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Exploring oxysterols and protein carbonylation in cervicovaginal secretions as biomarkers for cervical cancer development☆

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ABSTRACT

Cervical cancer, a major global health issue and the fourth most common cancer among women, is strongly linked to Human Papillomavirus (HPV) infection. Emerging evidence indicates that oxidative stress plays a critical role in the carcinogenesis of cervical tissue. This study investigates the relationship between oxidative stress markers—specifically oxysterols, lipid oxidation, and protein carbonylation—and the progression of cervical neoplasia.

Oxysterols, which are elevated in various inflammatory diseases and cancers, were measured in cervicovaginal fluid samples using LC-MS/MS. The targeted oxysterols included 27-hydroxycholesterol (27-OHC), 7β-hydroxycholesterol (7β-OHC), 7-ketocholesterol (7-KC), and 7α,27-dihydroxycholesterol (7α,27-diOHC). Among these, 7α,27-dihydroxycholesterol was significantly increased in correlation with the severity of neoplastic stages. In parallel, protein carbonylation, an indicator of cellular oxidative stress, was assessed. Results revealed higher levels of protein carbonylation in neoplastic samples compared to non-neoplastic controls. These modifications were further analysed through redox proteomics to identify the specific proteins affected.

The study demonstrates that elevated lipid oxidation and protein carbonylation in cervicovaginal secretions are linked to the development and progression of cervical cancer. Identifying these biomarkers may improve screening strategies, enabling the identification of individuals at increased risk for cervical neoplasia and guiding timely interventions.

Introduction

Human papillomavirus (HPV) infection is among the most prevalent sexually transmitted infections worldwide, posing significant public health challenges [1]. Despite significant advancements in vaccines designed to target multiple HPV strains, HPV remains the primary catalyst for cervical cancer (CxCa). Cervical cancer stands as the fourth most common cancer in women globally, predominantly manifesting as squamous cell carcinoma. Notably, specific HPV subtypes such as HPV-16, 18, 45, and 56 are closely linked to this malignancy [1,2]. The progression from virus-induced tissue dysplasia to cervical cancer is a gradual process that typically unfolds over a period of 2 to 10 years [3]. This transformation is particularly common among younger, sexually active women, who represent the demographic with the highest incidence and prevalence of HPV infection and subsequent cervical dysplasia.

Effective screening and accurate classification are crucial for the early detection and treatment of cervical cancer. Established protocols, such as the Bethesda System, provide a standardized approach to categorize cervical intraepithelial neoplasias (CINs) [4-7]. These are graded

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on a scale from 1 to 3, corresponding to "mild," "moderate," and "severe" dysplasia, respectively (CIN1, CIN2, and CIN3). This classification system is essential for identifying the severity of precancerous lesions and determining the appropriate course of treatment. However, current screening methods face several challenges. Patient discomfort, the invasive nature of procedures, and limited sensitivity and specificity of tests are significant barriers. These challenges highlight the critical need for more effective and less invasive screening techniques that can detect cervical cancer at its earliest stages, thereby improving treatment outcomes and the potential for a cure.

Oxidative stress plays a pivotal role in the development and progression of cervical cancer [8]. Elevated oxidative stress contributes to carcinogenesis in two primary ways. Firstly, it facilitates the integration of HPV into host cells through the accumulation of reactive oxygen species (ROS) [9]. These ROS induce DNA damage, which is crucial for the viral DNA to integrate into the host genome. Secondly, oxidative stress causes cellular toxicity by oxidizing essential biomolecules, disrupting their normal functions [8]. This dual impact of oxidative stress underscores its importance in the pathogenesis of cervical cancer.

Oxidative damage affects vital biomolecules such as proteins and lipids, leading to structural and functional alterations [10,11]. These changes can significantly impact cellular signalling pathways and overall cellular health. One of the key oxidative products, oxysterols—oxidised derivatives of cholesterol—plays a critical role in various cellular processes [12]. The levels of oxysterols are tightly regulated due to their cytotoxic effects. Increased levels of oxysterols can disrupt cellular redox balance, mitochondrial function, and trigger apoptosis. This disruption is implicated in the progression of HPV-associated cervical cancer [13], although a systematic evaluation of these effects on prognosis is still needed.

In the context of cancer, oxysterols have been implicated in several key processes that drive the development and progression of malignancies. Among these, specific oxysterols such as 27-hydroxycholesterol (27-OHC) have been extensively studied for their roles in cancer biology [14,15]. 27-OHC has garnered attention due to its ability to promote the proliferation of breast cancer cells [16,17]. Studies have shown that elevated levels of 27-OHC are associated with poor prognosis in breast cancer patients [14]. The pro-tumorigenic activity of 27-OHC is attributed to its dual role in modulating oestrogen receptor (ER) signalling and liver X receptor (LXR) pathways [17]. Beyond breast cancer, 27-OHC is implicated in other cancers such as prostate cancer cell proliferation and migration, colorectal cancers and liver cancers [15]. In prostate cancer, 27-OHC can activate LXR signalling, which may lead to increased expression of genes that promote cell survival and resistance to apoptosis [18]. This highlights the multifaceted roles of oxysterols in cancer biology, as their effects can vary depending on the tissue type and cellular context.

Another significant oxysterol, 7-ketocholesterol (7-KC), has been linked to oxidative stress and inflammation, both of which are hallmarks of cancer [19]. 7-KC is generated through the autoxidation of cholesterol and can induce cell death through oxidative damage and inflammatory responses. Elevated levels of 7-KC have been observed in various cancers, including colon and prostate cancer [20]. The presence of 7-KC in tumour tissues can exacerbate oxidative stress, leading to further DNA damage and promoting malignant transformation and progression. In colorectal cancer, oxysterols such as 7-KC and 27-OHC have been implicated in the modulation of the tumour microenvironment [21]. These oxysterols can influence the behaviour of cancer-associated fibroblasts (CAFs), which are key players in tumour progression and metastasis [21,22]. By altering the activity of CAFs, oxysterols can promote a pro-tumorigenic environment, enhancing cancer cell invasion and metastasis.

Protein carbonylation is another significant consequence of oxidative stress, resulting in irreversible covalent modifications induced by ROS or indirectly through lipid peroxidation. This modification serves as a key marker of oxidative stress [23]. Research has demonstrated that protein carbonylation leads to protein malfunction and cellular toxicity, potentially contributing to cancer development [24]. Despite its importance, the extent of protein carbonylation in cervical malignancies remains poorly documented. Few studies have explored its presence in cervical cancer cell lines and plasma samples. There is a need for comprehensive investigations into protein carbonylation content in cervical secretion material, comparing cancerous or precancerous stages to healthy controls.

In this study, specific oxysterols previously associated with various cancers, particularly breast tumours, were analysed in cervicovaginal fluid (CVF) samples. The targeted oxysterols included 27-hydroxycholesterol (27-OHC), 7β-hydroxycholesterol (7β-OHC), 7-ketocholesterol (7-KC), and 7α,27-dihydroxycholesterol (7α,27-diOHC). Using advanced liquid chromatography-tandem mass spectrometry (LC-MS/MS), these oxysterols were measured across non-neoplastic, low-grade neoplastic, and high-grade neoplastic groups.MATERIAL AND METHOD

Sample recruitment and collection

This study was conducted with the approval of the Acibadem Healthcare Institutions Medical Research Ethics Committee (registration number: 2022-07/23). All participants were provided written informed consent before sampling and experiments were performed following the approved institutional guidelines. Enrolment and sampling were performed at Acibadem Altunizade Hospital and Acibadem Maslak Hospital in Istanbul, Turkiye. Criteria of eligibility were women with (patient group) and without (control group) cervical neoplasia development. Exclusion criteria are unprotected sexual intercourse within 72 h of the sampling, or vaginal bleeding within the current or previous week before sampling, or any vaginal pomade or wick use within 72 h.

Alongside the neoplastic stages, non-neoplastic samples were also collected in the same way from women who undergo colposcopy-directed biopsy (CDB) or loop excision-based surgical procedure (LEEP) because of having persistent high-risk type HPV infection and abnormal Pap-smear results. The histopathological diagnosis by CDB or LEEP was used for sample classification.

During the pelvic examination, before any treatment (colposcopy imaging or any surgical excision), cervicovaginal area was rinsed with isotonic buffer to obtain the entire secretion with poured cells and discharge. The lavage liquid was aspirated back by a sterile plastic injector and collected into a centrifuge tube. Storage buffer (pH: 7.4; in 1X PBS: 0.05 M sodium azide and 1X proteinase inhibitor cocktail: Thermo #87785) was added to the samples as set up by 10 % final volume for protecting and stabilizing the biological material. Samples were divided into 3 aliquots, and they were stored at −20 °C until required for the experiment. The study groups were designed in accordance with the neoplasia development of patients: None-neoplastic (no intraepithelial lesion or dysplasia: NILM) cervixes were recruited to the “Control group” (n: 10); while cervixes with stage 1 of the CIN were recruited to the “CIN1” (n: 14); cervixes with stages 2 and 3 of CIN were grouped to “CIN2+” (n: 14) (see full patient list in supplementary file 1).

Lipid extraction of the samples

Lipids were extracted from the samples as described before [25]. Briefly, 500 μl of CVF sample was mixed with 1.5 ml of cold methanol containing 50 μg/ml Butylated hydroxytoluene (BHT), sonicated in an ultrasonic bath sonicator for 10 min (ThomasSci Branson CPX5800H), centrifuged at 14,000 × *g* for 10 min, and dried. Lipid extract resuspended in 40 μl 40 % aqueous methanol containing 0.5 % formic acid.

Cholesterol assay

The total cholesterol content was determined with Amplex® Red Cholesterol Assay Kit (Invitrogen™ #A12216). Briefly, 25 μl of the sample was mixed with 25 μl of reaction buffer and placed in microplate

wells. The working solution containing Amplex® Red reagent/HRP/cholesterol oxidase/ cholesterol esterase was added to wells. The plate is incubated for 30 min at 37 °C by protecting the light, and absorbance values were measured at 570 nm.

Target lipids and chemicals

Four hydroxy sterols: 7α,27-dihydroxycholesterol (7α,27-diOHC), 27-hydroxycholesterol (27-OHC), 7ß-hydroxycholesterol (7ß-OHC), and 7-ketocholesterol (7-KC) were measured by LC-MS/MS using a multiple reaction monitoring (MRM) based targeted MS method as described previously [25].

Authentic and deuterated standards were purchased from Avanti Polar Lipids (Alabama, USA) and Cayman Chemicals (MI, USA). Butyl acetate, hexane, isopropanol, methanol, and formic acid (HPLC/MS grade) were purchased from Fisher Scientific (Hampton, New Hampshire, USA). Butylated hydroxytoluene (BHT) was purchased from Merck (Dorset, UK).

Targeted lipidomics assay

The separation of the samples was performed using liquid chromatography (LC, DIONEX UltiMate 3000, Thermo Scientific UK Ltd., Hemel Hempstead, UK) using a reverse phase C18 column (NUCLEOSIL® C18, 100 mm, 5 μm pore size, Macherey-Nagel, Germany) coupled with a triple quadrupole mass spectrometer (QTRAP 5500, AB Sciex UK Ltd., Warrington, UK) using multiple reactions monitoring (MRM) methods. Data were analysed using Analyst software (version 7.1) quantification wizard to describe lipid abundance, and the acquired signal intensity (peak area) values of all targeted lipid species were normalized against the corresponding isotopically labelled internal standards and quantitation was done using the deuterated isotope/endogenous oxysterol ratio method with the internal standards. In the absence of deuterated 7,27-diOHC, the only target was calculated relatively by utilizing the standard curve. To ensure the reliability of acquired lipidomics data, quality control (QC) samples were used for the evaluation of data quality. Statistical analysis was carried out in SPSS and GraphPad Prism software and the significance of results between the study groups was revealed.

Protein carbonylation assay

Additional forty-three patient samples (control; $n = 10$), CIN1; $n = 17$) and CIN2+; $n = 16$) with the histopathologically confirmed diagnosis who visited the Department of Obstetrics and Gynaecological Oncology at Acıbadem Maslak and Acıbadem Altunizade Hospitals in Istanbul, Turkiye were recruited for the assay. Protein quantification was performed using Pierce Micro BCA™ Protein Assay Kit (ThermoFisher #23235). Samples were diluted 100-fold, mixed with working reagent, and incubated at 37 °C for 2 h, and absorbance values were measured at 562 nm.

Determination of protein carbonyl products in biological samples was performed according to the method described by Buss et al. [26]. Protein carbonyl content can be measured in unknown samples based on 2,4-dinitrophenylhydrazine (DNPH) derivatization of the carbonyl groups and detection of them through antibodies against DNP derivate. Unknown samples were diluted in carbonate buffer (pH: 9.6; 0.05 M) to be 20 μg/ml total protein concentration to normalize them. For the ELISA experiment, 1 μg protein with 50 μl volume of the samples was taken in a high-absorbant flat-bottom clear ELISA plate. Samples were treated with DNPH, the plate was blocked overnight with Tween-20, and after all, treated with incubated with conjugated antibodies and o-phenylenediamine dihydrochloride (OPD) substrate. Reaction was stopped with sulfuric acid (H_2SO_4) and spectrophotometric measurement was performed at 490 nm by a microplate reader (Tecan Life Science). The result sheet of the measurement has been given in the Supplementary file 1.

Shotgun proteomics analysis of the control and cancer samples

For proteomics analysis, 8 non-dysplastic control samples and 4 cancer samples that three of them with squamous cell carcinoma and one of them with primary diffuse large B cell lymphoma of the uterine cervix (pDLBUCx) were measured in duplicate (Supplementary file 2). For proteomics experiment, 0.1 % 100 μl mass spectrometry-compatible surfactant RapiGest which is dissolved in 50 mM ammonium bicarbonate (Waters, Milford, MA, USA) was added to 50 μl CVF samples. Mixture was homogenized through sonication and incubated at 95 °C for 10 min by shaking at 400 rpm. After reducing with dithiothreitol DTT and alkylation with iodoacetamide (IAA); 10 μl 100 ng/μL trypsin (Promega Trypsin Gold, Mass Spectrometry grade) enzyme was added, and tubes were left for overnight incubation at 37 °C. Digestion was ended with 0.1 % trifluoroacetic acid (pH: 2) and samples were centrifuged at 12.000 rpm for 30 min to debris any particle. A hundred μL of each sample was transported into LC glass vials. Each sample was injected with a volume of 5 μL and separated on an Ultimate 3000 HPLC system (Thermo Fisher Scientific, Germering, Germany), analysed in Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) in the data-dependent acquisition (DDA) mode.

For DDA measurements, samples were injected and loaded into a trap column (PepMap C18, 300 μm ID, 5 mm length, 5 μm particle size, 100 Å pore size; Thermo Fisher Scientific), and during the first 10 min, 0.1 % trifluoroacetic acid used as loading solvent at a flow rate of 20 μL/min. Afterward, the trap column was switched to the analytical column (PepMap C18, 75 μm ID × 250 mm, 2 μm particle size and 100 Å pore size, Thermo Fisher Scientific). Peptides were eluted with a 90-minute acetonitrile gradient ranging from 3 % to 30 % (and formic acid concentration from 0.1 % to 0.08 %, respectively) at 250 nL/min. The proteins in the samples were identified by exporting features with recorded MS/MS spectra using ProteoWizard software (version 3.0.9248). Proteomics data was uploaded in Pride (EMBL).

The resulting .mgf files were then submitted to Mascot (version 2.3.01, Matrix Science) for protein identification against the human UniProtKB/Swiss-Prot database (version 2012_12, Human Taxonomy, 20,395 entries) using trypsin digestion and fragment ion mass tolerance of 0.80 Da, parent ion mass tolerance of 10 ppm, and a maximum number of missed cleavages of two. Oxidation of methionine was specified as a variable modification and carbamidomethylation of cysteine as a fixed modification. The resulting peptide data from the Mascot search were processed using Scaffold (version 5.1.2, Proteome Software Inc.) for summarization and filtering. The number of proteins was determined based on the peptide data according to the criteria of the "Peptide Prophet" algorithm, with a probability of >95 % and protein identification with a probability of >99 %, and at least one peptide identification. Analysis of identified proteins was carried out in PANTHER (http://www.pantherdb.org/).

