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Serum Proteomic Analysis Using Gel-Based Liquid Chromatography Tandem Mass Spectrometry Reveals Differences Between Canine Oral Malignancies and Non-Malignant Conditions

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ABSTRACT

Background: Canine oral cancers are difficult to manage due to complex biology and a lack of non-invasive biomarkers. Proteomic approaches, particularly gel-based liquid chromatography–tandem mass spectrometry (GeLC–MS/MS), have been used on tissue and saliva, but serum remains obscure despite its clinical accessibility and ability to reflect systemic disease.

Objectives: This study evaluated GeLC–MS/MS for serum proteomic profiling in canine oral malignancies, compared to benign and healthy conditions.

Methods: We analysed 62 serum samples from dogs with oral melanoma (OM, $n = 28$), oral squamous cell carcinoma (OSCC, $n = 10$), benign tumours (BN, $n = 12$) and controls (healthy/periodontitis, n = 12) using GeLC–MS/MS-based proteomics.

Results: Significant protein expression differences emerged across groups. In OM and OSCC, phosphodiesterase 4D (PDE4D) was upregulated, while ornithine decarboxylase antizyme 3 (OAZ3), centriolar coiled–coil protein 110 (CCP110), non–specific serine/threonine protein kinase 8 (NEK8), receptor–type tyrosine–protein phosphatase F (PTPRF) and interleukin 23 receptor (IL23R) were downregulated. These proteins are linked to critical pathways, including insulin signalling, insulin resistance, adherens junctions and cell cycle regulation, highlighting their roles in cancer progression and showing potential interactions with common chemotherapy drugs like doxorubicin, cisplatin and cyclophosphamide.

Conclusions: This study demonstrates that GeLC–MS/MS-based serum proteomics can successfully identify candidate biomarkers for canine oral malignancies. The discovery of these protein signatures represents promising diagnostic and prognostic targets, with the potential to guide chemotherapeutic selection and improve clinical outcomes in dogs with oral cancer.

1 | Introduction

Oral cavity tumours and tumour-like lesions are frequently observed during routine clinical examinations in dogs. These lesions often cause significant discomfort, pain and difficulty in eating (Verhaert 2010). The World Health Organisation (WHO) defines oral potentially malignant disorders (OPMDs) as precancerous lesions with an elevated risk of progressing to oral cancer, encompassing various oral mucosal conditions susceptible to malignant transformation (Gupta et al. 2018). Previous studies have shown that oral cancer can develop without noticeable symptoms in its early stages and may arise either de novo or

through the malignant transformation of OPMDs (Coelho 2012; Sharma et al. 2018). Although OPMDs and oral cancers differ pathologically, their clinical presentations often overlap, complicating early diagnosis (Puttaraju and Nitin 2023). To address these diagnostic challenges, veterinary research has increasingly focused on characterising the molecular landscape of these lesions. Recent studies have identified dysregulated signalling pathways, such as PI3K/Akt/mTOR and MAPK, as critical drivers in the progression of both canine oral melanoma (OM) and oral squamous cell carcinoma (OSCC; Meuten et al. 2024; Mârza et al. 2025). Furthermore, the identification of potential therapeutic targets, including cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF), has provided deeper insight into tumour angiogenesis and the poor prognosis often associated with OSCC (Millanta et al. 2016; Files et al. 2024). In both humans and dogs, melanoma is a highly malignant tumour characterised by complex immune interactions within the tumour microenvironment. Specifically, tumour-infiltrating lymphocytes (TILs) and immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2) have been associated with immunosuppression, metastasis and poor overall clinical outcomes (Stevenson et al. 2023). Despite these advances in understanding specific malignancies, the molecular associations and transitions between inflammatory, hyperplastic, benign and malignant oral lesions remain poorly characterised. Moreover, the stepwise progression from normal oral tissue to OPMD and subsequently to malignancy has not been documented in dogs. This knowledge gap underscores the need to investigate the molecular characteristics of canine oral lesions to better understand their pathogenesis and potential parallels with human oral cancers. Although histopathology remains the gold standard for differentiating between benign and malignant oral lesions, relying solely on clinical examination is often insufficient for early detection and definitive differentiation at the initial presentation. (Fukuda et al. 2012).

