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Putative Detection of 4-Acetamidobutanoic Acid in an HPV-16-Positive Pooled Cervical Cytology Sample: An Exploratory LC–HRMS Study

Polar Metabolites in HPV-Genotyped Cervical Cytology Fluid

Ahmed Ali Abd Ali[1], Ali F. Hussein[2], Nada J. Dawood[3], Shivanshu Srivastava[4], Rakesh Rawal[5,6*]

[1] Department of Biochemistry and Forensic Science, School of Sciences, Gujarat University, Ahmedabad-380 009, Gujarat, India.
mscahmedali@gmail.com. ORCID 0009-0003-2817-2381
[2] Medical Technical Institute, Southern Technical University, Basrah, Iraq.
a.faisal@stu.edu.iq. ORCID 0000-0002-4104-593X
[3] Medical Technical Institute, Southern Technical University, Basrah, Iraq.
n.alkamil@stu.edu.iq
[4] YGen Healthcare Private Limited, Ahmedabad, Gujarat, India.
ygenlifesciences@yahoo.com
[5] Department of Biochemistry and Forensic Science, School of Sciences, Gujarat University, Ahmedabad-380 009, Gujarat, India.
[6] Professor and Head, Department of Medical Biotechnology, Gujarat Biotechnology University, Gandhinagar, Gujarat, India.
rakesh.rawal@gbu.edu.in
* Corresponding author: Rakesh Rawal, Professor and Head, Department of Medical Biotechnology, Gujarat Biotechnology University, Gandhinagar, Gujarat, India.
Email: rakesh.rawal@gbu.edu.in

ABSTRACT:

Objective: Residual cervical cytology material allows the biochemical environment of human papillomavirus (HPV) infection to be studied without additional sampling. We surveyed polar metabolites in this material, stratified by HPV genotype, to generate candidates for targeted testing.

Methods: Sixty residual cytology specimens were genotyped by multiplex real-time PCR and combined into four pools of fifteen: HPV-negative, HPV-16, HPV-18 and other high-risk. Cells were pelleted, washed twice in buffered saline, and extracted by a biphasic chloroform–methanol–water procedure. The aqueous phase was analysed by hydrophilic interaction chromatography with high-resolution tandem mass spectrometry, all four pools and two blanks in one sequence. The design yields four observations without replication, so no inferential statistics were computed. Three constraints qualify every result: no pooled quality control sample was injected, injections were not randomised, and the eluent was unbuffered, limiting retention of acidic analytes.

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Result: Annotation returned 291 features. After removing solvent additives, leachables, pharmaceuticals and non-specific matches, 77 were endogenous metabolites, of which 23 eluted in the void volume and were excluded. Choline, glutamate and a C6H13NO2 feature appeared in all four pools. A feature putatively annotated as 4-acetamidobutanoic acid (N-acetyl-GABA) was observed only in the HPV-16 pool, at 1.34×10^9 arbitrary units, with an MS/MS library match of 79.4 and a mass error of 0.15 ppm, it is resolved by 6.08 min from the only other C6H11NO3 feature present, supporting a putative annotation at Metabolomics Standards Initiative level 2. Pathway over-representation analysis returned no set surviving correction. Detection in one pool is not evidence of absence elsewhere, limits of detection being undetermined.

Conclusion: One putative annotation warrants follow-up: 4-acetamidobutanoic acid in the HPV-16 pool. Nothing reported is a validated biomarker. The report defines what this material yields under an untargeted workflow and what a confirmatory study must meet. Because each group was represented by a single non-randomized injection without pooled QC or technical replication, the study cannot distinguish biological differences from analytical variation.

1. INTRODUCTION

Cervical cancer remains among the most common cancers in women worldwide, with an estimated global burden that continues to fall disproportionately on low- and middle-income countries (Bray et al., 2024). Persistent infection with high-risk human papillomavirus (HR-HPV) is its principal cause, and HPV-16 and HPV-18 account for the majority of cases.

Transformation is driven by sustained expression of the viral oncoproteins E6 and E7, which inactivate p53 and pRb respectively. In the productive episomal state, E6 and E7 transcription is held in check by the viral E2 protein, which binds the upstream regulatory region and represses the early promoter. Integration of viral DNA into the host genome frequently disrupts or deletes the E2 open reading frame, and the loss of E2-mediated repression, together with stabilisation of the E6–E7 transcript, raises oncoprotein expression. Integration is therefore one route to deregulated expression rather than a precondition for it: episomal genomes can also sustain raised E6 and E7 levels through methylation of E2-binding sites and other promoter-level changes, and a substantial proportion of cervical carcinomas retain episomal virus (Oyervides-Muñoz et al., 2018). The distinction matters for the interpretation of metabolic findings, since episomal and integrated infections need not share a transcriptional programme, and the physical state of the viral genome was not determined in the present specimens.

Among the downstream consequences of E6 and E7 expression is metabolic reprogramming. In HPV-16-transformed cervical cells, E6 and E7 raise expression of the glutamine transporter SNAT1 together with GLS2 and glutamine synthetase, and proliferation becomes glutamine-dependent (Ortiz-Pedraza et al., 2023). Both oncoproteins also stabilise HIF-1α, favouring aerobic glycolysis (Arizmendi-Izazaga et al., 2021, Arizmendi-Izazaga et al., 2024), and HPV infection perturbs glycogen handling, the pentose phosphate pathway and fatty acid β-oxidation (Sitarz et al., 2021). Amino acid metabolism therefore sits close to the centre of HPV-associated metabolic change.

Studies of the cervicovaginal metabolome have begun to describe these changes in clinical material. Women with HR-HPV show lower concentrations of amino acids, lipids and peptides than women carrying only low-risk types (Borgogna et al., 2020), non-Lactobacillus vaginal communities perturb amino acid and nucleotide metabolism, with carnitine metabolism linking dysbiosis to genital inflammation (Ilhan et al., 2019), plasma profiling identifies glutamate, proline, hypoxanthine and pyroglutamate as discriminating between cervical neoplasia and healthy controls (Khan et al., 2019, Nam et al., 2020), and cervical mucus analysis reports decreased arginine with increased citrulline, proposed to weaken local tumour immunity (Kawasaki et al., 2024). Genotype-stratified comparisons remain few (Chen et al., 2022, Zhao et al., 2025).

Residual liquid-based cytology material is attractive for such work because it is already collected during routine screening, is genotyped as part of standard care, and requires no additional intervention. It is also demanding: the specimens are archived in an alcohol-based preservative, cell numbers vary between vials, and the cellular content is dominated by normal epithelium

even where disease is present. We therefore treated this study as an exploratory survey rather than a biomarker discovery exercise, and report it accordingly.

2. MATERIALS AND METHODS

2.1 Study design and specimens

This was an exploratory, cross-sectional, pooled-sample study of residual liquid-based cervical cytology specimens retained after routine cytological screening. Two preservative systems were represented across the series, ThinPrep and SurePath, individual specimens having been collected into whichever medium was in use for that patient at the time of screening, the series is therefore of mixed medium. Specimens were supplied by YGen Healthcare Pvt. Ltd., a private diagnostic laboratory group in Ahmedabad, Gujarat, India, and were collected at intervals through the calendar year 2024. They were transported to Gujarat University in cooled thermal containers and stored at −80 °C until analysis, which was performed in April 2025, the interval between collection and acquisition therefore ranged up to approximately thirteen months and differed between specimens. Sixty specimens were assigned to four groups of fifteen on the basis of HPV genotyping: HPV-negative, HPV-16-positive, HPV-18-positive, and positive for other high-risk genotypes. The cohort was unselected with respect to age and clinical status, and included women with cervical carcinoma who were receiving chemotherapy at the time of sampling. Specimens visibly contaminated with blood were excluded before processing. Demographic and cytological metadata, including age, cytological grade, smoking status, HIV status and contraceptive use, were not retrievable for the archived material. Because the specimens came from a private diagnostic laboratory and not from an organised screening programme, the specimens represent women presenting to private diagnostic services in one city and should not be read as a screening population.

2.2 Ethical approval

The material analysed was residual liquid-based cytology fluid remaining after routine diagnostic reporting and destined for disposal. It was irreversibly anonymised before transfer, no clinical or demographic record accompanied it, and no specimen was collected and no procedure performed for the purposes of this research. The full statement of the ethical position is given under Ethics Statement.

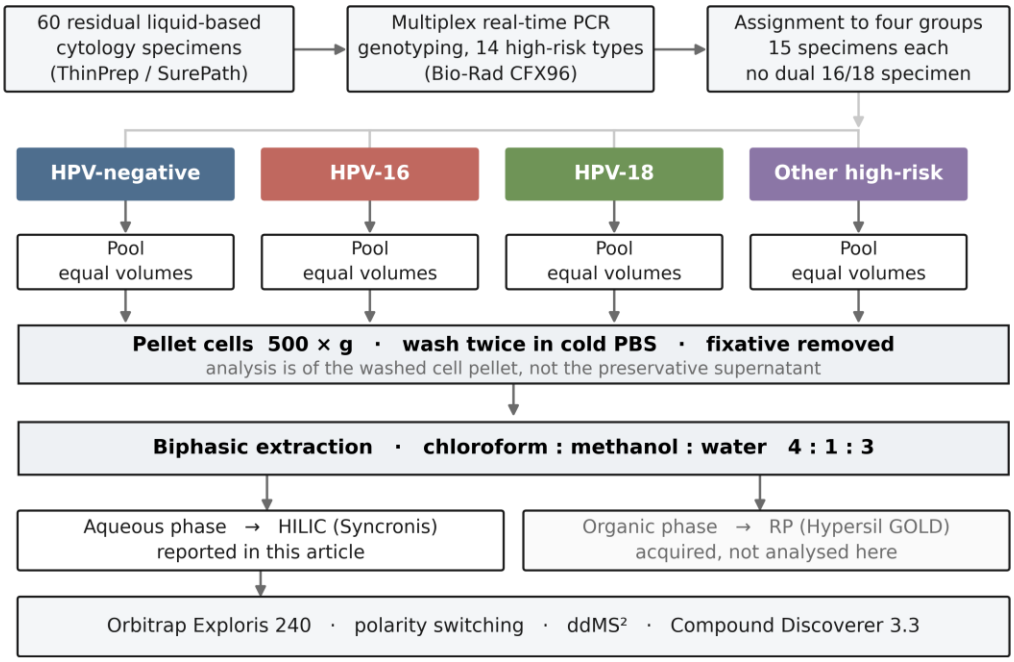

Figure 1. Study workflow. Sixty residual liquid-based cytology specimens were genotyped and assigned to four groups of fifteen, each group being combined into a single pool. Cells were pelleted and washed twice in buffered saline before extraction, so what was analysed is the washed cell pellet, not the preservative supernatant. Both phases of the biphasic extraction were recovered, only the aqueous phase is analysed in this article.

2.3 HPV detection and genotyping

Genomic DNA was extracted from a 200–400 μL aliquot of each vortexed specimen using a silica membrane-based kit with proteinase K lysis, sequential washes and elution in 50–100 μL of nuclease-free water. DNA concentration and purity were assessed spectrophotometrically, with A260/A280 between 1.8 and 2.0 accepted for downstream use, and extracts were stored at −20 °C. Genotyping used commercial multiplex real-time PCR assays targeting HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68, with genotype-specific hydrolysis probes and an internal amplification control, on a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Two commercial kits were used across the series: a Cosara kit (OEM, CODx, USA) and a Siemens HPV HR kit. Cycling comprised 95 °C for 10 min followed by repeated cycles of denaturation at 95 °C and combined annealing and extension at 60 °C with fluorescence acquisition. A specimen was called positive when a genotype-specific amplification curve reached threshold within the manufacturer's Ct interval and the internal control amplified, specimens negative for all targets with a valid internal control were called HPV-negative, specimens failing the internal control were re-extracted and retested. Each run included positive control DNA, a no-template control and an extraction blank, and pre- and post-PCR areas were physically separated. Specimens positive for HPV-16 were assigned to the HPV-16 group and those positive for HPV-18 to the HPV-18 group, irrespective of concurrent detection of other high-risk types, no specimen in this series was positive for both HPV-16 and HPV-18, so the question of assigning dual-positive specimens did not arise.

2.4 Pooling strategy

Sixty specimens were distributed equally across the four study groups, fifteen to each: fifteen HPV-negative, fifteen HPV-16-positive, fifteen HPV-18-positive and fifteen positive for other high-risk genotypes. An equal volume was taken from each individual specimen and the fifteen aliquots within a group were combined into one pooled sample before extraction. The procedure therefore produced four pooled samples from sixty specimens, each pool representing fifteen women, and each pool was extracted once and acquired once. The whole study consists of four analytical observations, one per group, and this is the basis on which every statement in the Results should be read.

Pooling was adopted to obtain a group-level profile at reduced analytical cost. No cell count, protein or nucleic acid normalisation was performed before pooling, so the contribution of each specimen to its pool is proportional to the cellular content of the volume taken, and was not equalised, and a specimen of unusually high cellularity may contribute disproportionately without that contribution being detectable. The consequences of these choices for interpretation are set out in the Limitations.

2.5 Metabolite extraction

Pooled samples were stored at −80 °C, thawed on ice and vortexed. Cervical cells were pelleted by centrifugation at 500 × g for 10 min at 4 °C. The supernatant was carefully aspirated and the pellet retained, the pellet was then resuspended in cold phosphate-buffered saline and re-pelleted, the wash being performed twice in order to remove residual fixative. The analysis reported here is therefore of the washed cervical cell pellet and not of the preservative supernatant.

Metabolites were extracted from the washed pellet with chloroform, methanol and LC–MS grade water in the proportions 4:1:3 (v/v/v). The mixture was vortexed for 1 min, held on ice for 10 min and centrifuged at 14,000 × g for 15 min at 4 °C, giving an upper aqueous phase and a lower organic phase. Both were collected into separate tubes and dried under nitrogen. The aqueous phase was reconstituted in water:acetonitrile (90:10, v/v) containing 5 mM ammonium acetate for hydrophilic interaction analysis and is the subject of this article, the organic phase was reconstituted in isopropanol:acetonitrile (50:50) for reversed-phase analysis and is not reported here. Reconstituted extracts were transferred to glass autosampler vials with inserts. Extraction was performed by the authors, the analytical facility operated the instrument and did not participate in sample preparation.

The washing step has consequences in both directions. Removing the cytology preservative before extraction means that the solvent proportions given above are effective as well as nominal, and that the metabolites recovered derive from the intracellular compartment, and not from a mixture of secreted and intracellular pools — an ambiguity that would otherwise attach to any measurement made on this matrix. Set against that, washing introduces phosphate-buffered saline, and salt carried through into the extract is a well-recognised cause of ion suppression and adduct formation in electrospray ionisation.

A further mismatch concerns the injection solvent, which bears directly on which features this study was able to retain. Hydrophilic interaction separation begins at high organic content — 90% acetonitrile in the present method — and the sample was introduced in a solvent of the opposite composition, 90% aqueous. Injecting a strongly aqueous plug onto a hydrophilic interaction column is a recognised cause of solvent-strength mismatch: the analyte band is not focused at the head of the column but is carried forward by the injection solvent itself, producing reduced retention, distorted or fronting peaks, and in the most polar analytes partial breakthrough at the void volume. The 10 µL injected corresponds to roughly 3% of the 350 µL column void volume, so the effect is partial, and it falls most heavily on the compounds least able to withstand it — the small, highly polar species this separation exists to retain.

2.6 LC–HRMS acquisition

All four pooled extracts and both procedural blanks were acquired in a single analytical sequence on 24 April 2025, between 15:37 and 17:30 local time, under one instrument method. Acquiring every group on the same day under the same method removes between-batch variation as a source of difference between the groups.

Chromatographic separation used hydrophilic interaction liquid chromatography on a Vanquish Flex UHPLC system (Thermo Fisher Scientific). The column compartment was held at 40 °C in still-air mode and the autosampler at 10 °C. Mobile phase A was water containing 0.1% (v/v) formic acid and mobile phase B was acetonitrile containing 0.1% (v/v) formic acid, delivered at a constant flow of 0.300 mL min^{-1}. The injection volume was 10.0 µL. The gradient is given in Table 1. Ultraviolet absorbance was monitored at 214 nm. Retention times later than 13.0 min fall within the re-equilibration window and do not represent retention under the gradient.

Table 1. Chromatographic gradient, hydrophilic interaction separation

Time (min)	Mobile phase B (%)	Flow (mL min^{-1})	Step
0.0	90.0	0.300	Initial condition
9.0	30.0	0.300	Linear gradient
12.0	30.0	0.300	Hold
13.0	90.0	0.300	Return to initial condition
18.0	90.0	0.300	End of re-equilibration

Mobile phase A, water containing 0.1% (v/v) formic acid, mobile phase B, acetonitrile containing 0.1% (v/v) formic acid. Column 40 °C, autosampler 10 °C, injection volume 10.0 µL.

Detection used an Orbitrap Exploris 240 (Thermo Fisher Scientific) with a heated electrospray ionisation source. Spray voltage was 3500 V in positive and 3000 V in negative mode, sheath, auxiliary and sweep gas were 40, 12 and 0 arbitrary units, the ion transfer tube was held at 300 °C and the vaporiser at 350 °C, the RF lens was set to 70%. Full scans were acquired at a resolving power of 60,000 over m/z 100–1500 in profile mode, with both polarities acquired within the same run by polarity switching, a standard automatic gain control target and a maximum injection time of 100 ms, EASY-IC internal calibration was applied scan to scan. Data-dependent MS/MS was acquired for the five most intense precursors per cycle using a 1.5 m/z isolation window, stepped normalised higher-energy collisional dissociation energies of 30, 50 and 150%, a resolving power of 30,000 and a maximum injection time of 22 ms. Dynamic exclusion removed a precursor for 2.5 s after a single occurrence, with a ±5 ppm tolerance and isotope exclusion enabled, and the intensity threshold for triggering was 5.0×10^3. External mass calibration was performed on a fifteen-day schedule as part of routine instrument maintenance. One acquisition was performed per pooled sample.

The eluent differed from the one specified in the study protocol, and the departure warrants explicit description. The protocol prescribed 5 mM ammonium acetate at approximately pH 6.5–7.0 in both mobile phases, acquisition was performed with an

unbuffered eluent containing 0.1% formic acid at approximately pH 2.7. The stationary phase is zwitterionic, and retention of ionisable analytes on such a phase depends on the ionic strength of the eluent, which shields the charged surface and stabilises the water layer through which partition occurs, without an ammonium salt that retention is weaker and less reproducible. At pH 2.7 carboxylic acids are additionally protonated and lose the ionic interaction on which their retention depends. Three observations reported below follow from this: the absence of several acidic metabolites from the dataset, the number of endogenous features eluting at or near the void volume, and the inconsistency of retention time between acquisitions for some annotations. The injection solvent was also not matched to the eluent, the extracts having been reconstituted in 90% acetonitrile containing 5 mM ammonium acetate at pH 6.8.

Separation used a Syncronis HILIC column, 150 × 2.1 mm (Thermo Fisher Scientific, part number 97505-152130), whose void volume is 350 µL. At the flow rate used this corresponds to an unretained elution time of 1.17 min, and the 2.0 min cut-off applied below to define the unretained region is therefore approximately 1.7 times the void time.

2.7 Quality control

Two procedural blanks containing extraction solvents only were carried through the same preparation and acquired within the analytical sequence, one as the first injection and one as the last. The sequence comprised six injections, given in Table 2.

Table 2. Composition of the analytical sequence, 24 April 2025

Position	Injection	Vial	Time
1	Procedural blank	A1	15:37
2	HPV-negative pool	E7	15:56
3	Other-HR-HPV pool	E8	16:15
4	HPV-16 pool	E9	16:34
5	HPV-18 pool	F1	16:53
6	Procedural blank	A1	17:12

Each pool was injected once. The organic-phase extracts were acquired in a second sequence of the same structure on the same day, those data are not analysed here.

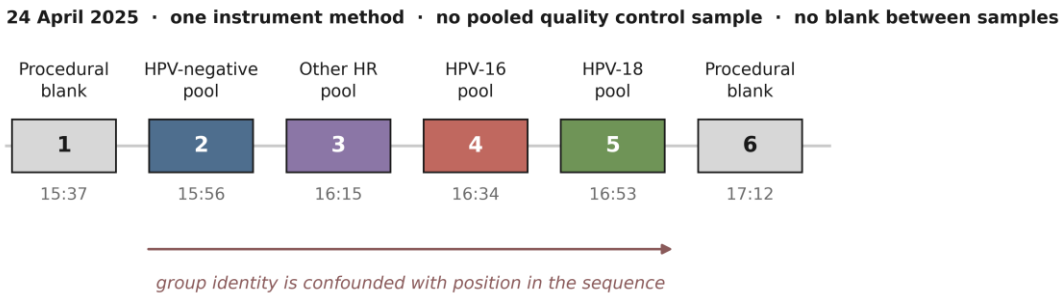

Figure 2. Composition of the analytical sequence. Each group was acquired once, at a fixed position, so group identity cannot be separated from position in the run. No pooled quality control sample was injected and no blank was placed between samples.

No pooled quality control sample was injected. This departs from published guidance for untargeted clinical metabolomics, which recommends a pooled quality control sample injected repeatedly through the sequence (Broadhurst et al., 2018), and it has a definite consequence: no estimate of analytical repeatability exists for this study. The coefficient of variation of feature

areas on repeated injection of identical material was not measured, and no threshold can therefore be set below which a difference between acquisitions is indistinguishable from instrumental variation. The two blanks bracket the samples and permit identification of background introduced during preparation, but because no blank was injected between samples, carry-over between consecutive injections was not assessed.

The injections were not randomised. Each group was acquired once, at a fixed position in the sequence, so group identity is completely confounded with position: any monotonic change across the sequence, whether from signal decline, accumulating carry-over or continuing column equilibration, would be indistinguishable from a difference between groups. This is carried into the Limitations and constrains every between-group comparison reported here.

2.8 Data processing, annotation and feature curation

Raw data were processed in Compound Discoverer 3.3 (Thermo Fisher Scientific) with annotation against the mzCloud spectral library, ChemSpider, Metabolika and an endogenous metabolite mass list. Exported feature lists were intensity-filtered by the analytical facility, the lowest peak area present in any export was 3.13×10^5. Annotation confidence was assigned according to the Metabolomics Standards Initiative (Sumner et al., 2007) and the complementary high-resolution mass spectrometry confidence scheme (Schymanski et al., 2014). Because no authentic reference standards were run, level 1 is not claimed for any feature. Level 2 was assigned where a data-dependent MS/MS spectrum was acquired and the library match score was at least 70, all remaining annotations were assigned level 3.

Features were curated before interpretation. An entry was retained only where the annotation corresponded to an endogenous human metabolite plausibly present in cervical epithelium or mucus. Entries were excluded, with the reason recorded, where they corresponded to solvent or mobile-phase additives, polymer and plasticiser leachables, pharmaceuticals, agrochemicals, plant, fungal or microbial metabolites, or where the mass error exceeded 3 ppm or the annotation was non-specific. Features eluting before 2.0 min, approximately 1.7 times the measured void time of the column, were treated as unretained, in that region an annotation cannot be distinguished from co-eluting isobaric species, and such features were flagged and excluded from interpretation. Several endogenous features eluted at or below the void time of 1.17 min itself. The complete inventory with every exclusion decision is provided as Supplementary Table S1.

2.9 Pathway over-representation analysis

The curated compound list was submitted to MetaboAnalyst (Pang et al., 2024) for over-representation analysis against the SMPDB metabolite set library, with hypergeometric testing and both Holm and false discovery rate correction. Compound names were cross-referenced to HMDB identifiers (Wishart et al., 2022). This analysis operates on a list of compound names and does not use peak intensities, no peak matrix was normalised at any point in this study.

2.10 Reporting standards

Annotation confidence is reported throughout according to the Metabolomics Standards Initiative (Sumner et al., 2007) and the complementary high-resolution mass spectrometry scheme (Schymanski et al., 2014). Instrument, column, eluent, gradient, source and scan parameters, the composition of the analytical sequence, the processing software version and the reference libraries interrogated are all stated above, so that the acquisition can be reproduced. Departures from the written study protocol are reported at the point where they arise, not gathered at the end, and are summarised in the Limitations.

2.11 Statistical considerations

The pooled design produces one analytical observation per group. Means and measures of dispersion cannot be estimated, inferential tests have no valid error term, and multivariate methods including principal component analysis, PLS-DA, OPLS-DA and machine-learning classifiers would fit four observations in a space of dozens of features and could only overfit. No such analyses were performed and none are reported. Peak areas are presented as raw, uncorrected values, because no between-sample normalisation was possible, they are semi-quantitative and comparisons of magnitude between acquisitions are not supported.

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3. RESULTS AND DISCUSSION

3.1 Feature inventory and curation

Automated annotation returned 291 features across the four acquisitions: 57 in the HPV-negative pool, 70 in the HPV-16 pool, 83 in the HPV-18 pool and 81 in the other-HR-HPV pool. Curation retained 77 entries corresponding to endogenous human metabolites and excluded 214. The excluded set was dominated by exogenous material. Trifluoroacetic acid was the single most intense feature in the other-HR-HPV acquisition at 2.97×10^{10} arbitrary units, exceeding the most intense endogenous feature in that acquisition by more than an order of magnitude. Neither mobile phase contained trifluoroacetic acid, so this feature did not originate in the eluent, it represents contamination of the sample or carry-over within the fluidic system, and since no blank was injected between samples its origin cannot be localised further. A nylon cyclic dimer and 11-aminoundecanoic acid, the corresponding polyamide monomer, appeared repeatedly among the highest-intensity features in every acquisition, together with di(2-ethylhexyl) phthalate, bis(4-ethylbenzylidene)sorbitol and triethanolamine. A hydroxy-melphalan feature was present in the HPV-16 and HPV-18 acquisitions, consistent with the documented inclusion of participants receiving chemotherapy.

Of the 77 endogenous entries, 23 eluted before 2.0 min in the unretained void volume and were excluded from interpretation. These included phytosphingosine, sphinganine, hexanoylcarnitine, 2-hexenoylcarnitine, suberoylcarnitine and succinic acid, none of which can be reliably annotated in that region under HILIC conditions.

The number of retained endogenous entries differed markedly between acquisitions: 23 in the HPV-negative pool, 14 in the HPV-16 pool, 29 in the HPV-18 pool and 11 in the other-HR-HPV pool. A more than two-fold difference in the number of endogenous metabolites recovered is unlikely to reflect biology alone and more plausibly indicates differences in acquisition quality, ionisation efficiency or matrix suppression between injections. The counts do not follow the order of injection, which was HPV-negative, other-HR-HPV, HPV-16, HPV-18: the lowest count belongs to the second injection and the highest to the fourth. Simple monotonic drift across the sequence is therefore not sufficient to explain them, although with one acquisition per group and no quality control injections the possibility cannot be excluded. This observation constrains every comparison that follows and is returned to in the Discussion.

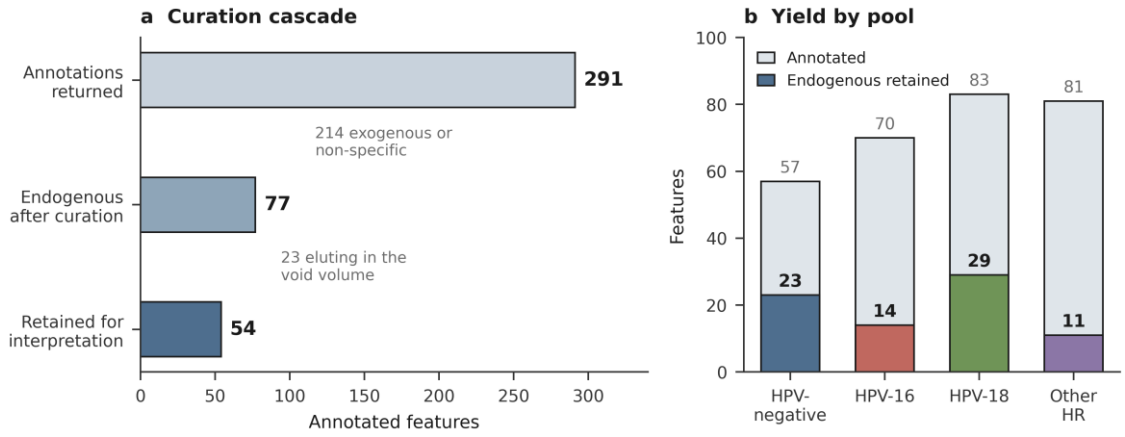

Figure 3. Curation of the annotated feature lists. (a) Of 291 annotations returned across the four acquisitions, 214 were exogenous or non-specific and a further 23 eluted within the void volume, leaving 54 features carried into interpretation. (b) Annotated and retained endogenous features by pool.

The 23 endogenous features that eluted within the void volume are not distributed at random across compound classes: they are predominantly small, highly polar species. Two conditions of the acquisition worked in the same direction upon precisely that group of compounds. The eluent contained no ammonium salt, so ionisable analytes were weakly retained, and the injection solvent was 90% aqueous against a mobile phase starting at 90% organic, so the sample band was carried forward instead of focusing at the column head. Void-region elution in this dataset is therefore better read as a systematic consequence of the injection and eluent conditions than as a property of the compounds themselves, and the decision to exclude those features from interpretation is correspondingly conservative rather than arbitrary.

The same reasoning strengthens the features that were retained. A compound that reached 7.652 min despite an injection solvent working against retention has demonstrated retention under adverse conditions, and its chromatographic behaviour is the more reliable for it.

3.2 Metabolites detected in all four pools

Three annotations were common to all four acquisitions. Choline was the most intense endogenous feature in three of the four, annotated at level 2 with library match scores between 99.3 and 99.9. Glutamate was detected in all four at retention times between 8.80 and 8.94 min, at level 2 in the HPV-negative pool and level 3 elsewhere. A feature of formula C6H13NO2 was detected in all four at retention times between 6.07 and 6.24 min, the automated annotation differed between acquisitions, returning L-norleucine in three and isoleucine in the fourth, with the isoleucine assignment carrying the lowest library match score in the dataset. These are the same chromatographic feature under different automated names, and we report it as an unresolved C6H13NO2 isomer — norleucine, leucine or isoleucine — rather than assigning it to a single compound.

Table 3. Endogenous metabolites detected in all four pooled acquisitions

Annotation	Formula	RT range (min)	HPV-neg	HPV-16	HPV-18	Other HR	Best MSI level
Choline	C5H13NO	2.55–2.68	7.61×10^8	2.00×10^9	1.38×10^9	8.67×10^8	2
Glutamate	C5H9NO4	8.80–8.94	9.37×10^7	5.56×10^7	5.00×10^7	4.99×10^7	2
C6H13NO2 isomer (Leu/Ile)	C6H13NO2	6.07–6.24	4.73×10^8	4.72×10^8	6.76×10^8	3.44×10^8	2

Values are maximum peak areas in arbitrary units from a single acquisition per group. They are semi-quantitative, were not normalised between acquisitions, and are not comparable in magnitude across columns.

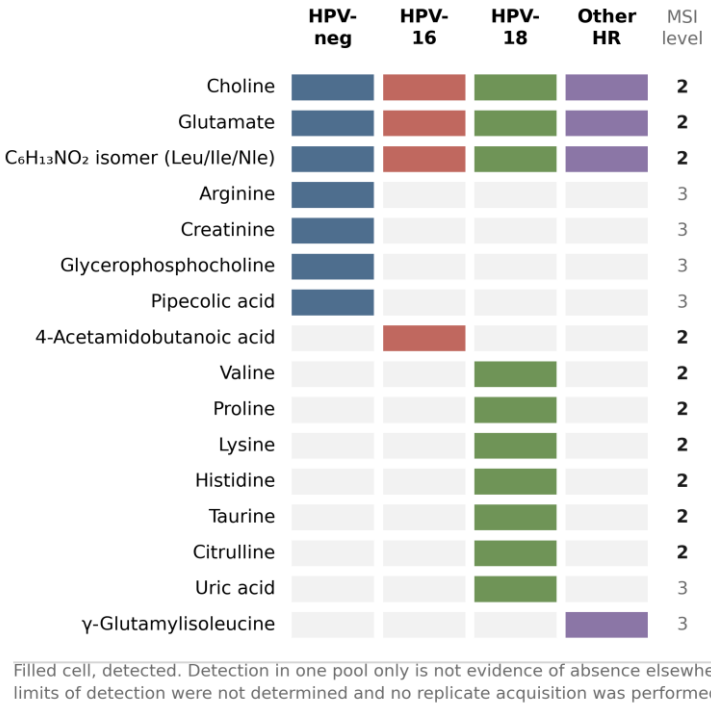

Figure 4. Endogenous metabolites by pool, with annotation confidence. Detection in a single pool reflects the reporting threshold applied to the feature lists and is not evidence of biological absence. The 4-acetamidobutanoic acid label denotes a putative annotation.

3.3 Detections restricted to a single pool

Several annotations appeared in only one acquisition. In the HPV-negative pool these included arginine, creatinine, glycerophosphocholine and pipecolic acid, in the HPV-16 pool, a feature putatively annotated as 4-acetamidobutanoic acid, in the HPV-18 pool, proline, valine, lysine, taurine, histidine, citrulline, uric acid and several lysine and phenylalanine derivatives, and in the other-HR-HPV pool, γ-glutamylisoleucine. With the exception of the putative 4-acetamidobutanoic acid annotation and the HPV-18 amino acids, most of these carry level 3 annotations based on accurate mass alone.

We state explicitly that restriction to a single pool is not evidence of absence. Limits of detection and quantification were not determined, technical replicates were not acquired, the exported feature lists were intensity-filtered, and pooling dilutes any signal contributed by a minority of the fifteen constituent specimens. A metabolite reported here as detected in one pool may be present below the reporting threshold in the others.

3.4 Putative 4-acetamidobutanoic acid annotation and the C6H11NO3 isomer question

A feature putatively annotated as 4-acetamidobutanoic acid (N-acetyl-GABA) was observed in the HPV-16 pool at 1.34×10^9 arbitrary units, the second most intense endogenous feature in that acquisition. The putative annotation rests on more than accurate mass: a data-dependent MS/MS spectrum was acquired and matched the mzCloud reference spectrum at 79.4, the mass error was 0.15 ppm on the $[M+H]^+$ ion at m/z 146.08119, and the retention time of 7.652 min is consistent with the other polar amino acid derivatives in the same run, which eluted between 6.59 and 8.94 min. This remains a putative annotation, without validation against an authentic reference standard or further confirmatory MS/MS fragmentation.

One further feature of formula C6H11NO3 was present in the dataset, in the HPV-negative pool. Since isobutyrylglycine and 4-acetamidobutanoic acid are structural isomers of identical monoisotopic mass, accurate mass alone cannot distinguish them, and the two features must be compared directly. They are separated by 6.08 min. The HPV-negative feature elutes at 1.570 min, within the unretained void volume alongside the polyamide and plasticiser features, where a polar acylglycine cannot be retained under HILIC conditions, no MS/MS spectrum was acquired for it, no library match was returned, and two independent processing runs assigned it two different identities. We therefore report a putative annotation of the HPV-16 feature as 4-acetamidobutanoic acid at MSI level 2, and make no identification of the HPV-negative feature.

Table 4. Annotation evidence for the two C6H11NO3 features

Evidence	HPV-16 pool	HPV-negative pool
Retention time (min)	7.652	1.570
Chromatographic region	Retained	Void volume (unretained)
Precursor ion	$[M+H]^+$ m/z 146.08119	$[M+H]^+$ m/z 146.08121
Mass error (ppm)	0.15	0.26
MS/MS spectrum acquired	Yes (data-dependent)	No
Spectral library match	79.4	None returned
Annotation consistent across two processing runs	Yes	No — two different identities returned
Peak area (arbitrary units)	1.34×10^9	1.24×10^8
Assigned confidence	MSI level 2 (putative annotation)	Not assigned, excluded from interpretation

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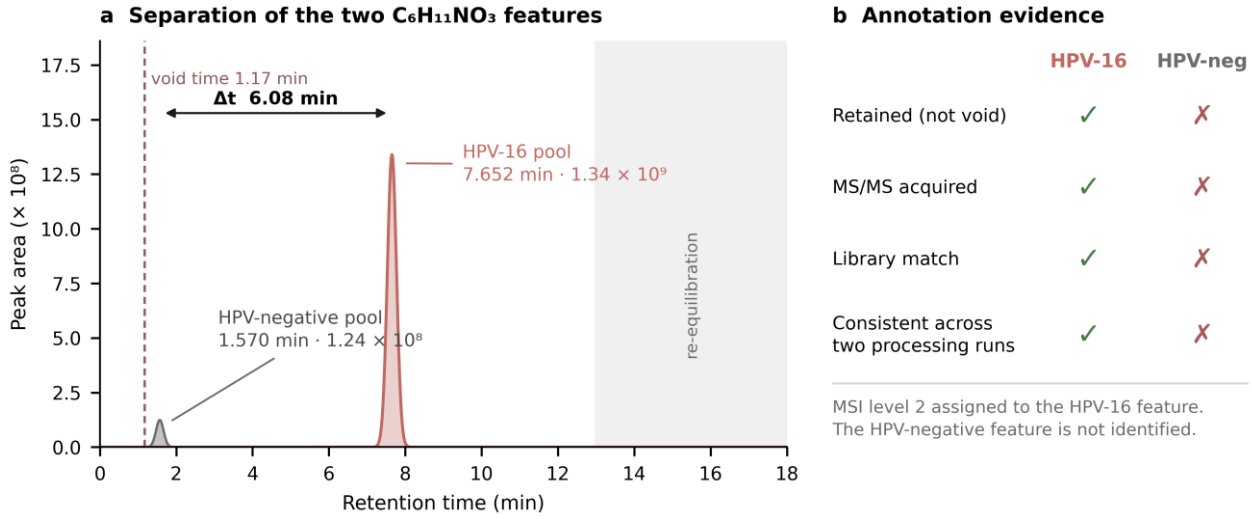

Figure 5. The two features of composition $C_6H_{11}NO_3$. (a) The HPV-16 feature, putatively annotated as 4-acetamidobutanoic acid, is retained and chromatographically resolved from the HPV-negative feature, which elutes close to the column void time of 1.17 min. (b) Annotation evidence for each. The two are separate features and are reported separately.

3.5 Pathway over-representation analysis

Over-representation analysis of the curated compound list against 38 SMPDB metabolite sets returned arginine and proline metabolism as the most enriched set (3 hits, raw p = 0.0024), followed by glycine and serine metabolism (3 hits, raw p = 0.0034) and carnitine synthesis (2 hits, raw p = 0.0066). No set retained significance after correction for multiple testing: the smallest adjusted values were 0.232 by the Holm method and 0.168 by false discovery rate. We report this result as a descriptive summary of which pathways the annotated compounds belong to, and not as evidence that any pathway is altered by HPV status.

Table 5. Over-representation analysis of the curated compound list (top five sets)

Metabolite set (SMPDB)	Set size	Expected	Hits	Raw p	Holm p	FDR
Arginine and proline metabolism	52	0.311	3	0.0024	0.232	0.168
Glycine and serine metabolism	59	0.353	3	0.0034	0.332	0.168
Carnitine synthesis	22	0.132	2	0.0066	0.629	0.207
Oxidation of branched-chain fatty acids	26	0.156	2	0.0091	0.866	0.207
Urea cycle	28	0.168	2	0.0105	0.991	0.207

No metabolite set reached significance after Holm or false discovery rate correction. The analysis uses compound identity only and does not incorporate peak intensity or group membership.

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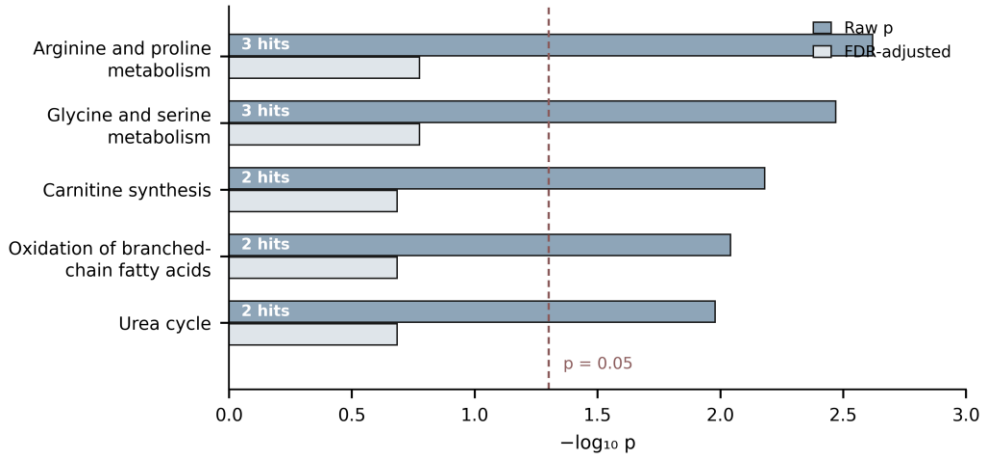

Figure 6. Over-representation analysis of the curated compound list. Raw p values for the five highest-ranked sets are shown against the values obtained after correction for multiple testing. No set survives correction, and the analysis is reported as a negative result.

3.6 Discussion

Before interpreting these observations we state what was not done. No functional, transcriptional or enzymatic measurement was made in this study. No mechanism proposed below was tested here. The design produces four analytical observations, and every comparison between groups is a comparison between single injections without replication. What follows is therefore an attempt to place a small number of observations in the context of published work, not a set of conclusions about HPV biology.

3.6.1 Putative 4-acetamidobutanoic acid annotation

The observation of a feature putatively annotated as 4-acetamidobutanoic acid in the HPV-16 pool is the observation from this dataset that is best supported analytically and most interesting biologically. In hepatocellular carcinoma, phosphomevalonate kinase stabilises glutamate decarboxylase 1, increasing synthesis of γ-aminobutyric acid, which acetyl-CoA acetyltransferase 1 then converts to 4-acetamidobutanoic acid, the released metabolite activates GABA-A receptors on CD8+ T cells, inhibits AKT1 signalling, and suppresses T-cell activation and intratumoural infiltration (Zhou et al., 2024). Independent work in lung adenocarcinoma shows that GAD1-derived GABA acts on the tumour immune microenvironment through macrophage polarisation (Dong et al., 2024). A plausible route therefore exists by which this metabolite could contribute to local immune suppression.

Two constraints must be stated alongside this. First, that axis was characterised in hepatocellular carcinoma and has not been demonstrated in cervical epithelium, the present study provides no evidence that it operates here. Second, the putatively annotated feature was observed in one pooled injection, and its absence from the other three lists is a statement about the reporting threshold, not about biology. What the observation supports is a targeted, quantitative measurement of 4-acetamidobutanoic acid in individually analysed HPV-16-positive and HPV-negative specimens, with authentic reference standards. It does not support describing the compound as a biomarker, and we do not do so.

3.6.2 Detection, non-detection and the composition of cervical specimens

A cervical cytology specimen is dominated by normal epithelial cells even where high-grade disease is present, and abnormal cells form a small minority of the collected material. It follows that common amino acids should be detectable in every specimen group, and that a reported complete absence of such metabolites from an entire group is more likely to be analytical than biological. Our data are consistent with this reasoning rather than against it. Arginine and creatinine, for example, appear only in the HPV-negative list, but both are annotated on accurate mass alone without MS/MS confirmation, and both fall in the lower half of that list by intensity. The exported lists were intensity-filtered, no limits of detection were established, and the pooling step dilutes contributions from individual specimens. We have accordingly removed any language of loss or depletion from this report.

Two further factors bear on this. The number of endogenous metabolites recovered differed more than two-fold between acquisitions, which points to variable acquisition quality, not to variable biology. And trifluoroacetic acid, a strong ion-suppressing agent in positive-mode electrospray, was the most intense feature in one acquisition and absent from another. Because neither mobile phase contained it, its presence is not a constant of the method but a variable contaminant differing between injections, and variable suppression of that kind is sufficient on its own to move polar amino acids above and below a reporting threshold between acquisitions.

3.6.3 Relation to published cervicovaginal metabolomics

Where our observations overlap with the literature, they do so in a limited and non-independent way. Glutamate was detected in all four pools, consistent with its identification as a discriminating metabolite in plasma profiling of cervical neoplasia (Khan et al., 2019) and with the glutamine dependence conferred by E6 and E7 through SNAT1 (Ortiz-Pedraza et al., 2023). Detection of arginine in the HPV-negative pool only is directionally consistent with the report of decreased arginine and increased citrulline in cervical mucus from women with cervical neoplasia (Kawasaki et al., 2024) and with the generalised reduction of amino acids in HR-HPV-positive women (Borgogna et al., 2020), though our data cannot test either. Carnitine and acylcarnitine features were present across the dataset, and carnitine metabolism has been linked to genital inflammation in the presence of vaginal dysbiosis (Ilhan et al., 2019), we did not characterise the vaginal microbiota, so we can add nothing to that relationship.

We draw particular attention to one claim we do not make. 5-Aminolevulinic acid features were present in three acquisitions, and it would be possible to construct an argument about heme biosynthesis and photodynamic therapy from that pattern. We reject that argument for two reasons. Analytically, the feature in the HPV-negative acquisition elutes at 1.580 min in the void volume while those in the HPV-18 and other-HR-HPV acquisitions elute at 8.51 min, these are different chromatographic peaks and cannot be compared. Retention behaviour of this kind is expected for an acidic analyte under the unbuffered conditions used here, and it illustrates the more general point that the separation, rather than the specimens, determines which acidic species this study could recover at all. Clinically, the published evidence points the other way: topical 5-ALA photodynamic therapy achieves high lesion clearance in HR-HPV-positive high-grade lesions (Hu et al., 2022), and a comparative study found no significant difference in HPV clearance between HPV-16/18 and non-16/18 groups after treatment (Chen et al., 2023). There is no basis in our data or in the literature for a genotype-stratified photodynamic therapy claim.

3.6.4 Exogenous background

Three quarters of the annotated features in this dataset were not endogenous human metabolites. Polyamide oligomers, phthalate plasticisers, a polymer clarifying agent and a mobile-phase additive were among the most intense features in every acquisition. This is a general caution for untargeted work on archived cytology material, where specimens have been stored in alcohol-based preservative in plastic vials for extended periods, and it is the reason a procedural blank is not an optional refinement but a requirement for interpreting any feature list of this kind (Broadhurst et al., 2018). We report the full inventory with exclusion reasons so that readers can assess these decisions rather than take them on trust.

3.6.5 Strengths

Two aspects of the design lend weight to the observations reported above. All four pools and both blanks were acquired on a single day within one sequence under one instrument method, so between-batch variation is excluded as a source of difference between the groups. And the complete annotated feature inventory, including every excluded entry with its reason for exclusion, is provided as supplementary material, so that the curation on which the endogenous list depends can be examined rather than taken on trust.

3.6.6 Limitations

The pooled design is the principal limitation and shapes every other one. Fifteen specimens were combined per group and one pooled sample per group was analysed, giving four analytical observations. Within-group variability cannot be estimated, the influence of individual specimens on a group profile cannot be assessed, and no inferential or multivariate statistic is computable. No power calculation is possible or presented.

Limits of detection and quantification were not determined and technical replicates were not acquired, so detection in one pool and not another cannot be interpreted as a difference in concentration. The exported feature lists were intensity-filtered by the analytical facility and the filtering parameters were not disclosed to us, the lowest area present in any list was 3.13×10^5.

No authentic reference standards were analysed, so no annotation reaches MSI level 1. Of the retained endogenous entries, most are level 3, supported by accurate mass alone. Only the aqueous phase is analysed here, so lipids and other non-polar metabolites are outside the scope of this report, the organic phase was acquired on the same day and remains available for separate analysis.

The groups differ by more than HPV genotype. The cohort was unselected across ages and clinical states and included women with cervical carcinoma receiving chemotherapy, evidenced by the presence of a hydroxy-melphalan feature in two acquisitions. Age, cytological grade, smoking status, HIV status and contraceptive use, all of which influence the cervicovaginal metabolome, were not available. Any difference observed between groups may reflect these variables rather than HPV status, and we cannot separate them.

The number of endogenous metabolites recovered varied more than two-fold between acquisitions, indicating differences in acquisition quality that are not accounted for and that constrain all between-group comparison.

No pooled quality control sample was injected in the analytical sequence. Analytical repeatability was therefore never measured, and there is no basis on which to judge how large a difference between acquisitions must be before it exceeds what the instrument would produce on re-injection of identical material. The two procedural blanks were placed at the beginning and end of the sequence, with none between samples, so carry-over between consecutive injections was not assessed, the trifluoroacetic acid feature reported above illustrates why that matters.

The injections were not randomised and each group was acquired once at a fixed position, so group identity is confounded with position in the sequence. We have no means of separating a genuine difference between groups from a systematic change across the run.

Four provisions of the written study protocol were not implemented, and we record them as deviations instead of passing over them. Stable isotope-labelled internal standards were not spiked into the samples before extraction, so peak areas could not be normalised to an internal reference and remain uncorrected. Technical and biological replicates were not acquired. The injection order was not randomised. And no pooled quality control mixture was injected. Each of these was specified in the protocol as good practice for untargeted work, and their absence is the reason the quantitative claims in this report are confined to statements of detection rather than of amount.

The specimens were collected into two different preservative media. ThinPrep is methanol-based and SurePath ethanol-based with additional buffering agents, so the two differ in their fixative chemistry and in the exogenous background they contribute. Because specimens were not stratified by medium before pooling, each pool may contain a different proportion of the two, and a difference between pools in the exogenous or partially extracted fraction cannot be excluded on this ground. The cells were washed twice in buffered saline before extraction, which removes the bulk of the preservative and limits the effect, but does not eliminate it.

The chromatography worked against retention in two independent respects, both bearing on the same class of compound. The mobile phases contained 0.1% formic acid and no ammonium salt or pH buffer, which is not the usual configuration for hydrophilic interaction separation of ionisable metabolites and gives weaker and less reproducible retention of acidic and strongly basic analytes. Independently of that, the extracts were reconstituted in a 90% aqueous solvent and injected onto a separation beginning at 90% organic, a solvent-strength mismatch that reduces retention and causes partial breakthrough of the most polar analytes. Nearly a third of the endogenous features recovered eluted within the void volume and were excluded from interpretation on that account, and the number would be expected to fall substantially were the extracts reconstituted in a high-organic solvent and the eluent buffered as the protocol specified. Several of the compounds reported in the cervicovaginal metabolomics literature are acidic, and their absence from this dataset is attributable to the separation, not to the specimens. The injection solvent was also not matched to the eluent.

The specimens were collected through 2024 and acquired in April 2025, so they were archived at −80 °C for periods of up to approximately thirteen months that differed between specimens and were not equalised between groups. Metabolite stability

during frozen storage is compound-dependent, and a difference in archival interval between groups is an alternative explanation for a difference in the features recovered from them.

4. CONCLUSION

This exploratory survey of pooled residual cervical cytology specimens produced one observation that we consider worth pursuing. A feature putatively annotated as 4-acetamidobutanoic acid was observed in the HPV-16 pool with MS/MS and spectral library support and clear chromatographic separation from the only other feature of the same elemental composition in the dataset, which supports a putative annotation but does not establish chemical identity. Published work describing a route by which this metabolite suppresses CD8+ T-cell activation makes the observation biologically interesting, though that route has not been demonstrated in cervical tissue and was not tested here. Beyond this, the study establishes little: four pooled injections cannot support statistical comparison, non-detection cannot be distinguished from concentration below the reporting threshold, and the groups differ by more than viral genotype. We therefore present these findings as a starting point for targeted quantitative measurement of 4-acetamidobutanoic acid in individually analysed, clinically characterised specimens with authentic reference standards, and not as evidence of genotype-specific metabolic reprogramming or as a candidate biomarker. Because each group was represented by a single non-randomized injection without pooled QC or technical replication, the study cannot distinguish biological differences from analytical variation.

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Approval: This work forms part of an approved doctoral thesis undertaken by the first author in the Department of Biochemistry and Forensic Science, School of Sciences, Gujarat University, Ahmedabad, under the supervision of the last author.

AUTHOR CONTRIBUTIONS

AAAA designed the study, selected and processed the specimens, performed DNA extraction and HPV genotyping, prepared the pooled extracts, curated and interpreted the metabolomic data, and drafted the manuscript. AFH and NJD contributed to data interpretation and to critical revision of the manuscript. SS provided the residual clinical specimens and the associated documentation. RR supervised the work, provided access to the analytical facility, and critically reviewed the analysis and the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests. The work reports results forming part of a doctoral thesis and no commercial interest is served by its publication. One author (SS) is affiliated with YGen Healthcare Private Limited, the diagnostic laboratory that supplied the residual specimens, that organisation had no role in the design of the study, in the analysis or interpretation of the data, or in the decision to publish.

ETHICS STATEMENT

Ethical Declaration: The material analysed in this study consisted entirely of residual liquid-based cytology fluid remaining after completion of routine diagnostic cytological examination, which would otherwise have been discarded as clinical waste. No specimen was collected for the purposes of this research, no additional clinical procedure was performed on any patient, and no intervention of any kind was undertaken. The specimens were irreversibly anonymised by the supplying diagnostic laboratory before transfer: no name, hospital number, date of birth, address or any code capable of restoring the link to an individual accompanied the material, and no clinical, cytological or demographic record was available to the investigators at any stage. Because that link had been destroyed before the material reached the investigators, the women concerned could not be identified or contacted, and individual informed consent for the secondary research use of the residual material was accordingly not obtainable. The specimens were supplied by YGen Healthcare Private Limited, Ahmedabad, under a No Objection Certificate (reference GUYG/12384, dated 1 March 2024), which records that specimen collection and sharing were conducted

in accordance with that organisation's standard informed consent and sample privacy protocols. The study was conducted in accordance with the principles of the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

Data Availability: The complete annotated feature inventory for all four acquisitions, with curation decisions and reasons for exclusion, is provided as Supplementary Table S1. The twelve raw acquisition files — six comprising the hydrophilic interaction sequence (four pooled samples and two procedural blanks) and six comprising the parallel reversed-phase sequence acquired on the same day — together with both instrument method files, are held by the authors and are available from the corresponding author on reasonable request. They will be deposited in a public metabolomics repository on acceptance and the accession number added at proof stage.

Study Registration: Not applicable. This is an exploratory laboratory study and not a clinical trial, guideline or meta-analysis, and it was not registered in any study registry.

Use of AI tools: An AI language model was used to assist with data curation decisions and with language editing only. All experimental data were generated by the authors, all interpretation is the authors' own, and all cited references were verified against PubMed by DOI.

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