Redox epiproteomics

To search for the carbonylation modification of certain amino acids, Mascot Daemon database search was used by using mass changes in the residues. Within the MS configuration parameters, amino acid modifications regarding mass shift because of the carbonylation by losing or gaining C, H, N, or O atoms were set up. For the transition of proline (C5H9NO2) to pyroglutamic acid (C5H7NO3), mass shift modification "H(+2) O" has been entered. For arginine (C6H14N4O2) to glutamic-5-semialdehyde (C5H9NO3), "C(−1) H(−6) N(−3) O" has been entered. For threonine (C4H9NO3) to 2-amino 3-oxobutanoic acid (C4H7NO3), "H(−2)" has been entered. Lastly, for lysine (C6H14N2O2) to 2-aminoadipic acid (C6H11NO4), a modification "H(−3) N(−1) O(2)" has been entered.

Statistical analysis

All analyses were performed using GraphPad Prism® software version 10. For the significance analysis, a one-way ANOVA was conducted to determine if there were statistically significant differences among the groups. Principal Component Analysis (PCA) was performed using ClustVis (https://biit.cs.ut.ee/clustvis/) to explore the data's underlying structure and identify potential clustering patterns based on the measured variables. Additionally, a Receiver Operating Characteristic (ROC) curve was generated using GraphPad Prism to assess the diagnostic performance of the biomarker, providing a measure of its sensitivity and specificity in distinguishing between non-dysplastic and CIN2+ patient samples. A p value of <0.05 was considered statistically significant in all the performed analyses.

Results

Analysis of cholesterol and oxysterol levels in cervical intraepithelial neoplasia

Patient demographics were compared, and one-way ANOVA was conducted to determine if there were statistically significant differences among the groups. There were no statistically significant differences between the groups in terms of age, body mass index (BMI), or the contraception method between groups selected for this study. Total cholesterol content was not different between control (395.28 ± 329.69 nM), CINI (455.25 ± 437.77 nM) and CIN2 (524.58 ± 613.25 nM) groups ($P = 0.3$). Absolute concentrations of 7α,27-diOHC was found to be significantly ($p = 0.02$) increased in the CIN2+ group compared to the control group (Fig. 1A). CINI group was not changed compared to the control group. Other oxysterols (27-OHC, 7β-OHC and 7KC) were not significant in patients compared to control samples.

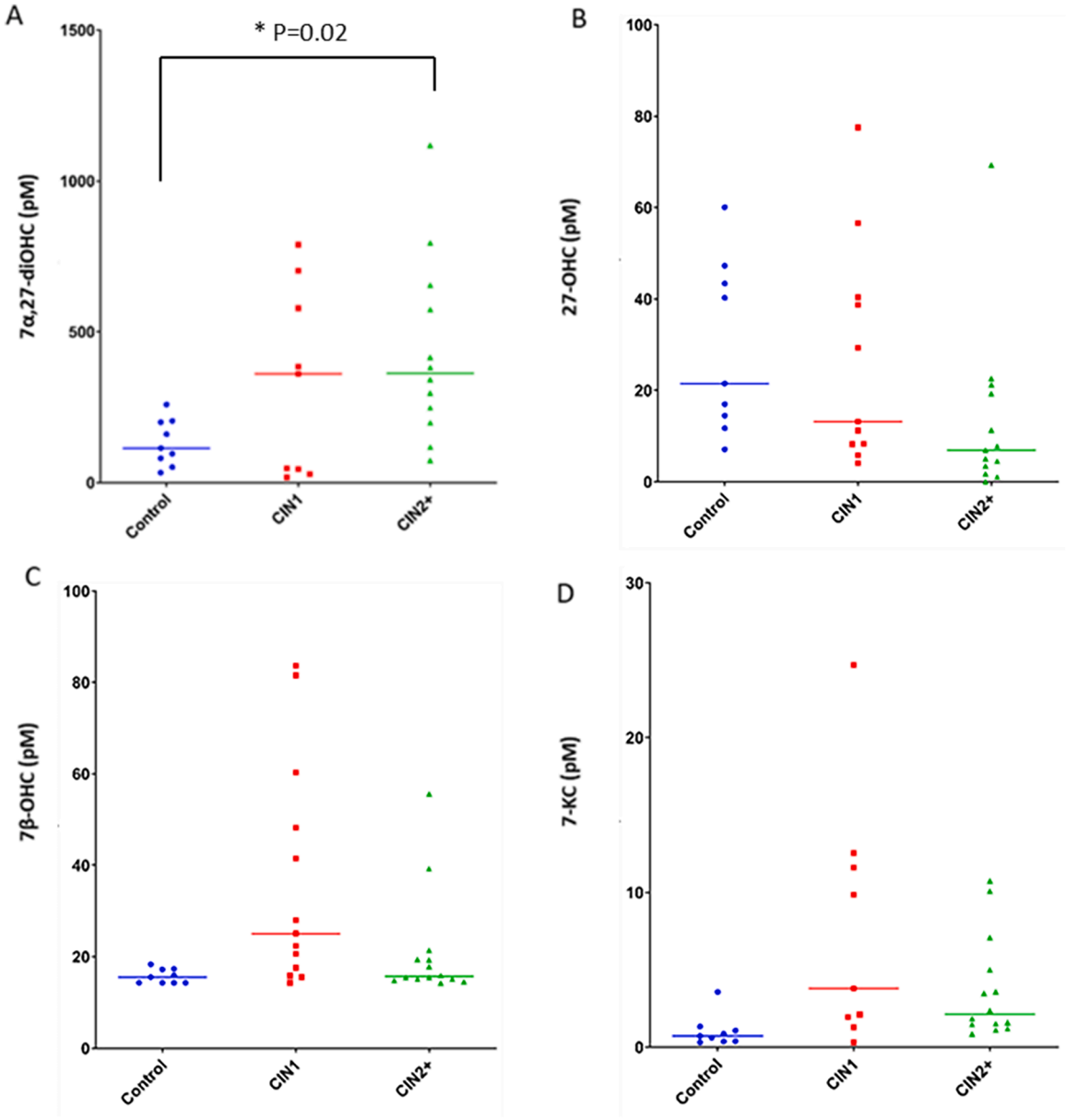

Fig. 1. Free oxysterol concentrations in CVC from patients with cervical cancer (control; $n = 10$, CIN1; $n = 14$, CIN2+; $n = 14$); (A) 7α,27-diOHC (B) 27-OHC (C) 7β-OHC (D) 7-KC. Data was analysed with One-way ANOVA on GraphPad Prism 10 software.

Protein carbonylation as a biomarker in cervical intraepithelial neoplasia

Protein carbonylation ELISA test was performed for 44 samples (11 of them overlapped with the previous experiment cohort and 33 of them are additionally collected new samples) that include a control group with no intraepithelial lesion or neoplasia, CIN1 group and CIN2+ group (CIN2; $n = 6$, CIN3; $n = 4$, and CxCa; $n = 6$). The power analysis confirmed that the study's sample size of 44 is sufficient to detect significant differences in protein carbonylation levels among the control, CIN1, and CIN2+ groups, given the large effect size observed ($\eta^2 = 0.30$). This ensures that the study is well-powered to identify meaningful differences, supporting the validity of the findings.

Total protein concentrations were not significantly different between sample groups (Control; 1624.27 ± 1265.14 μg/ml, CIN1; 2251.22 ± 1095.31 μg/ml and CIN2+; 2099.01 ± 1073.43 μg/ml). Fig. 2A presents no difference to protein carbonylation between control (1.10 ± 0.68 nmol/mg of protein) and CIN1 group (0.79 ± 0.46 nmol/mg of protein). However, there was a significantly higher ($P = 0.0007$) protein carbonylation observed in CIN2+ group (4.26 ± 3.25 nmol/mg of protein) compared to the control group. In addition, a receiver operating characteristic (ROC) curve was plotted based on CDB diagnosis. Non-dysplastic patient samples were assigned as "0″ and CIN2+ patient samples were assigned as "1″. A high clinical discriminant power was provided through a value of 0.92 area under the curve (AUC) with 93.75 % sensitivity and 77.78 % specificity. To further quantify the magnitude of the differences observed, effect sizes were calculated using eta squared (η^2) and omega squared (ω^2) from ANOVA results. An η^2 of 0.30 suggests that 30 % of the variance in protein carbonylation levels is explained by the differences between the groups. This represents a large effect size, indicating substantial differences in protein carbonylation among the groups. The ω^2 value of 0.44, which adjusts for sample size, confirms a large effect size, though slightly more conservative. These effect sizes highlight the significant impact of disease stage on protein carbonylation levels.

Redox proteomic analysis of carbonylated proteins in cervical intraepithelial neoplasia

Redox proteomics was performed in a randomly selected subset of samples. This subset had higher protein carbonylation in cancer group (2.84 nmol/mg of protein) compared to the control group (1.42 nmol/mg of protein) $p = 0.0021$. To identify proteins that are carbonylated and to investigate level of carbonylation, a label-free non-targeted proteomics analysis was designed. In total, 4282 proteins were identified, and 258 proteins have found to be statistically differently ($p < 0.05$) expressed between control and cancer samples. Among these, 51 proteins were found to be decreased in the cancer group and 207 proteins were found to be increased in the cancer group compared to the control group (see supplementary file 2). A principal component analysis (PCA) for differentially expressed proteins showed that control samples completely dissociated from the cancer samples with tight clustering (Fig. 3).

Regarding the analysis of carbonylation modification of the most prone amino acids, the "total unique peptide count" information was used, and 60 unique proteins containing at least one carbonylated arginine/lysine/proline/threonine were significantly identified between the groups, shown in Table 1. For carbonylation of arginine, 4 proteins were found to be at a higher amount of glutamic-5-semialdehyde in the cancer group. For carbonylation of proline, 33 proteins were found to be at a higher amount of pyroglutamic acid in the cancer group, and 7 proteins were at a higher amount in the Control group. For carbonylation of threonine, 24 proteins were found to be at a higher amount of 2-amino 3-oxobutanoic acid in the cancer group; only 7 proteins were at a higher amount in the Control group. For carbonylation of lysine, 6 proteins were found to be at a higher amount of 2-aminoadipic acid in the cancer group; only 1 protein was at a higher amount in the Control group.

Albumin was identified as overexpressed in the cancer group with high-level threonine carbonylation. Nevertheless, concerning both carbonylated and differentiated proteins in overall identification, those are 51.61 % of intracellular and cytoskeletal proteins according to the PANTHER analysis, while 20.97 % of extracellular, and 27.42 % of basement membrane with collagen-containing extracellular matrix

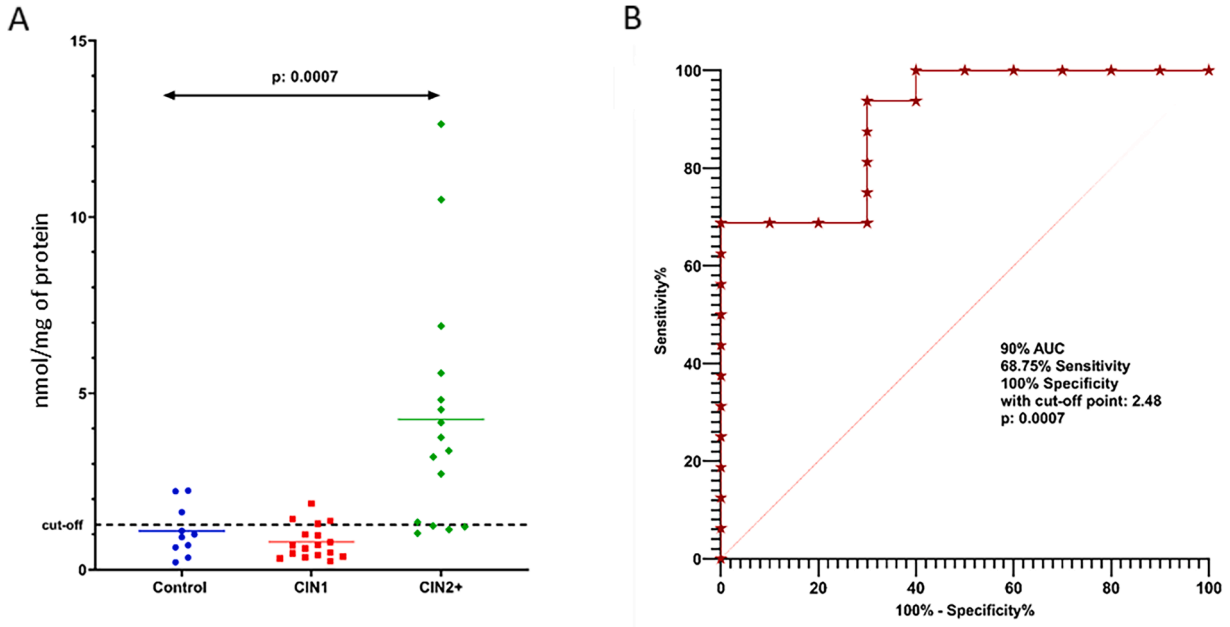

Fig. 2. Protein carbonyls in CVF. A. Control group (n: 10); CIN1 group (n: 17); CIN2+ group (n: 16) consisting of CIN2, CIN3, and CxCa diagnosed by CDB. B. ROC curve plotted for discrimination of protein carbonylation between the control and CIN2+ patients: 90 % AUC with 2.48 cut-off point and 100 % sensitivity, 68.75 % specificity.

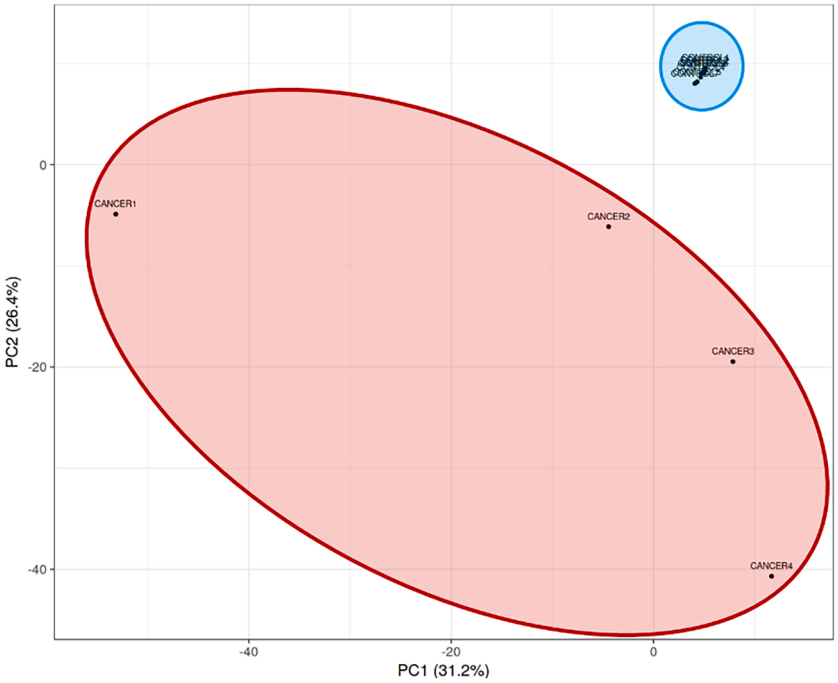

Fig. 3. PCA plot for proteins in CVF samples. The red cloud consists of cancer patient samples, and the blue cloud consists of control samples. (Created by biit.cs.ut.ee/clustvis).

which is an expected outcome since CVF contains plenty of excreted material.

In total, 41 unique proteins were found to be carbonylated at least once in one sample shown in Table 1. Six of the proteins were carbonylated in at least 1 patient sample for both proline and threonine; only complement C1q and tumour necrosis factor-related protein 9A was carbonylated for both proline and lysine, and 3 proteins were carbonylated for both arginine and proline.

Discussion

The findings of this study highlight the critical role of oxidative stress in the progression of cervical cancer. Elevated levels of specific cholesterol oxidation products and protein carbonylation in cervicovaginal secretions can serve as indicators of oncogenesis. The results revealed for the first time that 7α,27-dihydroxycholesterol levels increased with the severe neoplastic stage. Additionally, this study assessed protein carbonylation levels in CVF samples as a marker of increased cellular oxidative stress. The findings indicated that protein carbonylation was significantly higher in the neoplasia group compared to the non-neoplastic control group. The significant increase in protein carbonylation levels in the CIN2+ group, coupled with large effect sizes (η^2 and ω^2), provides strong evidence for the association of protein carbonylation with advanced stages of cervical intraepithelial neoplasia and cervical cancer. The high discriminative power indicated by the ROC curve analysis further supports the potential of protein carbonylation as a valuable biomarker for cervical neoplasia. Using sensitive redox proteomic approach, this study characterised carbonylated proteins to identify the specific proteins affected by oxidative modifications.

Previous studies demonstrated that oxysterols are increased in biofluids such as serum, plasma, or tissues in various types of malignancies [12,27]. CVF is an important biofluid with significant potential for biomarker identification, particularly in the context of gynaecological diseases CxCa. CVF, a complex mixture of secretions from the cervix, vagina, and associated glands, contains a diverse array of biological molecules, including proteins, lipids, nucleic acids, and metabolites. These constituents reflect the physiological and pathological state of the female reproductive tract, making CVF a valuable source for non-invasive biomarker discovery. The composition of CVF varies with hormonal cycles, age, and the presence of infections or malignancies. Changes in the cervicovaginal lipidome have been previously associated with preterm birth, genital infection, and cancer [28]. This study provides first analysis of oxysterols in CVF. Recent studies have highlighted potential role of 27-OHC in cancer biology as it can modulate key signalling pathways involved in cell proliferation, survival, and migration, making it a significant player in oncogenesis. 27-HC is a significant oxysterol produced mainly through the CYP27A1 enzyme. In the context of cervical cancer, 27-OHC is particularly noteworthy due to its ability to influence the tumour microenvironment [17]. It can promote the proliferation of cancer cells by activating liver X receptor (LXR) and oestrogen receptor (ER) pathways. The activation of these pathways can lead to enhanced cell growth and survival, contributing to the progression of cervical neoplasia [17]. Furthermore, 27-OHC has been shown to induce oxidative stress, a condition linked to DNA damage and genomic instability, which are crucial steps in the carcinogenic process [15]. This study shows low levels of 27-OHC in CxCa compared to healthy individuals. İnterestingly, di-hydroxylated oxysterol 7α,27-dihydroxycholesterol, a metabolic product of 27-OHC via the cytochrome P450 enzyme, oxysterol 7α-hydroxylase (CYP7B1) [29]was significantly increased. Oxysterols like 7α,27-diOHC are known to modulate signalling pathways and metabolic processes, potentially contributing to oncogenesis [30]. The presence and elevated levels of 7α,27-diOHC suggest its potential as a biomarker for Cxca diagnosis and prognosis. Further research is needed to fully elucidate the specific mechanisms by which 7α,27-diOHC contributes to cancer development and to explore its potential as a diagnostic and therapeutic target. Understanding the role of oxysterols like 7α,27-diOHC in cancer could lead to new strategies for the management and treatment of various malignancies.

One major challenge in oxysterol analysis is availability of analytical methods that are sensitive to detect very low levels. Using a targeted lipidomic approach this study revealed for the first time that oxysterol levels in CVF is 2000–3000 times lower compared to the levels in plasma. Using higher volume of starting materials, applying steps to enrich samples, and using sensitive instruments will be useful for future

Table 1
Carbonylated proteins in CVF. The difference in identified proteins with carbonylated amino acids was investigated based on total unique peptide counts using Scaffold viewer software.

Carbonylated amino acids	Protein name	Quantitative profile in cancer compared to control
Proline	Histone H1.5	↓
	Histone-lysine N-methyltransferase SETD5	↓
	Protein CDV3 homolog	↓
	Splicing factor 3A subunit 1	↓
	Immunoglobulin heavy constant gamma 3	↑
	Lysine-specific demethylase 6B	↓
	Collagen alpha-1(II) chain	↓
	Ankyrin repeat domain-containing protein 36C	↓
	Transcription elongation regulator 1	↓
	Voltage-dependent L-type calcium channel subunit alpha-1C	↑
	Parathyroid hormone/parathyroid hormone-related peptide receptor	↑
	Collagen alpha-3(V) chain	↓
	SH3 and multiple ankyrin repeat domains protein 3	↑
	Forkhead box protein O4	↑
	Pleckstrin homology domain-containing family G member 2	↑
	Synapsin-1	↑
	Reticulon-4	↑
	UBX domain-containing protein 11	↑
	Putative uncharacterized protein FLJ45275, mitochondrial	↑
	Low-density lipoprotein receptor-related protein 3	↑
	Mitogen-activated protein kinase 9	↑
	Fibronectin type III domain-containing protein 4	↑
	Collagen alpha-1(XV) chain	↑
	Collagen alpha-2(IV) chain	↑
	Rho guanine nucleotide exchange factor 17	↓
	Collagen alpha-1(IX) chain	↓
	Complement C1q and tumor necrosis factor-related protein 9A	↓
	Atherin	↓
	Glutamate receptor ionotropic, kainate 4	↑
	Myocardin-related transcription factor A	↓
Lysine	Immunoglobulin heavy constant gamma 1	↑
	Complement C1q and tumor necrosis factor-related protein 9A	↑
Threonine	Collagen alpha-1(IV) chain	↓
	Mucin-6	↓
	Albumin	↑
	Transcription elongation regulator 1	↓
	Immunoglobulin heavy constant gamma 3	↑
	Nuclear receptor-binding factor 2	↓
	Ankyrin repeat domain-containing protein 36C	↓
	Pleckstrin homology domain-containing family G member 2	↑
	Parathyroid hormone/parathyroid hormone-related peptide receptor	↑
	Growth factor receptor-bound protein 7	↑
	Zinc finger protein 624	↑
	Low-density lipoprotein receptor-related protein 3	↑
	YTH domain-containing family protein 3	↑
	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 3	↓
Arginine	Cluster of E1A-binding protein p400	↓
	E1A-binding protein p400	↓
	Voltage-dependent L-type calcium channel subunit alpha-1C	↑
	Fibronectin type III domain-containing protein 4	↑
	Histone-lysine N-methyltransferase SETD5	↓

CVF oxysterol analysis. CVF contains epithelial cells, immune cells, enzymes, cytokines, antibodies, lipids, and microbial products. İn this study, fluids were centrifuged to remove any debris or cellular materials before analysis. However, cytological scraping samples such as smear, rather than lavage aspiration, could give additional view for oxysterols within HPV-associated cervical malignancy.

Increased oxidative stress ultimately causes protein damage in cells [11]. İn line with oxidative stress hypothesis, this study showed increased protein carbonylation in the group consisting of dysplasia and cancer of the cervix. Carbonylation is a type of protein oxidation that involves the introduction of carbonyl groups (aldehydes or ketones) into protein side chains, typically at lysine, arginine, proline, or threonine residues. This modification is often irreversible and can significantly alter protein function, leading to cellular dysfunction and disease [31]. Here, we explore the use of mass spectrometry for identifying and characterizing carbonylated proteins, emphasizing its relevance and application in CxCa diagnosis. Data from randomly selected 8 control and 4 cancer samples aligned with protein carbonyl ELISA measurements for high carbonylation in cancer patients. Protein coverage revealed mostly intracellular carbonylated proteins and excreted matrix proteins. While protein carbonylation is a valuable marker, its interpretation in the context of cervical cancer needs more attention, considering its roles in quality control and redox signalling. Comprehensive oxidative stress evaluation typically requires additional antioxidant parameters such as glutathione (GSH), the GSH/GSSG ratio, superoxide dismutase (SOD), and catalase (CAT), which are not analysed in this study. This approach will ensure a more accurate understanding of the oxidative stress condition and the multifaceted roles of protein carbonylation in cervical cancer.

The major limitation in cervical cancer patient assessment is the classification. Even though CxCa screening technique primarily applied worldwide, Pap-smear test has a low sensitivity and specificity This could contribute to high false-negative and false-positive outcome ratios [32,33]. Therefore, the design of this study was based on histology information by the scores of mainly CDB or LEEP, if exist (see supplementary file 1). It should be noted that the Pap-smear test is a screening method, not a diagnostic method, hence, CDB method has better accuracy than the Pap-smear test [34]. Patients with a squamous intraepithelial lesion (LSIL of HSIL) according to the initial screening test Pap-smear, are frequently recruited for CDB, and 60 %–90 % of them are diagnosed with CIN [35,36], and a pathology report with ≥CIN2 requires LEEP to remove the transformed malignant tissue. Considering the LEEP procedure is the gold standard, the discrepancy between CDB and LEEP has been investigated and the rate of agreement of the diagnostic report is around ~60 % [37,38] which means that approximately half of the colposcopy patients had been misdiagnosed. In summary, colposcopy and particularly Pap-smear tests as current approaches have inherent inaccuracy, and this handicap affects statistical analyses. Therefore, we were unable to achieve the most ideal diagnosis for all of the patients. Also, the sample size was not adequate to determine the diagnostic performance of oxysterols with high accuracy. The reason for

this, we preferred to recruit only colposcopy patients for the study, however, the frequency of CDB application is 3.8 % in Turkye [39] which is another limitation to collect a larger number of samples. Additionally, we could not reach out for extensive information about the risk factors associated with the patients such as polygamy/monogamy status, parity, and smoking.

In the female genitourinary system, certain metabolites are distinctly associated with particular species of local floral bacteria. Hence, an altered metabolite composition may show a discomposed microbiome, and the discomposed microbiome eventually brings the deregulated local system health [40,41]. Therefore, analysing the microbiome in parallel with metabolic markers may warrant a more comprehensive understanding of the interplay between microbial communities and host metabolic pathways. Given that there is a relationship that has not been well-understood between oxidative stress, HPV persistence, neoplastic transformation, and chemical treatment of the cervix, our data shows for the first time a promising avenue for future investigations.

Conclusion

Cervical cancer remains a significant global health burden, closely linked to HPV infection. Despite advances in vaccination and screening, the disease continues to pose challenges due to the limitations of current detection methods. CVF is a promising biofluid for biomarker identification in cervical cancer and other gynaecological diseases. Despite the challenges, the non-invasive nature and rich molecular content of CVF make it an attractive target for research. This study underscores the importance of oxidative stress in the development and progression of cervical cancer. By exploring the roles of oxysterols, lipid oxidation, and protein carbonylation, we can gain a deeper understanding of cervical carcinogenesis. This, in turn, can inform the development of more effective screening tools and therapeutic strategies, ultimately improving outcomes for women worldwide.

CRediT authorship contribution statement

Busra Kose: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Serkan Erkanlı:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Alper Koçak:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Coskun Guzel:** Writing – review & editing, Supervision, Software, Resources, Methodology, Investigation, Data curation, Conceptualization. **Theo Luider:** Writing – review & editing, Supervision, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Irundika H.K. Dias:** Writing – review & editing, Supervision, Validation, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Ahmet Tarik Baykal:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare there is no conflict of interests.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.arres.2024.100111.

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