Proteomics presents a promising strategy to address these challenges by uncovering the molecular mechanisms underlying malignant transformation. Analysis of protein expression profiles through proteomic techniques has led to the identification of biomarkers capable of differentiating human OSCC from epulis (Papa et al. 2018). In veterinary research, biomarker studies in canine oral oncology remain limited, particularly in serum proteomics. Previous work successfully utilised matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography tandem mass spectrometry (LC MS/MS) to identify characteristic peptide mass patterns and specific biomarkers, such as peptidyl-prolyl cis-trans isomerase FKBP4, steroid hormone receptor ERR1 (ESRRA) and ATP-binding cassette subfamily B member 5 (ABCB5), which are overexpressed in both canine OM and OSCC. Despite the identification of these markers, the comprehensive serum protein profiles and the underlying biological functions driving these malignancies have yet to be fully elucidated (Ploypetch et al. 2022). Furthermore, chemical isotope labeling LC–MS–based amine/phenol submetabolomics was employed to identify candidate biomarkers in dogs with OM and OSCC, in comparison with benign tumours (BN) and healthy controls (Ploypetch et al. 2024). While gel-based liquid chromatography–tandem mass spectrometry (GeLC–MS/MS) has been used to profile proteins in tumour tissues and saliva, its application to serum samples remains unexplored (Pisamai et al. 2018; Ploypetch et al. 2020). Therefore, this study aimed to apply a GeLC–MS/MS approach—combining sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with LC–MS/MS—to explore serum biomarkers associated with canine oral cancers (OM and OSCC) and compare them with those found in dogs with oral non-cancerous condition, including BN and controls (healthy and periodontitis cases). Our objective was to elucidate the molecular alterations contributing to disease development and progression. By identifying differentially expressed proteins across these groups, we aimed to uncover candidate biomarkers that may facilitate non-invasive diagnosis and provide insights into the pathogenesis of canine oral cancers.

2 | Materials and Methods

2.1 | Sample Collection and Preparation

All serum samples were collected from client-owned dogs presented at the Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University, and the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The study protocol was approved by the Mahidol University–Institute Animal Care and Use Committee (MU–IACUC), Thailand (Protocol No. MUVS–2021–04–14).

A total of 62 serum samples were obtained from dogs across six groups: 10 diagnosed with OSCC (aged 10–14 years), 28 with OM (aged 9–14 years), 12 with BN (aged 8–12 years), seven healthy dogs with normal oral health (aged 6–9 years) and five dogs with periodontitis (aged 7–13 years; Table 1). Diagnoses were based on clinical oral examinations, with periodontitis characterised by gingivitis, dental tartar and loss of periodontal attachment (Ploypetch et al. 2022). Dogs without detectable oral masses (i.e., healthy and periodontitis cases) were grouped together as controls (CTRL). Only dogs with no history of surgery, chemotherapy or radiation therapy were included. Approximately 3–4 mL of whole blood was collected from each dog and centrifuged at 3000 ×g for 10 min at 4°C. A volume of 500 µL serum was aliquoted, mixed with protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –20°C until further analysis. Total protein concentrations in the serum samples were determined using a modified Lowry assay, with bovine serum albumin employed as the reference standard (Winters and Minchin 2005). Additionally, tumour tissue samples from dogs diagnosed with OM, OSCC and BN were collected and histologically confirmed.

2.2 | Analysis of Serum Proteins by GeLC–MS/MS

Serum protein profiles were analysed using GeLC–MS/MS as previously applied to canine oral tumours in saliva and tissue samples (Pisamai et al. 2018; Ploypetch et al. 2020). Pooled serum samples from each group (OM, OSCC, BN and CTRL) were used for analysis. A total of 50 µg of pooled serum samples was mixed with a loading buffer containing dithiothreitol (DTT), SDS, Tris–HCl, glycerol and bromophenol blue dye. The mixture was heated at 90°C for 5 min and separated on a 12.5% SDS–PAGE

(Atto, Tokyo, Japan). After electrophoresis, the gel was fixed, silver-stained and scanned using a GS–710 scanner (Bio–Rad Laboratories, Benicia, CA, USA). The gel lane was divided into 17 segments, each cut into small pieces (1 mm^3). These gel fragments were dehydrated, followed by reduction and alkylation of cysteine residues. Trypsin digestion was performed overnight at 37°C. Peptides were extracted from the gel using a solution containing 50% acetonitrile (ACN) in 0.1% formic acid. For LC–MS/MS analysis, extracted peptides were separated by reversed-phase high-performance liquid chromatography (HPLC), using an LC system coupled to an HCTUltra PTM Discovery System (Bruker Daltonics, Billerica, CA, USA). Separation was carried out on a PepSwift monolithic capillary column (100 µm internal diameter × 50 mm; Thermo Fisher Scientific) using a linear gradient of ACN over 7.5 min, with a total run time of 20 min, including regeneration and equilibration steps. Peptide fragmentation spectra were acquired in data-dependent Auto MS mode. The scan range was set to 400–1500 m/z and narrowed to 200–2800 m/z when more than five precursor ions were detected. Raw data were converted to mzXML format using CompassXport software (Bruker Daltonics). Protein quantification was performed using DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare, Chicago, IL, USA; Johansson et al. 2006; Thorsell et al. 2007).

2.3 | Statistical Analysis

Both multivariate and univariate statistical analyses were conducted to evaluate the differential protein expression. Multivariate analysis was visualised using two-dimensional partial least squares discriminant analysis (2D–PLS–DA) score plots. Univariate analysis was based on a fold-change (FC) threshold of ≥ 2.0 and a false discovery rate (FDR) with $Q < 0.05$, to identify significantly differentially expressed proteins in comparisons of OM versus CTRL, OM versus BN, OSCC versus CTRL and OSCC versus BN. All statistical analyses were conducted using MetaboAnalyst 6.0. (http://www.metaboanalyst.ca/; Ewald et al. 2024). Venn diagram analysis was performed using Jvenn (http://bioinfo.genotoul.fr/jvenn/example.html) to identify overlapping significant proteins among groups (Bardou et al. 2014). The Kruskal–Wallis test was used to determine statistically significant differences ($p < 0.05$) among the datasets.

2.4 | Bioinformatics Analysis

MaxQuant version 2.2.0.0 was employed for protein quantification within individual samples, utilising the Andromeda search engine to match MS/MS spectra against the *Canis lupus familiaris* UniProt database (Tyanova et al. 2016). Protein annotation and functional analysis were performed using UniProtKB/Swiss–Prot entries to explore biological processes and sequence characteristics (UniProt Consortium 2018). Protein–protein interaction networks were analysed using STITCH 5.0 (accessed on 16 February 2025), to evaluate potential interactions among identified proteins and previously reported candidate biomarkers in canine oral cancers analysed via GeLC–MS/MS in saliva and tissue (Pisamai et al. 2018; Ploypetch et al. 2020). This analysis included previous potential proteins such as protein tyrosine phosphatase non-receptor type 5 (PTPN5), PTPN1, BRCA2 (DNA repair associated), WW domain binding protein 2 (WBP2), purinergic receptor P2Y1 (P2RY1) and proteasome activator subunit 4 (PSME4), as well as commonly used chemotherapy drugs including doxorubicin, cisplatin, toceranib and cyclophosphamide (Szklarczyk et al. 2016). Functional enrichment analysis was performed

TABLE 1 | Patients' history.

Group	Clinical staging	Clinical examination/ histological finding	Breed	N	Ages (years) Mean ± SD	Sex
CTRL	N/A	Normal gingiva	7 Beagles	5	7.85 ± 1.46	4 M and 3 Fs
		Periodontitis	2 Poodles and 3 mixed	7	8.67 ± 1.21	3 Mc and 2 Fs
BN	N/A	Peripheral odontogenic fibroma	2 Poodles, 3 Golden Retrievers, 2 Siberian Huskies, 1 mixed and 1 Shih-Tsu	12	8.75 ± 3.14	4 M, 2 Mc, 1 F and 2 Fs
		Acanthomatous ameloblastoma	1 Shih-Tsu, 1 Labrador Retriever and 1 Golden Retriever		10.67 ± 0.58	2 F and 1 Fs
OM	III	Melanotic melanoma	3 Poodles, 5 Golden Retrievers, 4 mixed, 1 Pug, 2 Shih-Tsus, 1 Labrador retriever, 1 Beagle, 1 Cocker Spaniel, 1 Schnauzer and 1 Pomeranian	20	12.61 ± 2.15	14 M, 2 F and 4 Fs
		Amelanotic melanoma	1 Poodle, 1 Cocker Spaniel, 5 mixed and 1 Dachshund	8	10.67 ± 1.75	5 M, 2 Mc and 1 F
OSCC	III	OSCC well differentiated	1 Poodle, 1 Cocker Spaniel, 2 mixed and 1 Pug	6	12 ± 1.26	2 M, 1 F and 3 Fs
		OSCC poorly differentiated	1 Poodle, 1 Mixed, 1 Shih-Tsu and 1 Bangkeaw	4 4	11.75 ± 2.5	2 M, 1 Mc and 1 F

Abbreviations: BN, benign tumours; CTRL, control; F, female; Fs, female spray; M, male; Mc, male castration; N/A, not available; OM, oral melanoma; OSCC, oral squamous cell carcinoma.

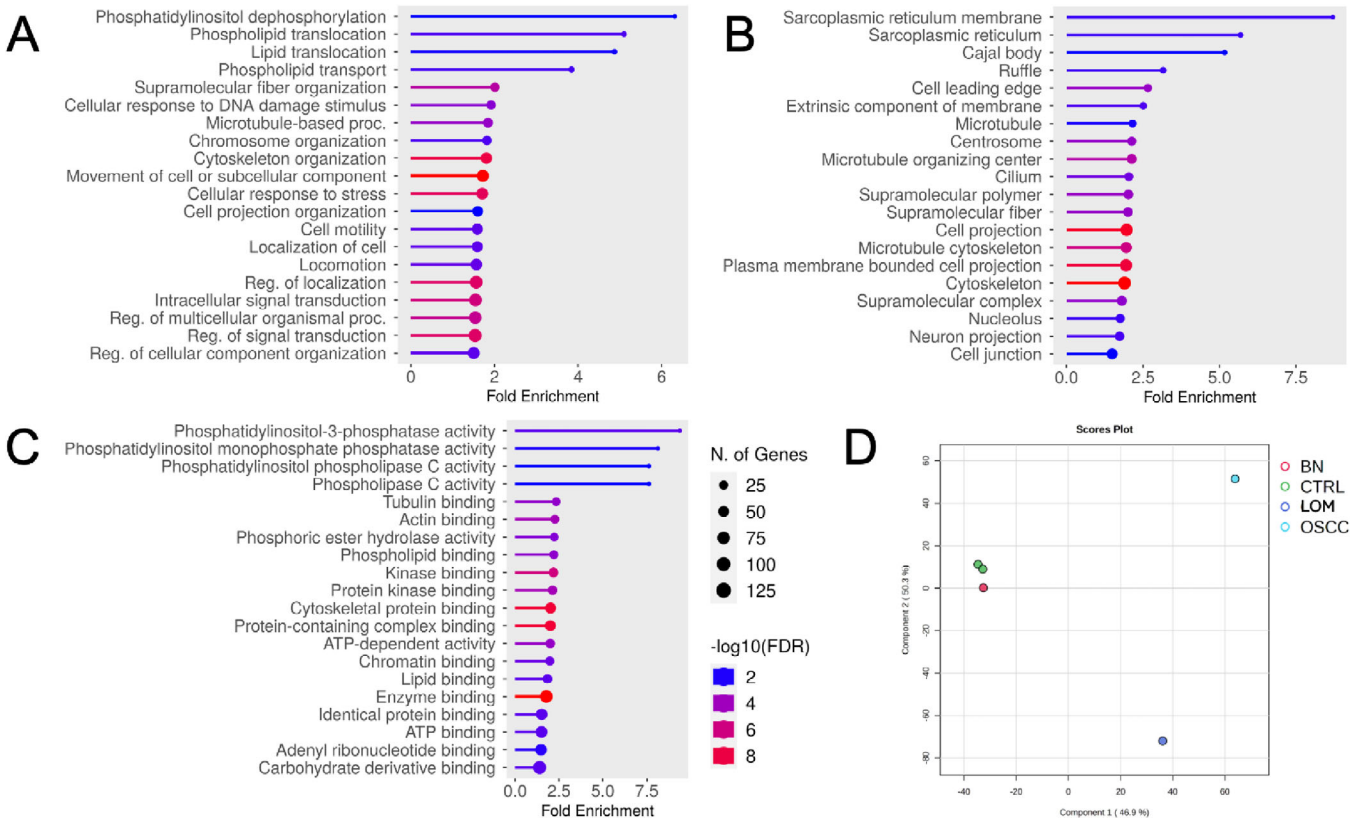

FIGURE 1 | Functional enrichment and multivariate analysis of differentially expressed proteins across four canine sample groups: Oral melanoma (OM), oral squamous cell carcinoma (OSCC), benign oral tumours (BN) and control (CTRL). (A–C) Bar plots depict the top enriched Gene Ontology (GO) terms classified by biological processes (A), cellular components (B) and molecular functions (C). Dot size represents the number of associated genes, while colour intensity (–log10(FDR)) reflects the statistical significance of enrichment, with darker colours corresponding to more significant results. Partial least squares discriminant analysis (PLS–DA) illustrates clear separation among the four groups, indicating distinct serum proteomic profiles (D). Each point represents a sample: OM (purple), OSCC (blue), BN (red) and CTRL (green).

using ShinyGO v0.80, based on the *Canis familiaris* database, incorporating the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Categories included biological process, molecular function and cellular component. The following settings were applied: a false discovery rate (FDR) adjusted p-value (Q) < 0.05, a pathway size between 2 and 5000, with 'remove redundancy' and 'abbreviate pathway names' options enabled (Ge et al. 2020).

3 | Results

3.1 | Proteome Diversity and Abundance

A total of 1240 proteins from *Canis lupus familiaris* were successfully identified (full details are provided in the Supporting Information, Table S1). To characterise the dataset, all identified proteins were annotated using Gene Ontology (GO) via ShinyGO v0.80 across three categories: biological process, cellular component and molecular function. GO enrichment analysis provided insight into the functional implications of these differentially expressed proteins. Altered biological processes included phosphatidylinositol dephosphorylation, cellular response to DNA stimulus and cytoskeleton organisation, key pathways involved in cell structure, signalling and stress responses (Figure 1A, Table S2). The affected cellular components included the sarcoplasmic reticulum membrane, cell projections and cytoskeleton, which are essential for maintaining cell architecture, communication and transport (Figure 1B, Table S2). In terms of molecular function, significant changes were observed in phosphatidylinositol-3-phosphatase activity, cytoskeleton protein binding and protein-containing complex binding, highlighting roles in signaling and structural integrity (Figure 1C, Table S2). To visualise group separation based on proteomic profiles, partial least squares discriminant analysis (PLS–DA) was conducted (Figure 1D). The score plot revealed clear discrimination among OM, OSCC, BN and CTRL groups, suggesting distinct proteomic signatures associated with each condition.

3.2 | Identification of Potential Canine Oral Cancer Biomarkers

A Venn diagram was constructed to visualise potential biomarkers identified through significantly different protein expression levels across group comparisons, including OM versus CTRL, OM versus BN, OSCC versus CTRL and OSCC versus BN (FDR with Q < 0.05 and FC > 2.0 or < 0.67; Figure 2; Table S3).

Across these comparisons, 17 proteins were identified as commonly significant candidate markers. Additionally, 64 significantly altered proteins were shared between the OM versus

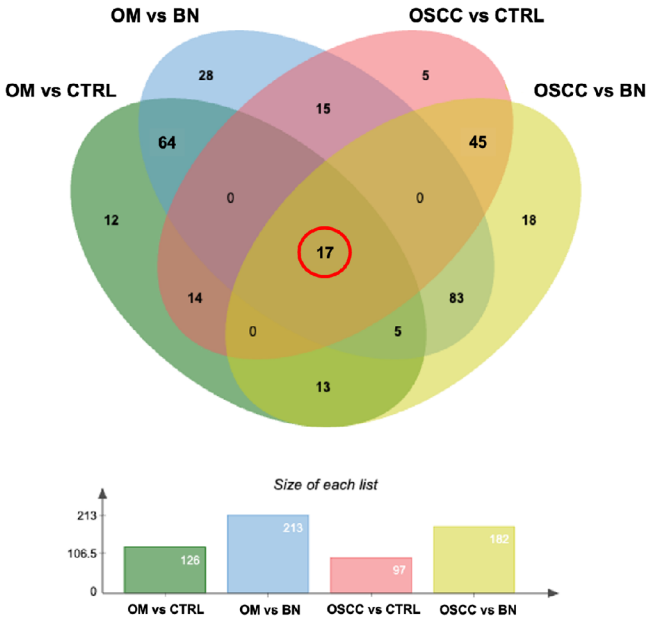

FIGURE 2 | