was based on the known biological roles of the two proteins. EIF4A1 participates in messenger RNA translation initiation in eukaryotes, (Lu et al., 2014; Nielsen & Trachsel, 1988; Rogers et al., 1999) while HNRNPA1 associates with RNA transcripts and promotes alternative translation initiation.(Bonnal et al., 2005; Dreyfuss et al., 1993; Jo et al., 2008)

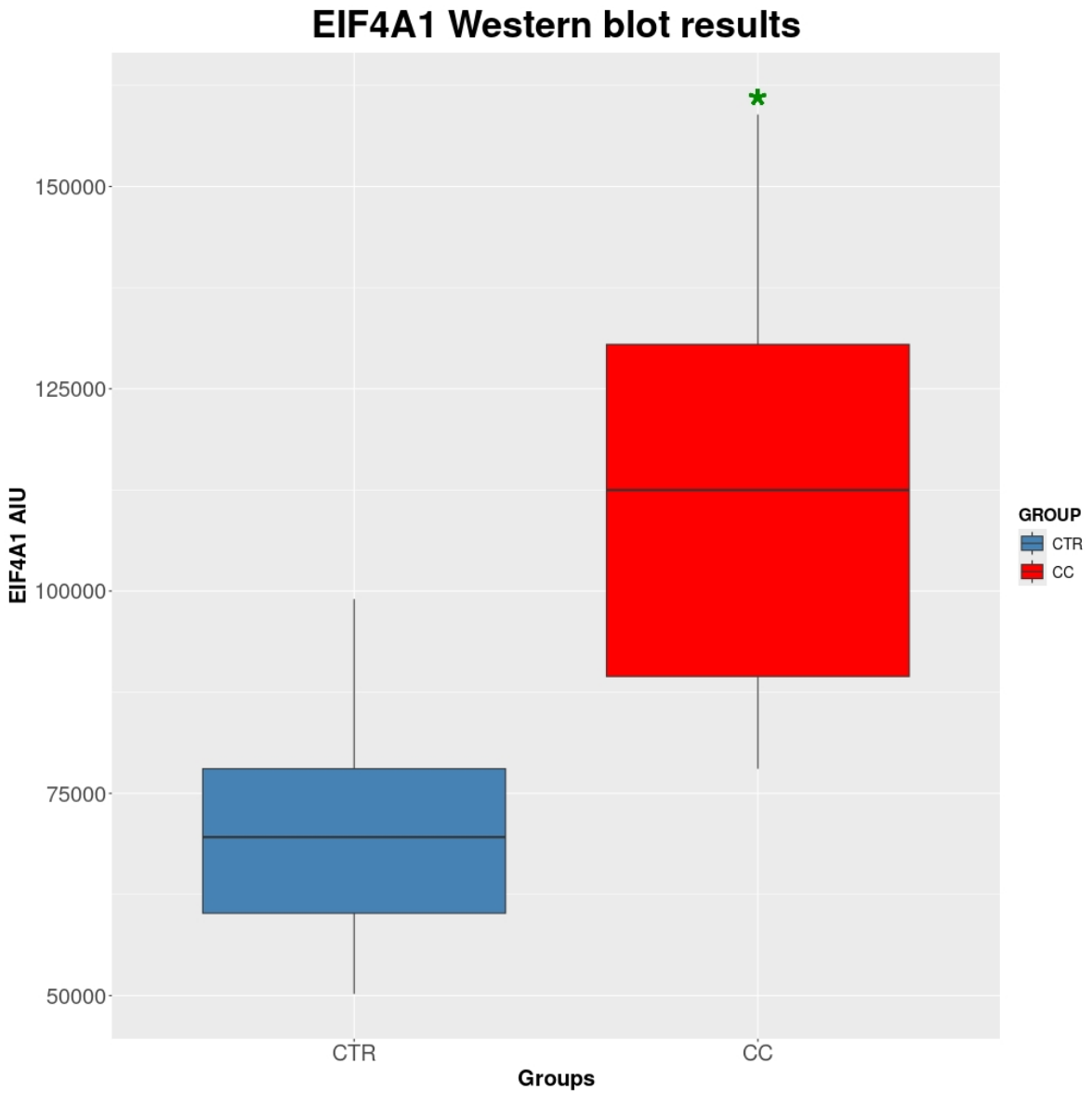

Figure 1. Boxplot of EIF4A1 expression detected by Western blotting. Each sample was tested at least twice, and the final intensity represents the mean. The membrane exposure time was 10 seconds. The green asterisk denotes the group showing significant differences with the control group (Wilcoxon test, $p < 0.05$). Abbreviations: AIU, arbitrary intensity units in pixels; CTR, control (blue, n=13); CC, cervical cancer (red, n=11)

Consistent with the dot blotting results, EIF4A1 expression levels differed significantly between the control and CC groups as detected by Western blotting (Wilcoxon test, $p < 0.05$). A limitation of these analyses lies in the number of technical replicates, i.e., two per biological sample. This is because processing multiple samples by Western blotting can be laborious and time-consuming.

