Title: Proteomic alterations in salivary exosomes derived from human papillomavirus-driven oropharyngeal cancer.

Running head: Salivary biomarkers for HPV-driven oropharyngeal cancer

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Abstract:

Background: Increasing evidence supports the notion that human papillomavirus (HPV) DNA integration onto the human genome can influence and alter the molecular cargo in the exosomes derived from head and neck cancer cells. However, the molecular cargo of salivary exosomes derived from HPV-driven oropharyngeal cancer (HPV-driven OPC) remains unelucidated.

Methods and Materials: Salivary exosomes morphology and molecular characterizations were examined using the nanoparticle tracking (NTA), western blot analysis, transmission electron microscopy (TEM) and mass spectrometry analysis.

Results: We report that HPV16 DNA was detected (80%) in isolated salivary exosomes of HPV-driven OPC patients. Importantly, we demonstrate an elevated protein levels of six main glycolytic enzymes (i.e. Aldolase (ALDOA), Glyceraldehye-3-phosphate dehydrogenase (GAPDH), Lactate dehydrogenase A/B (LDHA and LDHB), Phosphoglycerate kinase 1 (PGK1) and Pyruvate kinase M1/2 (PKM)) in isolated salivary exosomes of HPV-driven OPC patients, suggesting a novel mechanism that underlies the potential role of salivary exosomes in mediating the reciprocal interplay between glucose metabolism and HPV-driven OPC.

Conclusion: Our data demonstrate that the potential diagnostic value of HPV16 DNA and glycolytic enzymes in salivary exosomes in discriminating healthy controls from HPV-driven OPC patients, thereby opening new avenues in the future for clinical translation studies.

Key Points:

- The presence of HPV16 DNA in salivary exosomes derived from HPV-driven OPC.
- Glycolytic enzymes are enriched in salivary exosomes derived from HPV-driven OPC.
- The diagnostic potential of glycolytic enzyme in salivary exosomes for detection of HPV-driven OPC.

1. Introduction:

Oropharyngeal cancer (OPC), one of the common sub types of head and neck cancer (HNC), arising in the tonsillar region, the base of tongue or the soft palate, accounts for an estimated 97,000 deaths every year globally [1]. In addition to the traditional risk factors (i.e. tobacco and alcohol use), high-risk human papillomavirus (HPV), the etiological factor of cervical cancer has been associated with HNC carcinogenesis, particularly in OPC [2]. Unlike non HPV-driven HNC, patients with HPV-driven OPC are younger and have a high number of lifetime sexual partners as well as engaged in high-risk sexual behaviours (i.e. oral sex) [3, 4]. Given the escalating incidence of HPV-driven OPC in the high income countries including Australia [5-7], there is an increasing number of reports evaluating the epidemiology and clinical manifestation of oral HPV infection in the general population [8-10].

More than 200 different types of HPV have been identified to date, however, only some HPV types defined as "high-risk" could potentially contribute to the oncogenesis [11]. Among these, HPV16 and HPV18 are the most commonly detected types in about 70 percent of all cervical cancers, as well as other cancers of the anus, penis, vulva, and vagina [12]. Notably, over 90 percent of OPC are attributable to only HPV16 infection [13, 14]. Most HPV infections are transient and asymptomatic which have been reported to be cleared naturally within one year or two years [15]. Meanwhile, only a small proportion of remaining infections have a high potential for persistence and progression to malignancy.

It has been well established that the presence of persistent high-risk HPV infection is a notable precursor to HPV-associated cancers, including cervical, anogenital and OPC [16]. Moreover, persistent infection with high-risk HPV types can also promote genomic instability and subsequently trigger the viral DNA integration into the host genome [17]. Integration often results in the disruption of the viral El or E2 gene region and thereby leads to the upregulation of E6 and E7, two viral proteins

which are found to deregulate the tumour suppressor genes p53 and RB1, respectively [18]. In agreement with previous findings, a large proportion with a mixed HPV16 (episomal and integrated) status has been detected in both tumour and saliva samples from HPV-driven OPC cases [19, 20].

Ample evidence suggests that HPV DNA integration into the human genome can significantly contribute to the changes observed both at the genomic and transcriptomic levels [21]. Also, it has been associated with the alterations of molecular cargo in exosomes derived from HNC cells [22]. A recent study by Kannan et al [23] reported the presence of E7 oncoprotein in serum exosomes collected from HPV-driven OPC patients. Importantly, exosomal proteins and microRNAs (miRNAs) have been suggested as potential biomarkers for discerning the HPV status in HNC [24, 25]. However, current knowledge on the molecular cargo of salivary exosomes with respect to oral HPV16 infection is still in its infancy. The aims of this study were threefold: firstly, to determine whether the salivary exosome as a carrier of HPV16 DNA. Secondly, to profile the protein cargo within the enriched salivary exosomes collected from healthy controls and HPV-driven OPC patients. Thirdly, to identify potential mechanism underlying the interplay between the salivary exosomes and HPV-driven OPC tumours.

2. Methods and Materials:

2.1 Study design

This study was approved by the Medical Ethical Institutional Board of University of Queensland [HREC No: 2014000862]; Queensland University of Technology [HREC No: 1400000617 and 1400000641] and by the Princess Alexandra Hospital (PAH) Ethics Review Board [HREC Number: HREC/12/QPAH/381], following the Declaration of Helsinki 1964 and its later amendments or comparable ethical standards. Ten p16INK4a (p16) positive OPC patients from the PAH have been recruited. Additionally, 20 healthy controls (age and gender-matched) were also recruited. All participants provided written informed consent, prior to saliva sample collection. p16 positive OPC patients' clinical staging were based on the 8th edition of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) tumour–node–metastasis (TNM) staging system. p16 positivity (nuclear and cytoplasmic staining > 70% in tumour cells) for OPC patients was evaluated using CINtec® p16 Histology Kit (E6H4 clone) (Roche MTM Laboratories, Heidelberg, Germany).

2.2 Unstimulated saliva samples collection and processing

Unstimulated saliva samples were collected from participants as stated in our previously published research [26]. Briefly, participants were requested to tilt their heads down and to pool saliva in their mouths before drooling into a 50 mL falcon tube kept on ice and transported to the laboratory on dry ice for further analysis.

2.3 DNA extraction

Isolation of DNA from saliva samples was carried out with QIAmp DNA Mini Kit (Qiagen, Germantown, MD, USA) as described previously [27].

2.4 Salivary exosomes isolation and quantification.

Exosomes were isolated from unstimulated saliva (300-400 μL) collected from participants as described previously [28]. Saliva was first centrifuged at 16,000 g for 20 minutes at 4 ℃ and subsequently, the supernatant was ultracentrifuged at 100,000 g for 2 hours at 4 °C. The resulting pellet was resuspended with 50 μL PBS and stored at -80^{0}C freezer until further analysis. The salivary exosomes' size and concentration were measured by Nanoparticle Tracking Analysis (NTA) using a NanoSight NS300 system (Malvern Technologies, Malvern, UK).

2.5 Transmission Electron Microscopy (TEM)

The purified salivary exosomes were fixed with 3% glutaraldehyde in 0.1M sodium cacodylate buffer and subsequently, were loaded onto formvar carbon-coated grids. Next, these were stained with uranyl acetate and visualized by transmission electron microscope (TEM) (JEM-1400 series 80-100kV, JEOL USA Inc., Pleasanton, CA, USA).

2.6 HPV16 E6/7 DNA qPCR Analysis

For HPV16 DNA detection, diluted DNA from saliva (50 ng) and salivary exosomes (2 μL) were used in qPCR analysis as described previously [29]. Both HPV16 E6/7 primer (Forward: ACCGGTCGATGTATGTCTTGTTG; Reverse: GATCAGTTGTCTCTGGTTGCAAATC) and human β-globin primer (Forward: CAACTTCCACGGTTCACC; Reverse: GAAGAGCCAAGGACAGGTAC) were used.

2.7 Western blot

The experimental procedures of western blot are described in detail in our previously published work [30]. Briefly, protein concentration of salivary exosomes was quantitated using BCA Protein Assay Kit purchased from Thermo Fisher Scientific (Rockford, IL, USA). Then, 5 μg salivary exosomes were used for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and subsequently,

transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was then probed with the primary antibodies (CD9 (D8O1A) and GM130 (D6B1) (Cell Signalling Technology, Danvers, MA, USA); CD63 (H-193) and CD81 (B-11) (Santa Cruz Biotechnology, Dallas, TX, USA)) for overnight at 4°C. After washing thrice with Tris-buffered saline (TBS)-Tween 20 (TBST), the membrane was probed with secondary antibodies at room temperature for 1 hour. The protein signals were visualized using a ChemiDoc™ XRS Gel Documentation System (Bio-rad, Hercules, CA, USA) prior to Immobilon Western Chemiluminescent HRP Substrate incubation.

2.8 Salivary exosome samples preparation for Mass Spectrometry (MS) Analysis

Diluted total protein (12.5 µg) of salivary exosome samples were used for the MS analysis as described in our previous study [31]. For the protein denaturation, 15 µL of SDS-Tris buffer containing 4% sodium dodecyl sulphate, 100 mM Tris-HCl buffer pH 8.5 and 100 mM Dithiothreitol (DTT) and 200 µL of DTT Urea buffer containing 25 mM DTT and 8M urea in 100 mM Tris_HCl pH 8.5 were added to the samples within a 30 kDa Microcon YM-30 centrifugal filter device (Millipore) and incubated at room temperature for 60 minutes on agitator. The filter units were then centrifuged at 14,000 g at 21ºC for 15 minutes to deplete off the denaturing buffer. To wash away remaining denaturing buffer, the filters were washed with 200 µL Urea buffer by centrifuging the filters at 14,000 g at 21°C for 15 minutes. While, for the cysteine alkylation, 100 µL of 50 mM iodoacetamide (IAM) in Urea buffer was added to the filter units and incubated another 20 minutes at room temperature in the dark. To quench the alkylation, the filter units were centrifuged at 14,000 g for 10 minutes and washed with 200 µL Urea buffer by centrifuging the filters at 14,000 g at room temperature for 15 minutes. The filter units were then washed with 100 µL of 100 mM ammonium bicarbonate 2 additional times to remove any remaining urea. The proteins were then digested at 1:50 enzyme to protein ratio with 0.25 µg proteomics grade trypsin (Sigma-Aldrich, St. Louis, MI, USA) at 37 ^{0}C for 16 hours. The filter units were centrifuged at 14,000 g at 21 °C for 15 minutes to collect the digested peptides. The samples were dried using a Speed Vac vacuum concentrator and

reconstituted in 10 µL of 2% acetonitrile (CAN)/0.1% trifluoroacetic acid (TFA) and transferred into STAGE tips for sample clean up. with double SCX membrane (Empore, part no: 2251, 3M, Maplewood, MI, US) [32].

2.9 Salivary exosomal protein content using SWATH-MS

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS) was carried out using a Prominence nanoLC system (Shimadzu, Kyoto, Japan) connected to a TripleTof 5600 mass spectrometer with a Nanospray III interface (SCIEX) (SCIEX, Framingham, MA, USA) as described in our previous studies [31, 33]. Briefly, 2 µg of peptides were extracted and separated by a Vydac EVEREST reversed-phase C18 HPLC column with both buffer A (1% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile with 0.1% formic acid) for LC-MS/MS. Peptides were analysed using a MS-TOF scan followed by high-sensitivity information-dependent acquisition (IDA) and were identified by the Protein Pilot 5.0 software (SCIEX) using the UniProt's human proteome database (download date: 22122016) as previously published work.

2.10 Statistical analysis

Fisher's exact test was used to measure the significance of difference in lifestyle factors between healthy controls and p16-positive OPC patients. The difference of protein abundances between HPV-driven OPC patients and healthy controls were analyzed using MS stats [34] by applying a linear mixed-effects model. Partial least squares discriminant analysis (PLS-DA) was performed to investigate relation between the protein abundance data generated by SWATH-MS and the cohort of a given sample and classify the samples into groups. The protein abundance of exosomes in saliva from healthy controls and HPV-driven OPC patients was compared using the Mann Whitney U- test for non-parametric analysis. All statistical tests were two-sided and p values < 0.05 were considered significant. Receiver operating characteristic (ROC) curves were generated for the individual salivary exosomal protein to determine their clinical utility. All statistical analysis was performed using

GraphPad Prism 9 software version 9.1.0 (GraphPad Software Inc., La Jolla, CA, USA) and R (R Development Core Team, Vienna, Austria).

3. Results:

3.1 Population characteristics

Healthy controls and p16-positive OPC patients were recruited in this study as listed in Table 1. The median age for healthy controls and p16-positive OPC were 58.5 years (range from 45 – 74 years) and 62 years (range from 50 – 78 years), respectively. Nearly all healthy controls (80%) and p16-positive OPC (100%) were Caucasian men. The majority of p16-positive OPC patients were ever-smokers (including both current and former smokers) (90%) and ever-drinkers (50%). Whilst healthy controls were more commonly never-smokers (35%) and never-drinkers (55%). In addition, no significant differences in lifestyle factors were observed between healthy controls and p16-positive OPC patients. All cases of p16-positive OPC had tumours in the tonsillar region and were diagnosed at Stage II based on the 8^{th} edition staging system.

3.2 Isolation and characterization of salivary exosomes

Exosomes were isolated from unstimulated saliva samples collected from healthy controls and p16-positive OPC patients using differential centrifugation. As shown in Figure 1A and B, the majority of particle sizes in isolated salivary exosomes were smaller than 200 nm in diameter. Further evaluation of salivary exosomes morphology was examined by transmission electron microscope. Isolated salivary exosomes showed unique “cup-like” shape appearances (Figure 1C). In addition, a battery of exosome-related protein markers were then examined by western blotting. CD9, CD63 and CD81 proteins were found to be abundant in salivary exosomes isolated from both cohorts as shown in Figure 1D. To further confirm the purity of salivary exosomes, GM130, a Golgi apparatus marker was used as a negative control in this study. Strikingly, no significant difference in size, concentration and morphology of isolated salivary exosomes was observed between both cohorts.

3.3 Detection of HPV16 DNA in both saliva and salivary exosomes

Previous studies as well as work from our group have shown that saliva can be used as a non-invasive medium for detection of HPV infections [15, 35]. qPCR analysis revealed that the presence of HPV16 E6/7 DNA in saliva of patients with p16-positive OPC lesion (10/10; 100%) (Table 2). More importantly, HPV16 E6/7 DNA was only detected in salivary exosomes derived from p16-positive OPC cases (8/10; 80%) and not from healthy controls. Surprisingly, HPV16 E6/7 DNA was undetectable in exosomes of saliva from two p16-positive OPC cases, and this may be due to the low concentration of salivary exosomes. HPV16 E6/7 DNA was then confirmed in CD9 positive salivary exosome isolated using Invitrogen Dynabeads magnetic bead technology, further supporting the presence of HPV16 in salivary exosomes.

3.4 Proteomics analysis of salivary exosomes derived from HPV-driven OPC patients

To identify the protein content of the salivary exosomes, 12.5 µg of exosomal protein were used for SWATH mass spectrometry analysis. By using this approach, a total of 226 proteins were identified in healthy controls' salivary exosomes; while, 270 proteins in the HPV-driven OPC patients' salivary exosome. Meanwhile, 208 proteins (Yellow) were found in both samples (Figure 2A). PLS-DA revealed that the salivary exosomal protein signatures were able to discriminate between healthy controls (Blue) and HPV-driven OPC patients (Orange) as shown in Figure 2B. To further determine the key differentially expressed proteins, the fold change >1.5 and p-value <0.05 were used as selection criteria for the identification of upregulated and downregulated proteins in isolated salivary exosomes of HPV-driven OPC patients. 36 were predicted as differentially expressed proteins where the red dots regarded as upregulated proteins (18); while, the blue dots regarded as downregulated proteins (18) in isolated salivary exosomes of HPV-driven OPC patients as depicted in a volcano plot (Figure 2C).

3.5 Dysregulation of glucose metabolism by HPV-modified salivary exosomes

To investigate the biological and molecular interactions in the identified differentially expressed proteins of HPV-modified salivary exosomes, Ingenuity Pathway Analysis (IPA) analysis for protein interactions, functional networks, upstream regulatory analysis and canonical pathway was performed. The top-most network consisting of 19 focus molecules as in our dataset (red and green represented as upregulated and downregulated proteins, respectively) were found to be associated with carbohydrate metabolism, hematological disease and immunological disease (Figure 3A). There were a few subnetworks within this complex protein network such as hypoxia-inducible factor 1 α (HIF-1α) related proteins, immunoglobulin complex MAPK/ERK family proteins and lactate dehydrogenase (LDH) complex. As shown in Figure 3B, the activation/inhibition of upstream regulatory molecules on the basis of the identified differentially expressed proteins were predicted according to the Z-score. The activated molecules in HPV-modified salivary exosomes including HIF-1α (z-score 2.4), Serine/Threonine Kinase 11 (STK11) (z-score 2.4), Hepatocyte Nuclear Factor 4 (HNF4) (z-score 2.3), Prostate cancer gene expression marker 1 (PCGEM1) (z-score 2.2) and MYC (z-score 2.2); whereas the following molecules were inhibited: mTOR (mammalian target of rapamycin) inhibitor, torin1 (z-score -2.2), the mitogen-activated protein kinase (MEK) inhibitor, PD98059 (z-score -2.2) and TP53 (z-score -2.0). Meanwhile, canonical pathway analysis of our dataset revealed that glycolysis was the only enriched canonical pathway (z-score 2.0) in HPV-modified salivary exosomes (Figure 3C). Further, six main glycolytic enzymes including Aldolase (ALDOA), Glyceraldehye-3-phosphate dehydrogenase (GAPDH), Lactate dehydrogenase A/B (LDHA; LDHB), Phosphoglycerate kinase 1 (PGK1) and Pyruvate kinase M1/2 (PKM) were significantly upregulated ($p > 0.05$) in exosomes of saliva from HPV-driven OPC patient (Figure 4). Most importantly, using ROC curve analysis, LDHB had shown the highest area under the curve (AUC) which was 0.93; whilst the lowest AUC belonged to PGK1, approximately 0.73 (Figure 5).

4. Discussion:

Multiple previous studies have suggested utilising salivary exosomes as biomarkers in cancer [36-38]. Owing to its close proximity to the oropharynx and oral cavity, HNC tumour cell-specific biomolecules may shed or release directly into the human saliva, and as such the use of saliva has gained attention in recent years [39, 40]. Accumulating evidence suggests that exosomes may play a significant role in regulating both virus transmission and HPV-associated carcinogenesis, as in OPC [41, 42]. However, there has been very little research to investigate the alteration of molecular cargo in salivary exosomes with respect to oral HPV infection. In this study, we have demonstrated the presence of HPV16 DNA in exosomes isolated from saliva samples from HPV-driven OPC. Moreover, the salivary exosomal protein signature was able to discriminate between healthy controls and HPV-driven OPC. Importantly, glycolytic enzymes were enriched in salivary exosomes isolated from HPV-driven OPC, suggesting a novel mechanism that underlies the potential role of salivary exosomes in mediating the reciprocal crosstalk between HPV-driven OPC and glucose metabolism.

Growing evidence indicates that HPV-driven OPC and non HPV-driven HNC are distinct from molecular, pathological, and clinical perspectives [2]. In fact, HPV-driven OPC tumours, generally pathologically classified by p16 positivity, are more likely to be smaller, and at diagnosis, these tumour have already spread to the lymph node when compared to non HPV-driven HNC tumours [43]. Indeed, HPV-driven OPC have a favourable prognosis with a better response to both surgical and non-surgical treatment approaches. Consequently, overall survival rates for HPV-driven OPC are three times higher than for those with non HPV-driven HNC [44]. Given the rapid increase in the incidence of HPV-driven OPC [5], and the likelihood of recurrence within 2 years [45], there is now a need to develop low-cost non-invasive biomarkers for early detection of HPV-driven OPC as well as to discriminate between HPV-driven OPC and non HPV-driven HNC. There are now a number of studies demonstrating the presence of HPV in saliva as a non-invasive biomarker for early detection, screening and treatment response monitoring of HPV-driven OPC [15, 46]. Strikingly, our work

corroborates with the findings from a recent study [35] that observed the presence of HPV16 E6/7 DNA in salivary exosomes derived from HPV-driven OPC (80%), further supporting the usefulness of saliva for early detection of HPV-driven OPC.

A number of studies have now proposed that high-risk HPV strains promote the oropharyngeal malignancy by disrupting the cell cycle checkpoint control through the degradation of tumour suppressor proteins, p53 and retinoblastoma (RB) [18]. In addition, HPV E6 and E7 oncoproteins have been found to enhance the release of angiogenic factors in HPV-infected cells [47] . In particular, HIF-1α, an angiogenic master switch involved in the upregulation of numerous genes associated with glycolysis, angiogenesis, extracellular matrix metabolism and cellular proliferation has been reported to be induced by HPV infection [48]. This is further suggested by a recent study unveiling the mechanism in which HPV contributes to tumour angiogenesis by promoting the HIF-1α activity [49]. Furthermore, the increased expression of HIF-1α was found in HPV16 DNA positive OPC tumours, particularly in stage I-II and pT1-pT2 tumours, further suggesting the role of HIF-1α in early development of HPV-driven OPC. In agreement with previous studies, p53 and HIF-1α were the predicted inhibited and activated regulators, respectively in exosomes of saliva from HPV-driven OPC patients.

Recent research has demonstrated that HPV plays a salient role in the regulation of hypoxia-enhanced glycolysis or the Warburg effect by preventing the association and degradation of HIF-1α by von Hippel-Lindau tumour suppressor (VHL) [50]. Meanwhile, viral proteins E6 and E7 have also been shown to promote the glucose transporter 1 (GLUT1) expression via the induction of HIF-1α in lung cancer [51]. Further evidence is suggested by Crusius et al., where they have shown that viral protein E5 could indirectly stimulate the Warburg effect in HNC cells by activating the epidermal growth factor receptor (EGFR) signalling pathway [52]. It has been further proposed that viral proteins E6 and E7 can induce the expression of two essential enzymes, hexokinase and pyruvate

kinase associated with glycolytic pathway, as well as other proteins involved in the production of lactate, like lactate dehydrogenase [53]. These results are in concordance with our current finding showing that six key glycolytic enzymes such as ALDOA, GAPDH, LDHA, LDHB, PGK1 and PKM were significantly overexpressed in isolated salivary exosomes of HPV-driven OPC.

5. Conclusion:

In summary, our findings suggest a novel mechanism that underlies the potential role of salivary exosomes in mediating a reciprocal crosstalk between glycolytic pathway and HPV-driven OPC, which may synergistically promote oropharyngeal malignancy. One of the key pieces of evidence is the presence of HPV16 in isolated salivary exosomes of HPV-driven OPC, which in turn further promotes the viral transmission and carcinogenesis, thus impacting the protein content of exosomes. More importantly, in this pilot study, we have demonstrated the diagnostic potential of glycolytic enzymes in salivary exosomes in discriminating HPV-driven OPC patients from healthy controls. The major limitations of the present study are the limited sample size and the single-site participants recruitment. A future longitudinal study with a larger cohort from various geographic regions is warranted to determine the diagnostic value of these salivary biomarkers.

6. References:

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8. Ethics declarations:

8.1 Funding

CP is receiving funding currently from the Cancer Australia (APP1145657), NHMRC Ideas Grant (APP 2002576), NIH (1R21EB030349-01), Garnett Passes and Rodney Williams Foundation and Royal Brisbane Women's Hospital Foundation.

8.2 Conflict of Interest

The authors declare that there is no conflict of interest.

8.3 Ethics approval

This study was approved by the Medical Ethical Institutional Board of University of Queensland [HREC No: 2014000862]; Queensland University of Technology [HREC No: 1400000617 and 1400000641] and by the Princess Alexandra Hospital (PAH) Ethics Review Board [HREC Number: HREC/12/QPAH/381], following the Declaration of Helsinki 1964 and its later amendments or comparable ethical standards.

8.4 Consent to participate

All participants provided written informed consent, prior to saliva sample collection.

8.5 Consent for publication

Not applicable.

8.6 Availability of data and material

All datasets generated for this study are included in the article.

8.7 Code availability

Not applicable.

8.8 Author Contributions

All authors have read and agree to the published version of the manuscript. KDT, YX and CP: conceptualization. All authors: methodology, validation, formal analysis, data curation, investigation, and writing—review and editing. KDT: writing—original draft preparation. CP: funding acquisition.

Table and Figure Legend

Table 1: Participant Demographics.

		Controls (N =20)		OPC (N = 10)		P-value
		No.	%	No.	%	
Age (years)	<=55	6	30.0	2	20.0	0.682
	>55	14	70.0	8	80.0	
Gender	Male	16	80.0	10	100.0	0.272
	Female	4	20.0	0	0.0	
Race/Ethnicity	Caucasian	20	100.0	10	100.0	>0.999
	Others	0	0.0	0	0.0	
Smoking status	Ever	9	45.0	9	90.0	0.099
	Never	7	35.0	1	10.0	
	Unknown	4	20.0	0	0.0	
Drinking status	Ever	8	40.0	5	50.0	0.420
	Never	11	55.0	3	30.0	
	Unknown	1	5.0	2	20.0	
Anatomical Site	Tonsil	0	0.0	10	100.0	NA
	Others	0	0.0	0	0.0	
8th AJCC TNM classification	Stage I	0	0.0	0	0.0	NA
	Stage II	0	0.0	10	100.0	
	Stage III	0	0.0	0	0.0	
	Stage IV	0	0.0	0	0.0	
p16INK4a Status	Positive	0	0.0	10	100.0	NA
	Negative	0	0.0	0	0.0	

Table 2: The presence of HPV16 DNA in both saliva and salivary exosomes derived from HPV-driven OPC patients.

Sample	HPV16 DNA in saliva	HPV16 DNA in salivary exosome
OPC 1	Yes	Yes
OPC 2	Yes	No
OPC 3	Yes	Yes
OPC 4	Yes	Yes
OPC 5	Yes	No
OPC 6	Yes	Yes
OPC 7	Yes	Yes
OPC 8	Yes	Yes
OPC 9	Yes	Yes
OPC 10	Yes	Yes

Figure 1: Salivary exosomes isolation and characterization. A-B: Representative image of size distribution (the majority ranged from 50 nm to 200 nm) in salivary exosomes derived from healthy controls and HPV-driven OPC by Nanoparticle Tracking Analysis (NTA). C: Representative transmission electron microscope (TEM) image of salivary exosomes derived from HPV-driven OPC (scale bar = 200 nm) confirmed with "cup-shaped" appearances. D: Representative western blot of common exosomal marker proteins CD9, CD63 and CD81 enriched in salivary exosomes derived from healthycontrols and HPV-driven OPC and not for the Golgi marker, GM130.

Figure 2: Proteomic analysis of exosomes in saliva collected from healthy controls and HPV-driven OPC patients. A Venn diagram showing overlaps of identified proteins (Yellow) in salivary exosomes derived from healthy controls and HPV-driven OPC patients. **B** Partial Least Squares Discriminant Analysis (PLS-DA) score plot of proteome signatures in salivary exosomes derived healthy controls (Blue) and HPV-driven OPC (Orange) patients. **C** Volcano plot showing the differential expression of salivary exosomal proteins in HPV-driven OPC patients (Red represented as upregulated proteins while blue represented as downregulated proteins; fold change >1.5 and p-value <0.05).

Figure 3: Ingenuity Pathway Analysis (IPA) of 36 differential expressed proteins in salivary exosomes derived from HPV-driven OPC patients. A: The top scoring IPA protein network was associated with carbohydrate metabolism, hematological disease and immunological disease. B: Predicted upstream activated and inhibited regulators based on the identified expressed proteins in salivary exosomes derived from HPV-driven OPC patients. C: Glycolysis was the top most canonical pathway enrich in HPV-modified salivary exosomes.

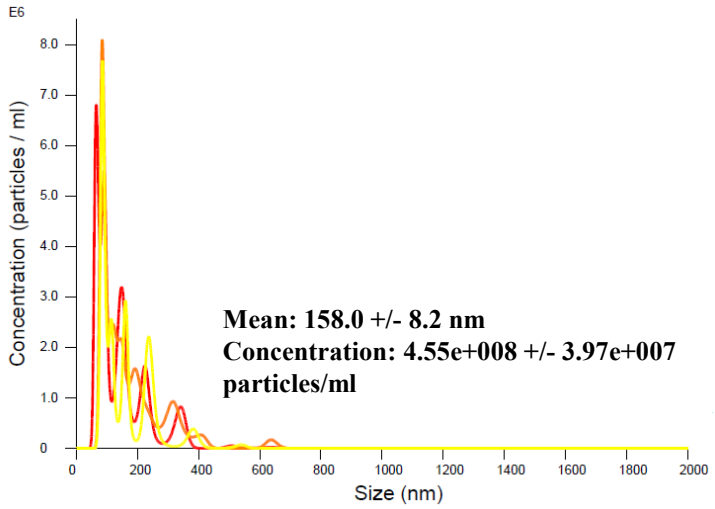
Figure 4: The overexpression of salivary exosomal proteins (glycolytic enzymes) in HPV-driven OPC patients. Data are presented as the logarithm (1og 10) of protein proportion. Statistically

significant differences were determined using Mann-Whitney U-test. (p values: * < 0.05 and **** < 0.0001).

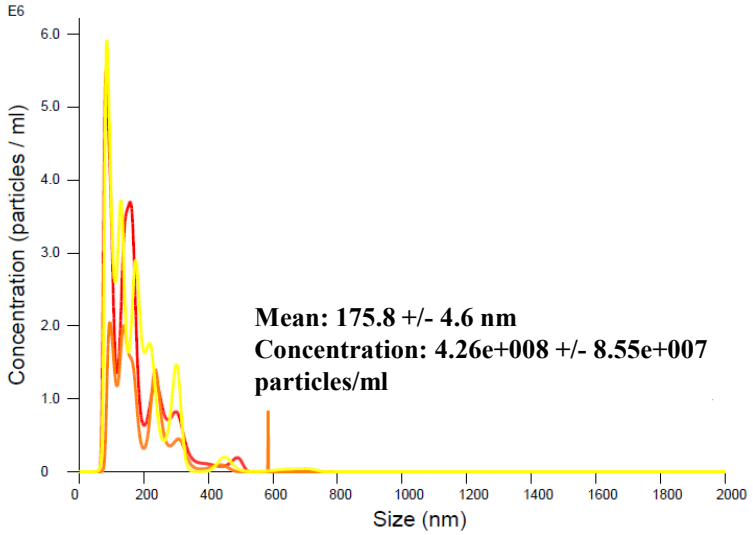
Figure 5: The receiver operator characteristic (ROC) curve analysis of glycolytic enzymes in salivary exosomes derived from patients with HPV-driven OPC.

Figure 1

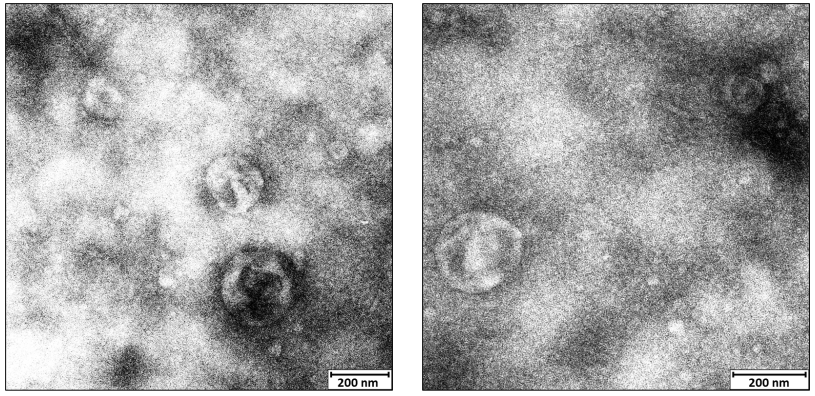
A. **Control**



B. **HPV-driven OPC**



C.



D.

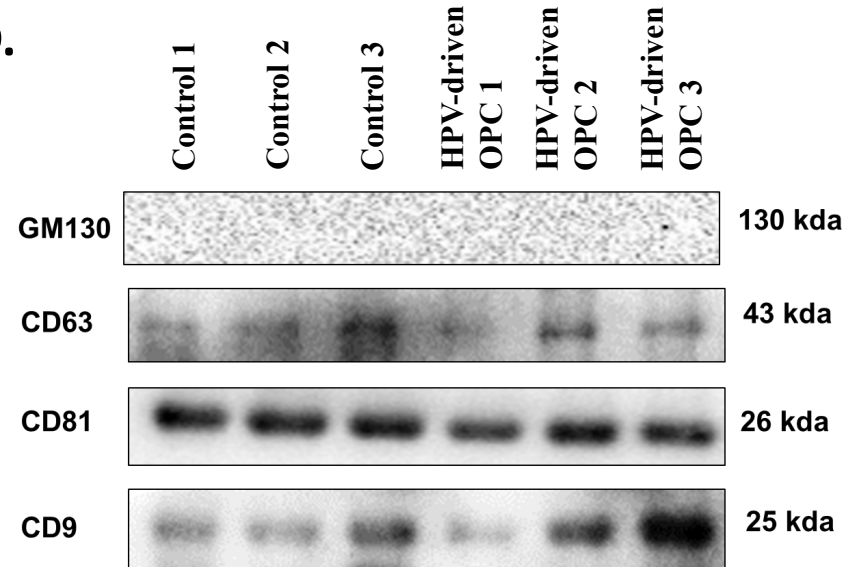
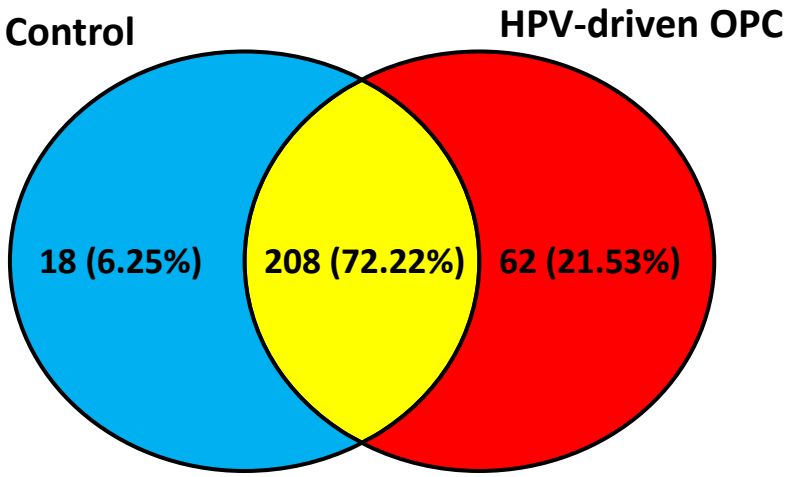
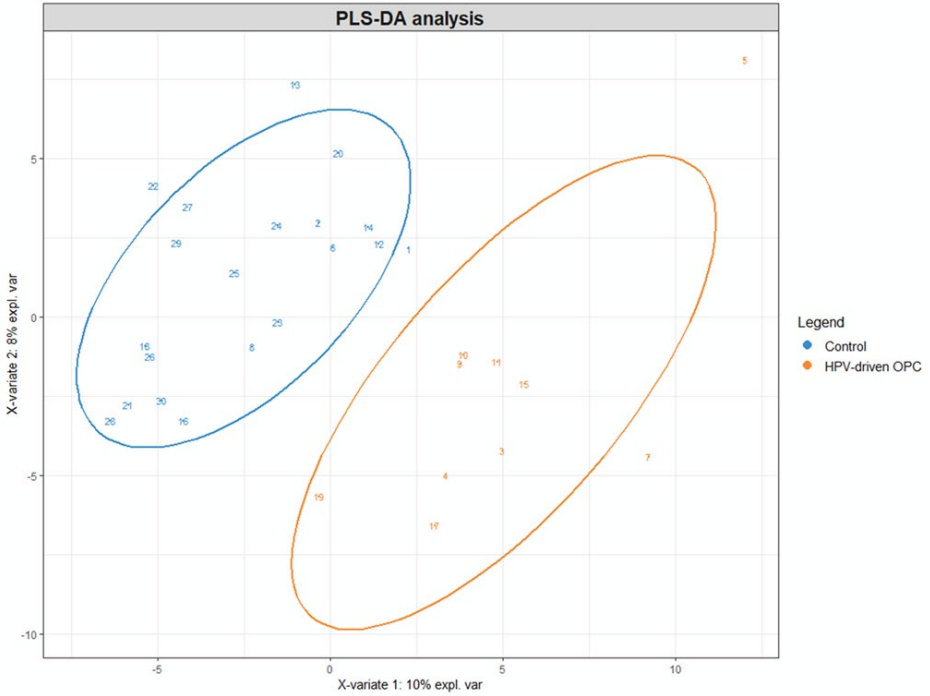


Figure 2

A.


Control
HPV-driven OPC
18 (6.25%)
208 (72.22%)
62 (21.53%)

B.


PLS-DA analysis
X-variate 2: 8% expl. var
X-variate 1: 10% expl. var
Legend
Control
HPV-driven OPC

C.

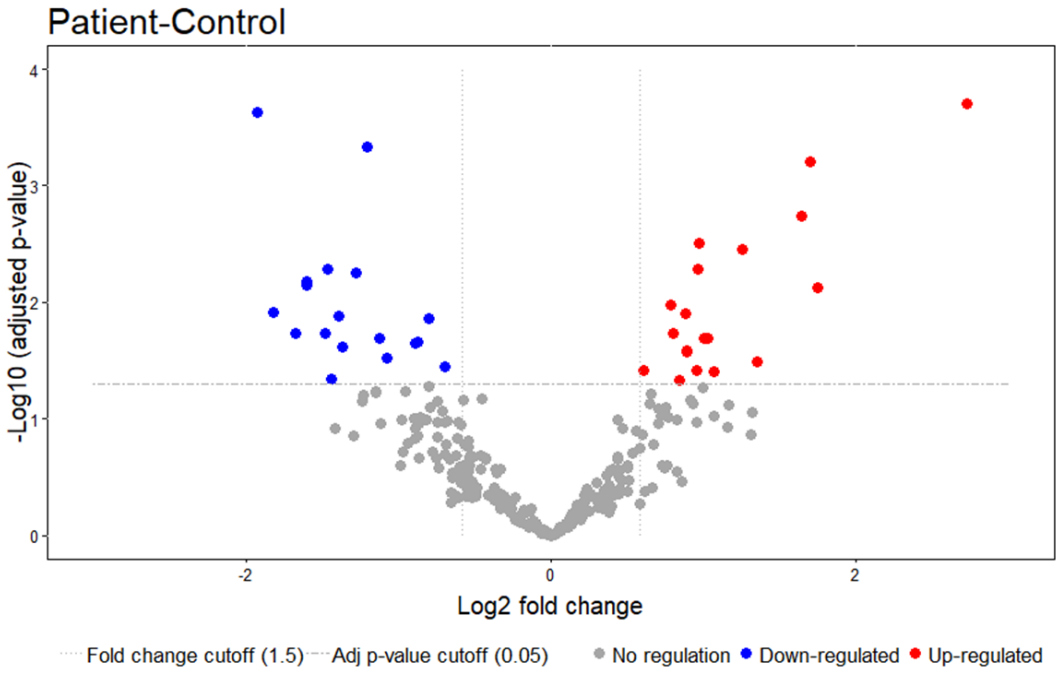
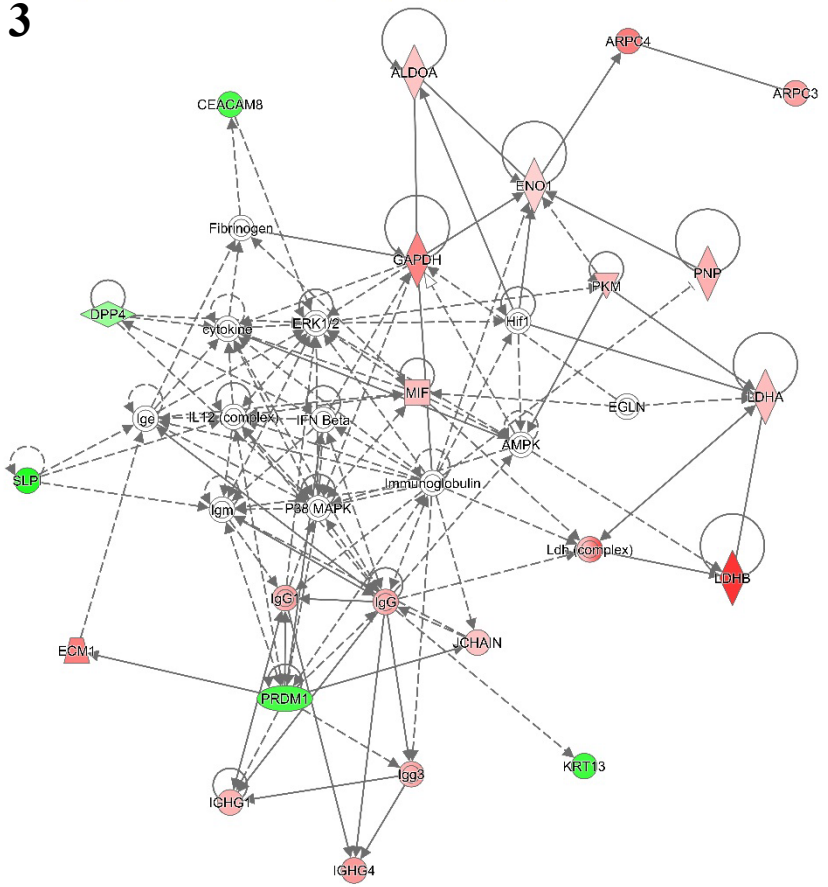

Patient-Control
-Log10 (adjusted p-value)
Log2 fold change
Fold change cutoff (1.5)
Adj p-value cutoff (0.05)
No regulation
Down-regulated
Up-regulated

Figure 3

A.



B.

Upstrem Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap
PCGEM1	other	Activated	2.201	4.71E-09
HIF-1α	transcription regulator	Activated	2.354	3.79E-05
HNF4α	transcription regulator	Activated	2.236	1.34E-02
STK11	kinase	Activated	2.449	3.65E-06
MYC	transcription regulator	Activated	2.060	4.51E-06
deferoxamine	chemical drug	Activated	2.213	1.13E-05
metribolone	chemical reagent	Activated	2.236	8.75E-05
torin 1	chemical reagent	Inhibited	-2.200	3.54E-07
TP53	transcription regulator	Inhibited	-2.010	3.49E-04
PD98059	chemical - kinase inhibitor	Inhibited	-2.200	1.48E-03

C.

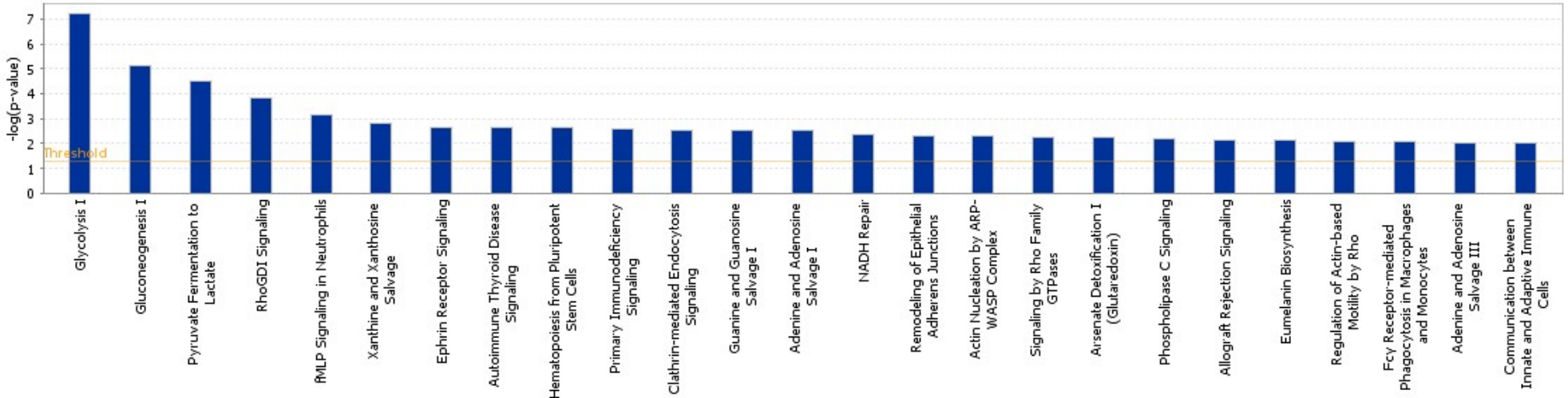


Figure 4

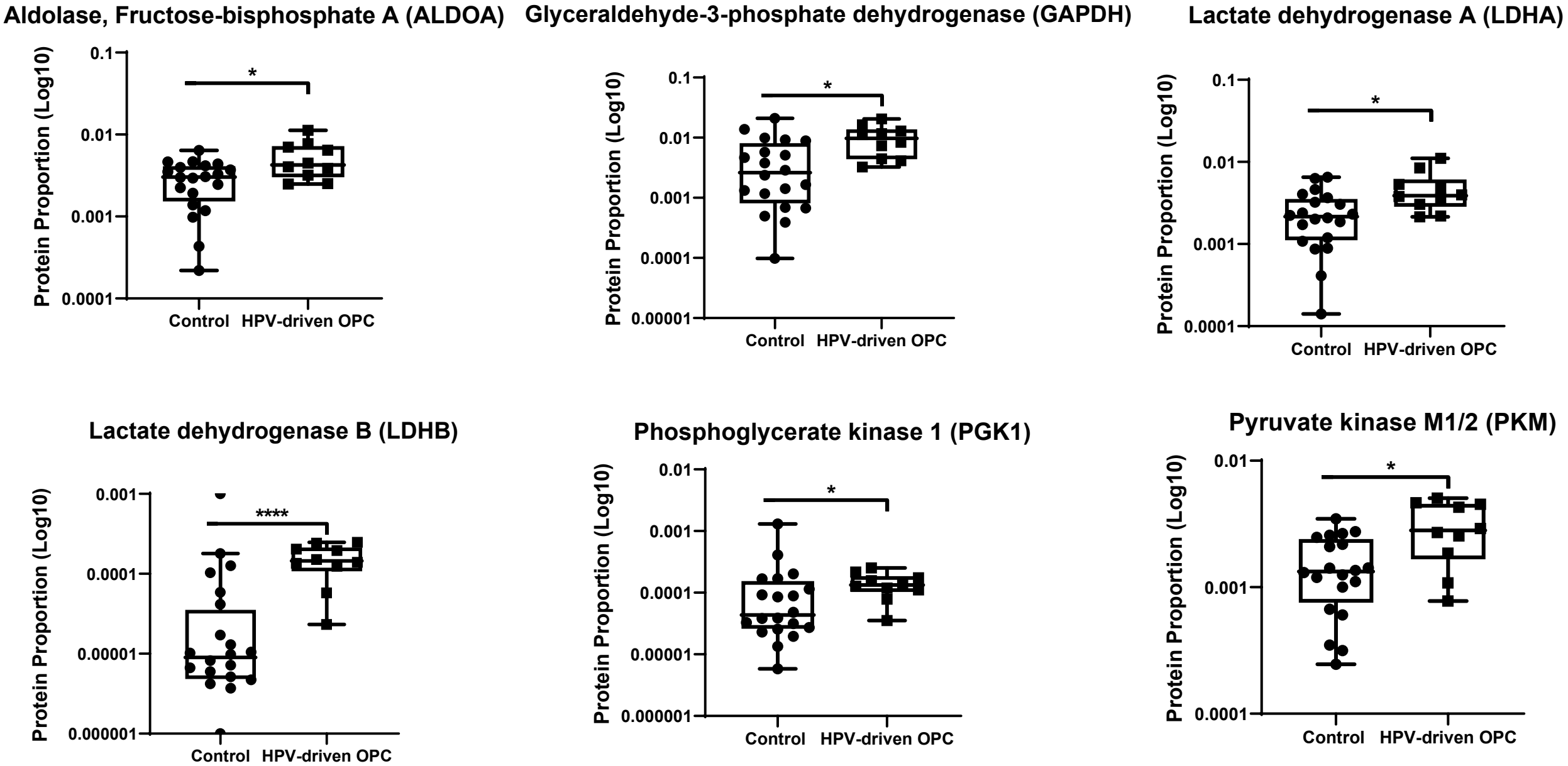
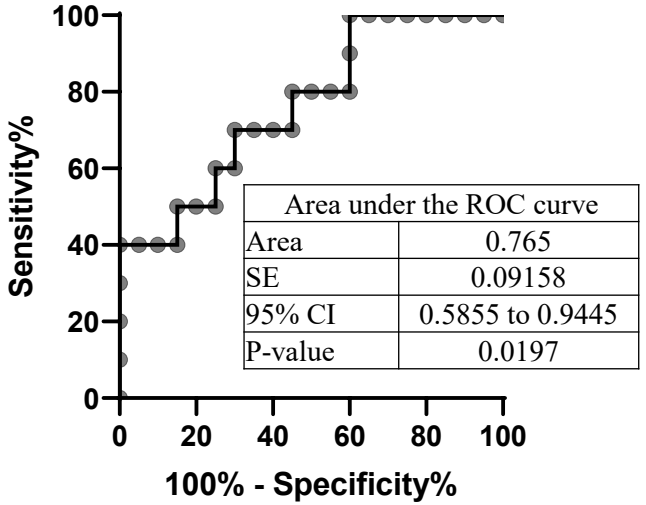

Aldolase, Fructose-bisphosphate A (ALDOA)
Protein Proportion (Log10)
0.1
0.01
0.001
0.0001
*
Control
HPV-driven OPC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Protein Proportion (Log10)
0.1
0.01
0.001
0.0001
0.00001
*
Control
HPV-driven OPC
Lactate dehydrogenase A (LDHA)
Protein Proportion (Log10)
0.1
0.01
0.001
0.0001
*
Control
HPV-driven OPC
Lactate dehydrogenase B (LDHB)
Protein Proportion (Log10)
0.001
0.0001
0.00001
0.000001
****
Control
HPV-driven OPC
Phosphoglycerate kinase 1 (PGK1)
Protein Proportion (Log10)
0.01
0.001
0.0001
0.00001
0.000001
*
Control
HPV-driven OPC
Pyruvate kinase M1/2 (PKM)
Protein Proportion (Log10)
0.01
0.001
0.0001
*
Control
HPV-driven OPC

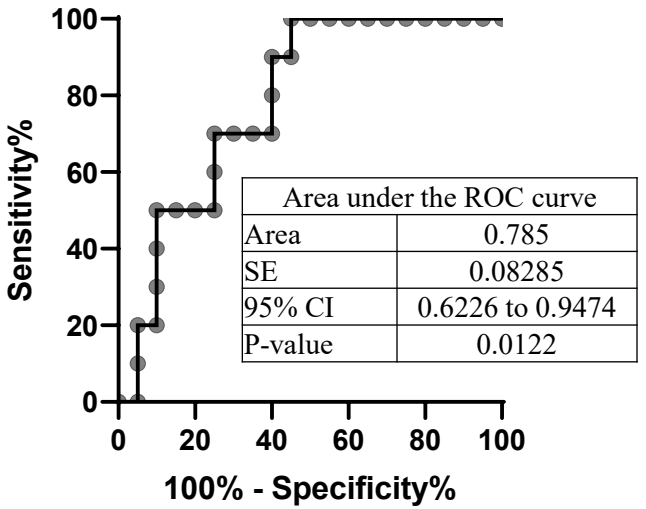
Figure 5

Aldolase, Fructose-bisphosphate A (ALDOA)



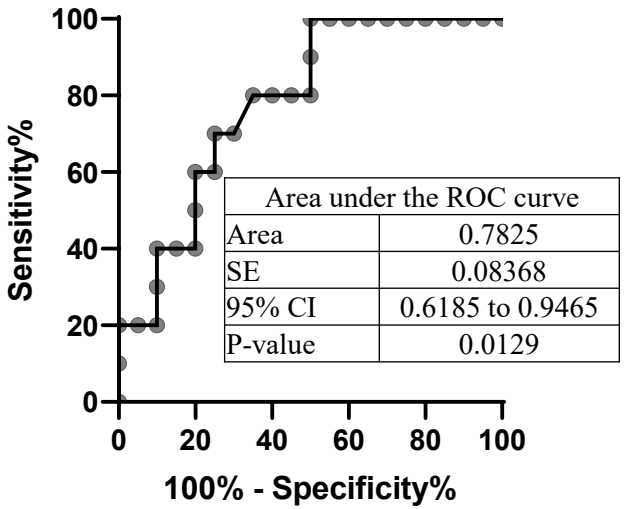
Area under the ROC curve	
Area	0.765
SE	0.09158
95% CI	0.5855 to 0.9445
P-value	0.0197

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)



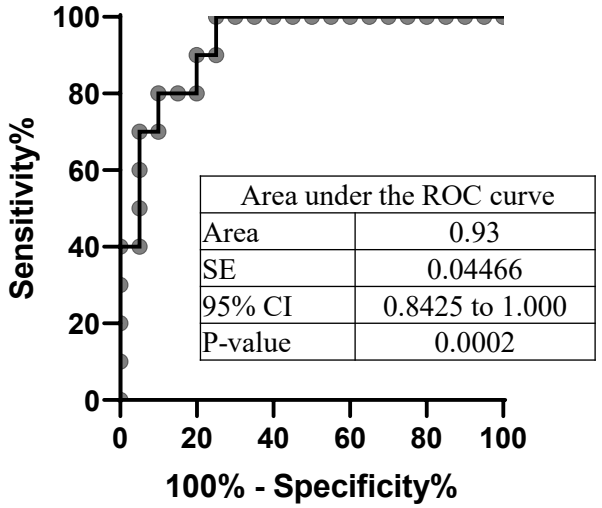
Area under the ROC curve	
Area	0.785
SE	0.08285
95% CI	0.6226 to 0.9474
P-value	0.0122

Lactate dehydrogenase A (LDHA)



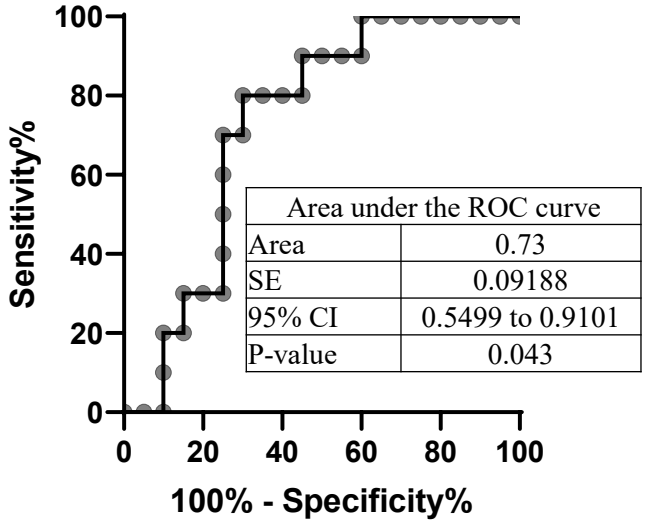
Area under the ROC curve	
Area	0.7825
SE	0.08368
95% CI	0.6185 to 0.9465
P-value	0.0129

Lactate dehydrogenase B (LDHB)



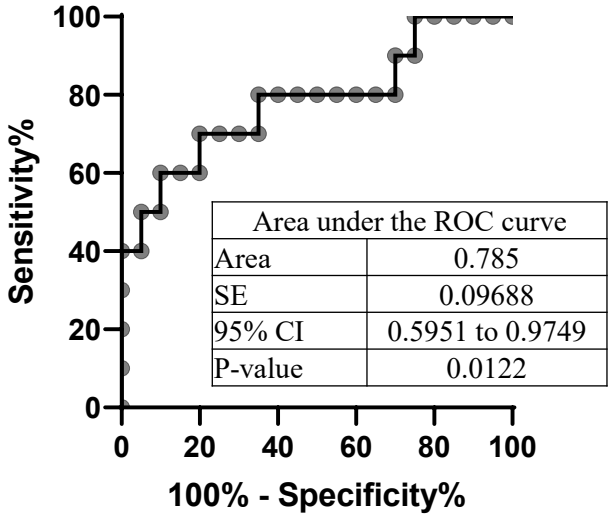
Area under the ROC curve	
Area	0.93
SE	0.04466
95% CI	0.8425 to 1.000
P-value	0.0002

Phosphoglycerate kinase 1 (PGK1)



Area under the ROC curve	
Area	0.73
SE	0.09188
95% CI	0.5499 to 0.9101
P-value	0.043

Pyruvate kinase M1/2 (PKM)



Area under the ROC curve	
Area	0.785
SE	0.09688
95% CI	0.5951 to 0.9749
P-value	0.0122