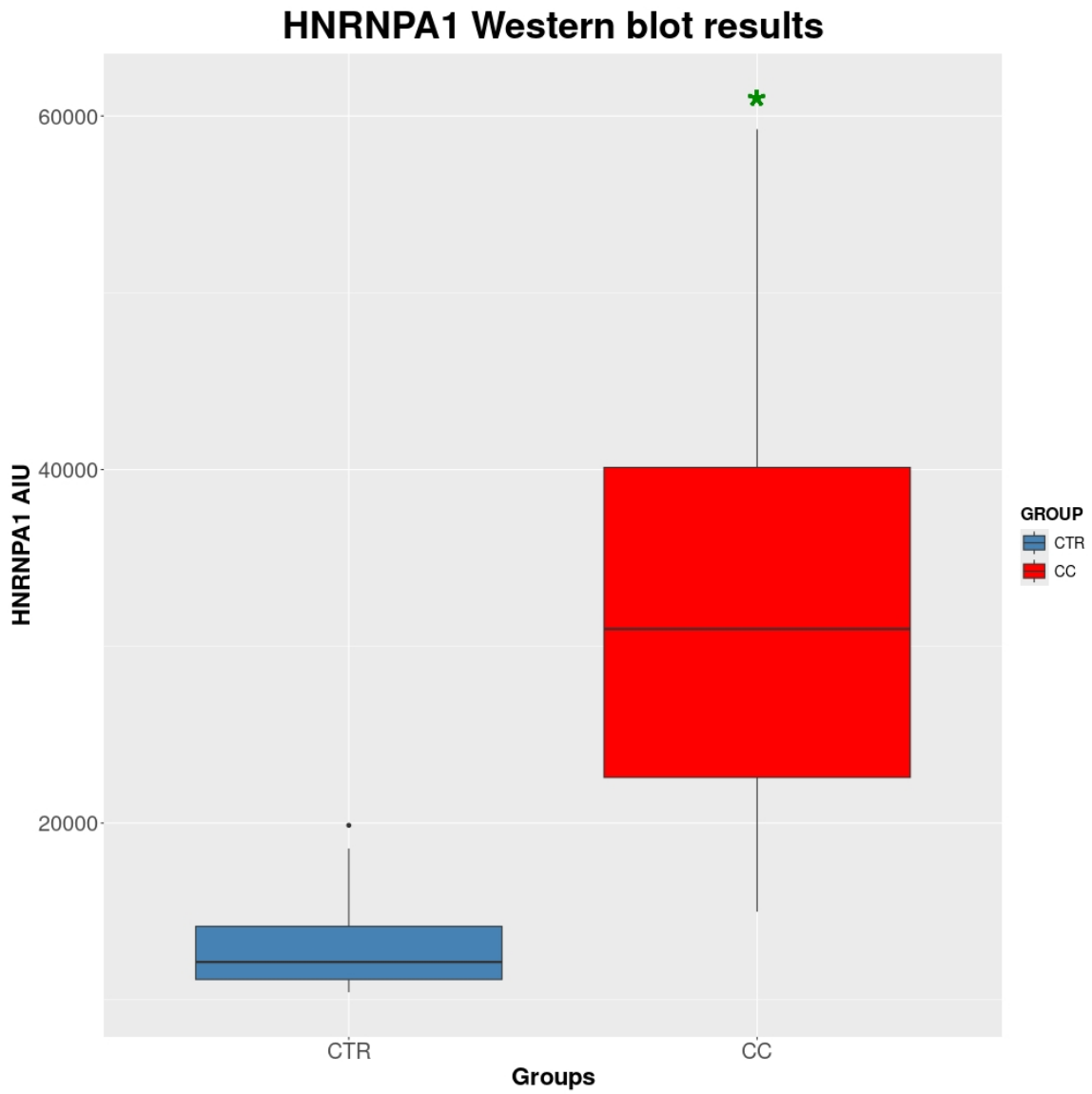

Figure 2. Boxplot of HNRNPA1 expression detected by Western blotting. Each sample was tested at least twice, and the final intensity represents the mean. The membrane exposure time was 10 seconds. The green asterisk denotes the group showing significant differences with the control group (Wilcoxon test, $p < 0.05$). Abbreviations: AIU, arbitrary intensity units in pixels; CTR, control (blue, n=11); CC, cervical cancer (red, n=11)

Similarly, significant differences in HNRNPA1 expression levels were found between the control and CC (Wilcoxon test, $p < 0.05$). As stated in the EIF4A1 Western blotting results, this analysis is limited to two technical replicates per biological sample.

3.1.3 Enzyme-Linked Immunosorbent Assay Results

Enzyme-linked immunosorbent assay (ELISA) is commonly used for clinical analyses, where immunodetection occurs in liquid phase. Given that EIF4A1 and HNRNPA1 biomarkers are associated with cervical cancer, we aimed to compare the control group with the precursor lesions groups (LSIL and HSIL). Biomarkers for these precancerous stages are crucial for early disease detection. We selected GAPDH for this comparison based on the findings of Xu, et al, who reported that serum auto-antibody against GAPDH decreases with increasing severity of cervical lesions. (Xu et al., 2019)

The concentration of GAPDH protein (in nanograms per milliliter) was estimated by ELISA in the control, LSIL, and HSIL clinical groups (Figure 3).

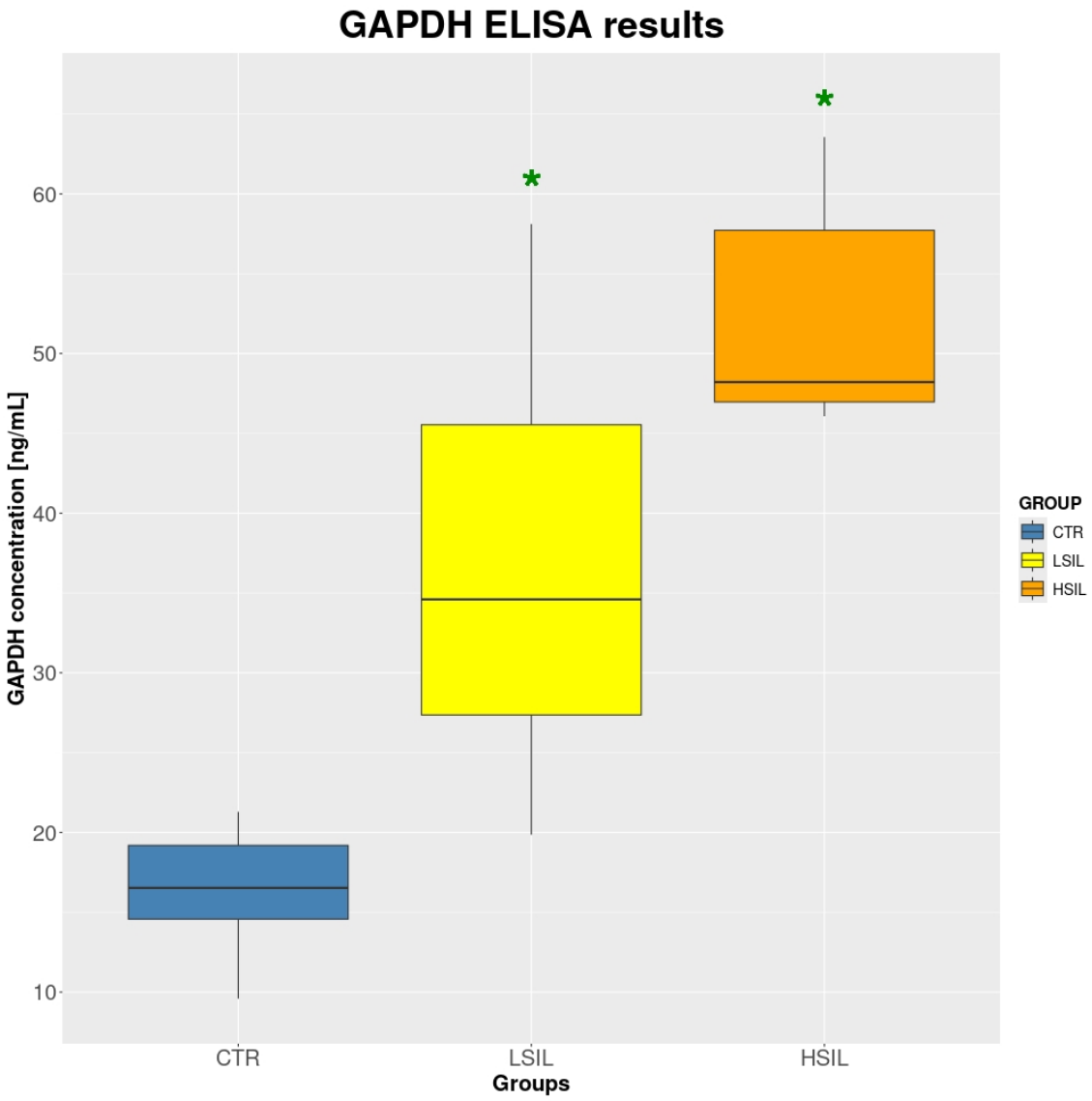

Figure 3. Boxplot GAPDH concentration (in nanograms per milliliter) in the control (CTR; blue, n=19), LSIL (yellow, n=19), and HSIL (orange, n=14). The green asterisk denotes the groups that showed significant differences with the control (pairwise Wilcoxon test, $p < 0.05$). Abbreviations: CTR, control; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions

A Kruskal-Wallis test indicated that there was at least one pair of groups showing significant differences ($p < 0.05$). Subsequent pairwise Wilcoxon tests confirmed significant differences between all three groups ($p < 0.05$), i.e., CTR vs. LSIL, CTR vs. HSIL, and LSIL vs. HSIL.

3.1.4 Surface Plasmon Resonance Results

Surface plasmon resonance (SPR) is an optical technique used to study interactions between molecules, such as protein-protein, protein-membrane, protein-drug, or protein-nucleic acid interactions using microfluidics.(Drescher et al., 2018; Fabini & Danielson, 2017) Building on the previous FDPS results, we estimated FDPS protein concentration in the control and cervical cancer groups using SPR (Figure 4).

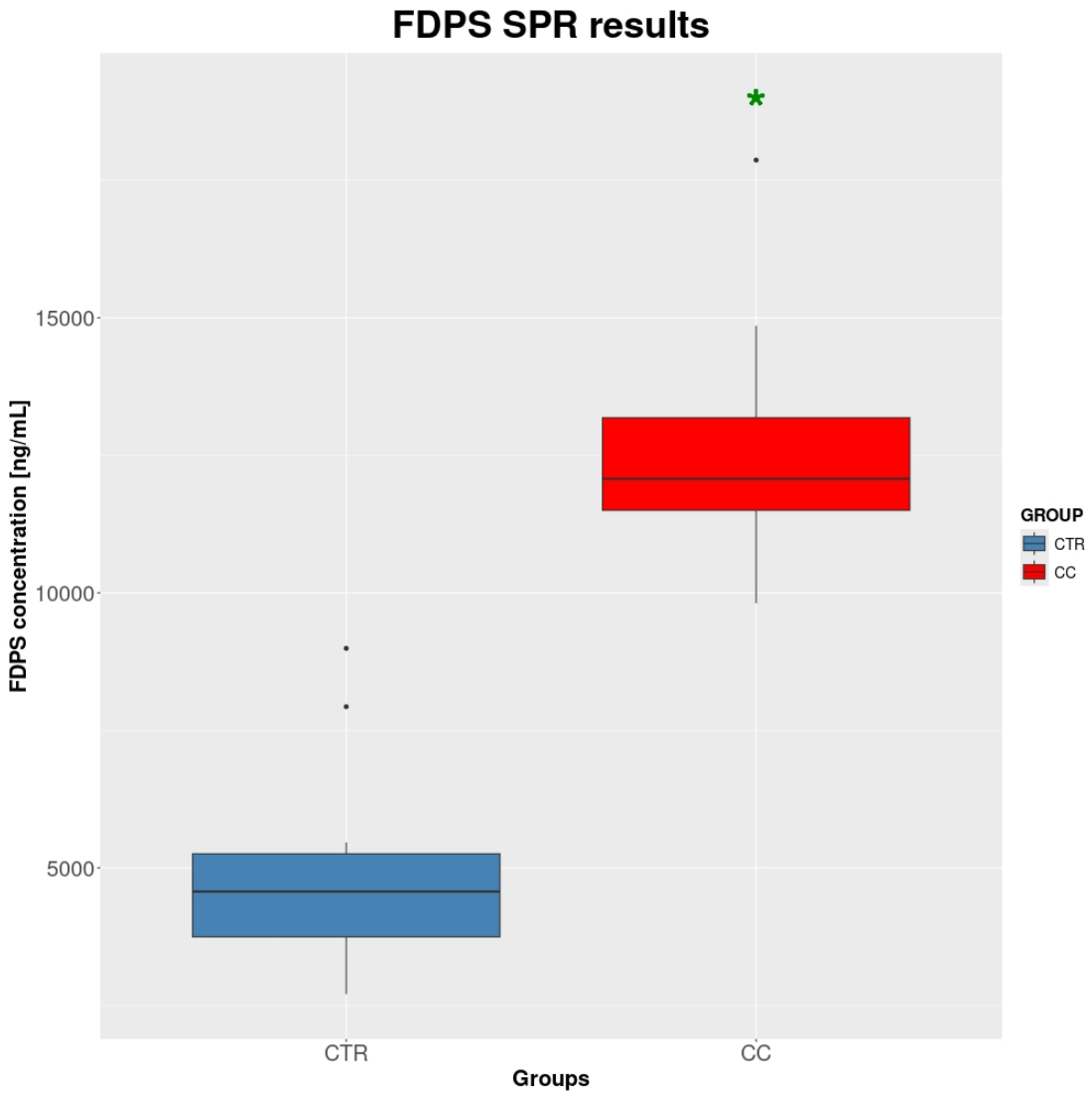

Figure 4. Boxplot of FDPS protein concentration estimated by SPR in the control and cervical cancer groups. The green asterisk denotes the group showing significant differences with the control (Wilcoxon test, $p < 0.05$). Abbreviations: CTR, control (blue, n=13); CC, cervical cancer (red, n=10)

A Wilcoxon test further confirmed that FDPS protein concentration significantly differed between the control and cervical cancer groups ($p < 0.05$).

3.1.5 Lateral Flow Assay Results

Lateral flow assays (LFA) are low-cost, portable, reliable, and rapid paper-based screening tests.(O'Farrell, 2015; Posthuma-Trumpie et al., 2009) We developed an LFA to detect FDPS as a proof of concept, testing sera from the four clinical groups. FDPS was selected for the prototype because: i) we have tested this biomarker with the most sensitive technology (SPR) of this study, and ii) we have visually tested it in one Western blotting experiment (data not shown). The results of both methodologies were concordant. SPR has been used by others to identify fast-interacting antibodies for LFA development. (Ross et al., 2018) Serum samples were mixed with control venous blood to assess the final prototype. The FDPS-LFA was conducted in collaboration with DCN^{DX} (Carlsbad, California, USA). After standardizing the prototype, we observed FDPS bands in HSIL and CC sera, both by visual inspection and using a LFA reader (Figure 5).

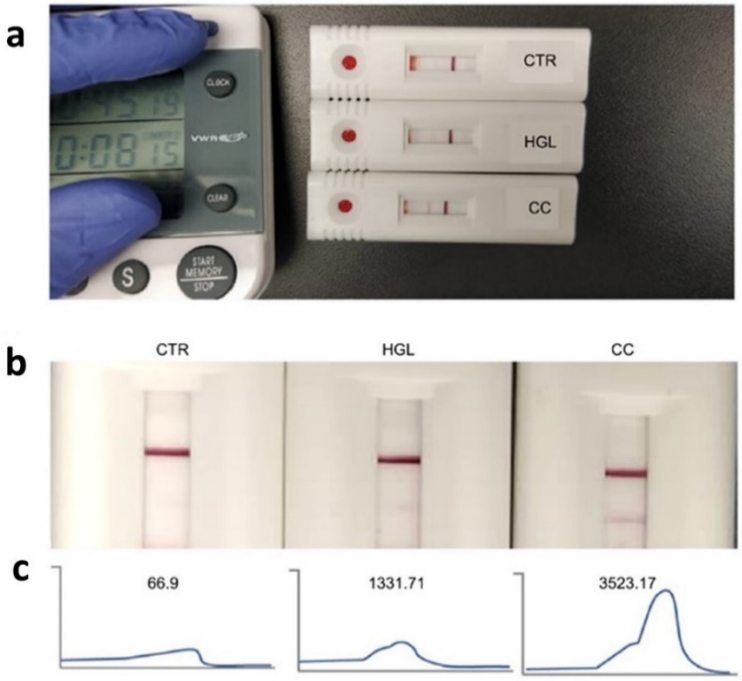

Figure 5. FDPS-LFA prototype. a) Final cassettes. The test line (FDPS antibody) is positioned closer to the sample well, while control line (secondary antibody) is located near the sample labels. b) Close up of the results window of the FDPS-LFA prototype. The strong red line indicates the control line (upper part) and the lighter one represents the test line (FDPS detection) in three samples: control (CTR), high-grade squamous intraepithelial lesion (labeled as HGL), and cervical cancer (CC). c) FDPS line intensities (in pixels) obtained by the DCN^{DX} LFA reader

As depicted in Figure 5, we successfully detected the test bands in HSIL and CC sera. The observed low intensity in the FDPS bands can be attributed to the type of sample used, namely control venous whole blood spiked with sera from different clinical groups. Further experiments are necessary to evaluate the prototype's performance with fresh blood samples, both venous and capillary drawn. Despite these limitations, these results show promise for an over-the-counter device for precancer and cervical cancer screening. More experiments are needed to improve the detection limit.

3.2 Sensitivity and Specificity of Candidate Biomarkers for Precancer and Cervical Cancer

The comparison of different screening tests typically relies on their sensitivities and specificities. We generated the receiver operating characteristic (ROC) curves for each biomarker using Western blotting, ELISA, and SPR methods to determine the optimal cutoffs, areas under the curve (AUC), sensitivities, and specificities (Table 2).

Table 2. ROC curve analysis for the precancer/cervical cancer biomarkers

Biomarker	Method	Groups	Cutoff	Sensitivity	Specificity	AUC
EIF4A1	Western blot	CTR vs CC	86,100	0.9091	0.9231	0.9441
HNRNPA1	Western blot	CTR vs CC	21,310	0.9091	1.00	0.9835
GAPDH	ELISA	CTR vs LSIL|HSIL	25.09	0.9697	1.00	0.9955
FDPS	SPR	CTR vs CC	9,810.02	1.00	1.00	1.00

Abbreviations. AUC, area under the curve; CTR, control; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CC, cervical cancer; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance. Western blot cutoff values are expressed in arbitrary intensity units (AIU). ELISA and SPR cutoff values are expressed in nanograms per milliliters. The optimal cutoff maximizes both sensitivity and specificity.

ROC curve analysis was conducted exclusively on clinical groups that exhibited significant differences. Individual ROC curves were generated for each biomarker due to the use of different sample sets, thus, the same subset of samples was not tested for all four biomarkers. As shown in Table 2, the sensitivity and specificity of most individual biomarkers were notably high, ranging from 91%-100% and 92%-100%, respectively.

4. Discussion

Precancerous cervical lesions (LSIL and HSIL) and cervical cancer (CC) represent significant public health concern.(World Health Organization, 2020) Despite the widespread use of cytology for early detection, its limitations in accuracy, acceptance, and coverage are notable, especially for women living in remote communities. (Akinlotan et al., 2017; Bravington et al., 2022; Bruni et al., 2022; Coleman, 2001; Kitchener et al., 2006; Mayrand et al., 2007; Petersen et al., 2022; Sadat Najib et al., 2020; Shin et al., 2021; L. Zhang et al., 2023) Many women prefer less-invasive screening options, such as blood tests.(Shin et al., 2021) Developing such tests require research on circulating biomarkers associated with the disease, particularly those related to precancerous stages.

The present study validated four human proteins as potential circulating biomarkers for precancerous cervical lesions and CC. We found GAPDH protein to be overexpressed in both LSIL and HSIL groups, while EIF4A1, HNRNPA1, and FDPS proteins showed higher abundances in the CC group.

Consistent with our findings, these proteins are known to be overexpressed in other cancers: GAPDH in pancreatic adenocarcinoma, breast, lung, and cervical cancers; EIF4A1 in hepatocellular, renal, and gastric carcinomas, lymphoma, prostate, endometrial, and cervical cancers; HNRNPA1 in hepatocellular carcinoma; and FDPS in liver, hepatocellular, and cervical carcinomas, pancreatic, colon, and rectum adenocarcinomas, lymphoma, thymoma, kidney chromophobe, acute myeloid leukemia, glioma, prostate and colorectal cancers. (Chen et al., 2020; C. Gao et al., 2020; S. Gao et al., 2021; Kayastha et al., 2022; Liang et al., 2014; Lomnytska et al., 2012; Notarnicola et al., 2004; Révillion et al., 2000; Schek et al., 1988; Seshacharyulu et al., 2019; Steinmann et al., 2023; Sung et al., 2003; Todenhöfer et al., 2013; Tokunaga et al., 1987; C. Wang et al., 2022; L. Wang et al., 2023; Woo Kim et al., 1998; Xu et al., 2019; L. L. Zhang et al., 2022; Zhou et al., 2013)

These biomarkers can individually distinguish between control and diseased samples. Their average sensitivity (94.7%) and specificity (98%) demonstrated promising results. GAPDH was overexpressed in squamous intraepithelial lesion samples, while EIF4A1, HNRNPA1, and FDPS were more abundant in cervical cancer sera. We suggest that GAPDH and one of the cancer biomarkers should be included during screening. Whether to include more biomarkers to achieve better performance remains to be elucidated. Other cancer screenings have benefitted from detecting multiple biomarkers.(Kang et al., 2022; Wu et al., 2022) Nevertheless, we cannot exclude that the simultaneous screening of these four biomarkers can lower the overall sensitivity and specificity. One practical implication of a multi-biomarker panel is that it may require higher sample volumes, longer testing times, and/or more expensive tests, depending on the methodology. Further experiments are required to assess this hypothesis. The reported average sensitivity for cytology is 51.3%. Screening for these four biomarkers could complement cytology by improving the identification of women with the disease. Biomarkers would enable less-invasive screenings that do not require extensive infrastructure. We believe these findings contribute to the development of a high-performance screening test for CC, which could help reduce gender and income disparities in human healthcare access.

To our knowledge, there are currently no available options for detecting precancerous cervical lesions or CC using human biomarkers in blood. We successfully developed an LFA for human FDPS detection as a proof of concept. This rapid test illustrates the feasibility of a timely, cost-effective, and noninvasive screening method for cervical pathologies. Rapid tests are crucial for accessing hard-to-reach populations, particularly those lacking specialized facilities, staff, or healthcare services. Implementing a rapid screening method could reduce overall costs, extend screening accessibility, and offer self-testing options. Our future experiments will include evaluating the simultaneous detection of all four biomarkers and further developing a rapid test.

The use of circulating biomarkers for CC screening could have a significant impact on reducing mortality by promoting early detection, which is critical as it allows for timely treatment, as well as high sensitivity and specificity that can complement current methods.

Limitations of this study include the sample size (n = 212), individual biomarker testing, the number of technical replicates, and screening of a single ethnic group (Mexican women living in cities). These limitations can contribute to some of the variation observed in the data and weaken the reliability of the comparisons. Therefore, our future research aims to expand screening to larger and more diverse populations in Mexico, to increase the

number of replicates and to investigate different biomarker combinations.

In conclusion, we provide evidence of the immunodetection of four CC biomarkers in human sera. We identified protein GAPDH associated with precancerous cervical lesions (LSIL and HSIL), and EIF4A1, HNRNPA1, and FDPS proteins associated with cervical cancer. While these protein's roles in cancer are not novel, this study represents the first detection of these biomarkers in human sera. We believe that this biomarker panel offers a valuable option for screening precancerous and cervical cancer.

5. Conclusion

Four out of five candidate biomarkers were detected in higher abundance in the sera of patients with a cervical disease compared to the control group. GAPDH was correlated with squamous intraepithelial lesions, while EIF4A1, HNRNPA1, and FDPS were associated to cervical cancer. A lateral flow assay for detecting FDPS was developed as a proof of concept. Collectively, these findings lay the groundwork for the development of an over-the-counter device to detect precancerous lesions and cervical cancer. Based on our findings and analysis, EIF4A1, HNRNPA1, GAPDH, and FDPS proteins hold promise as biomarkers for enhancing early detection and complementing screening methods. Their high sensitivity and specificity make them valuable candidates for further validation and potential integration into cervical cancer screening programs. If further characterized, these biomarkers could significantly contribute to reduce cervical cancer globally.

Supplementary Materials

The following supporting information can be downloaded at: https://ccsenet.org/journal/index.php/gjhs/article/view/0/50473, Table S1: Clinical groups; Table S2: List of candidate CC biomarkers with selection criteria.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the National Ethics Committee of Mexican Social Security Institute (IMSS, protocol code R-2019-785-070 and date of approval August 08, 2019).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the present study.

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Data Availability Statement

The data that support the findings of this study are available on request.

Competing Interests Statement

The authors declare that there are no competing or potential conflicts of interest.

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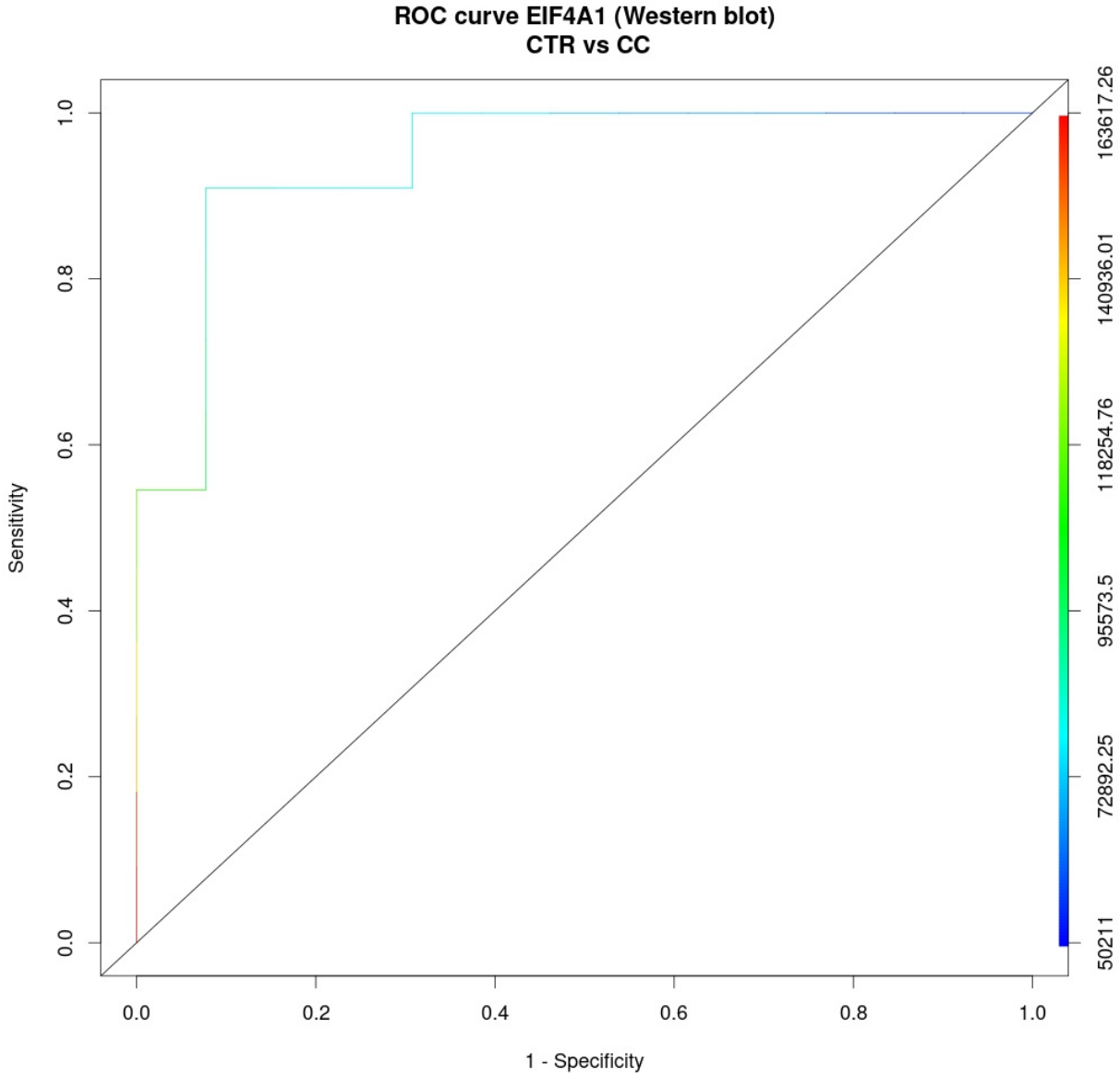
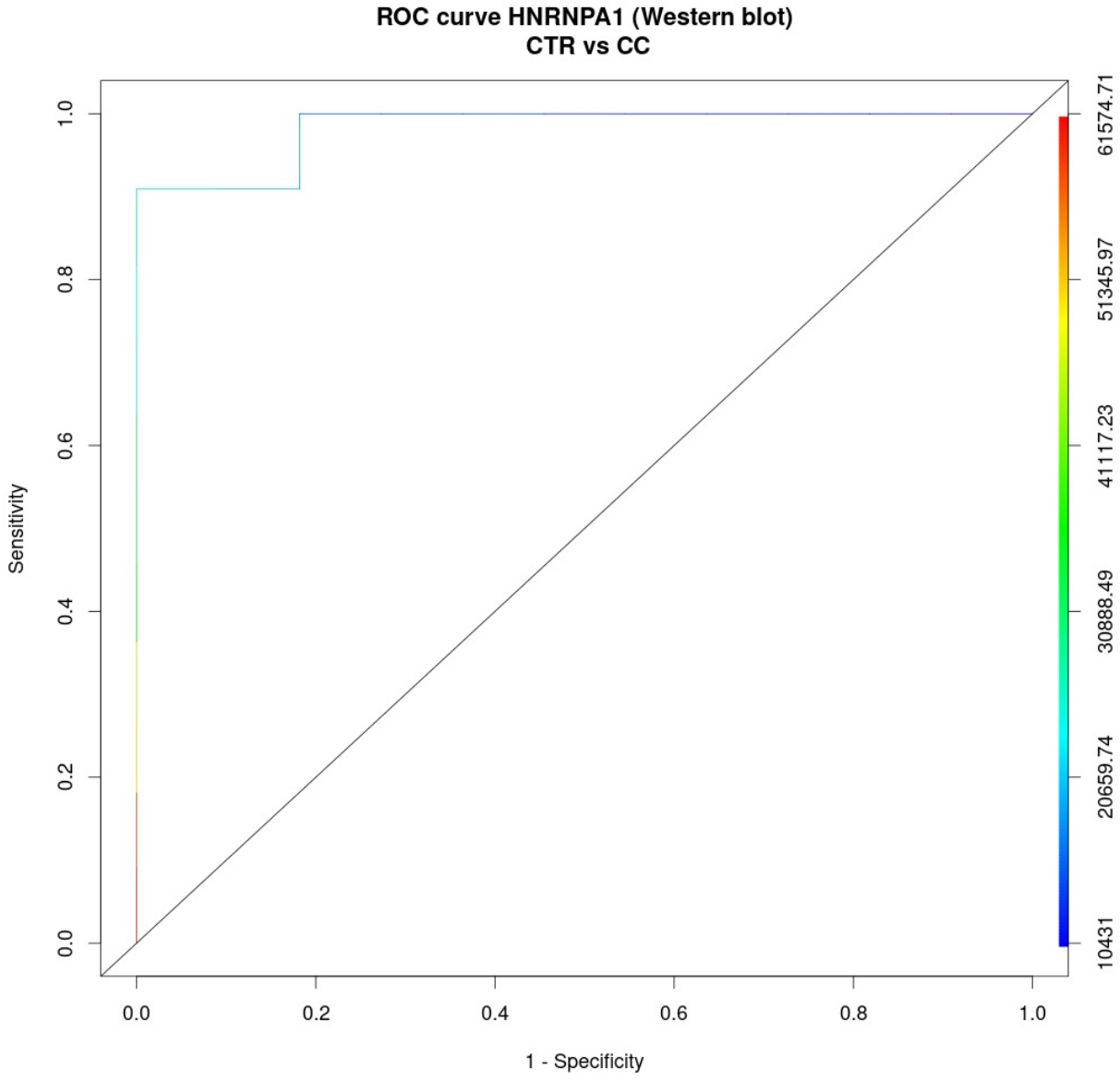
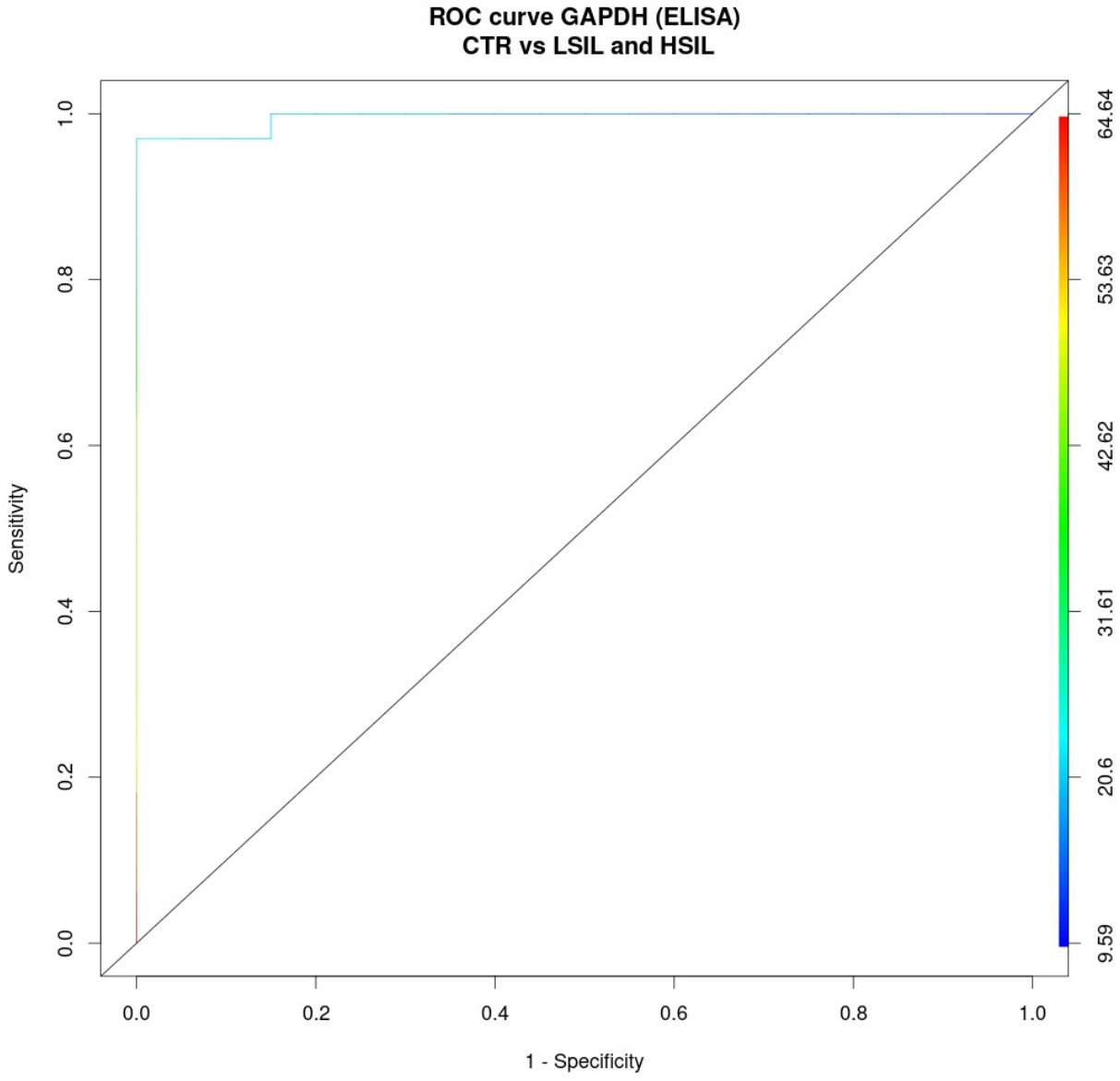
Appendix



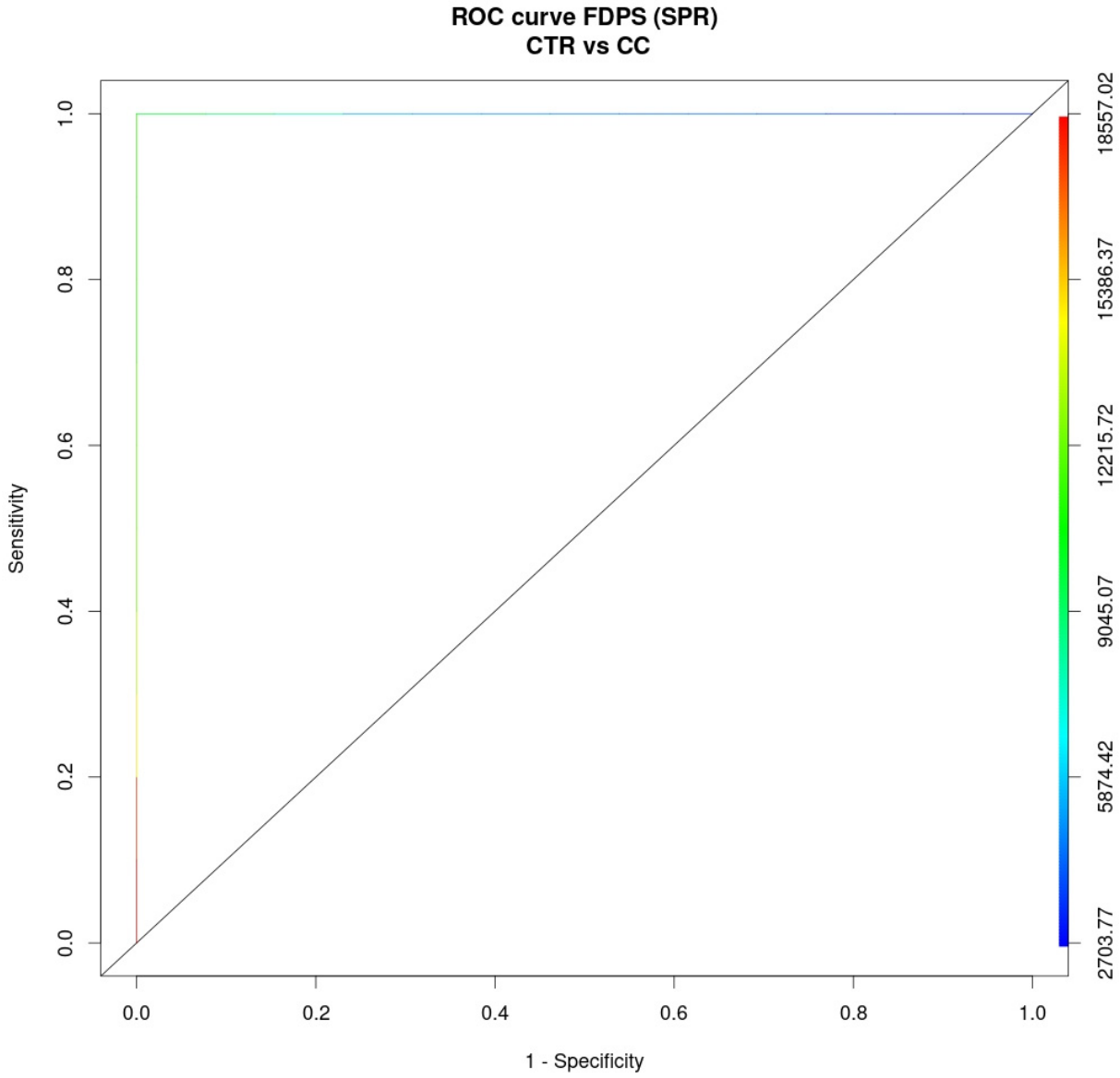
Supplementary Figure 1. ROC curve EIF4A1 (Western blot) CTR vs CC



Supplementary Figure 2. ROC curve HNRNPA1 (Western blot) CTR vs CC



Supplementary Figure 3. ROC curve GAPDH (ELISA) CTR vs LSIL and HSIL



Supplementary Figure 4. ROC curve FDPS (SPR) CTR vs CC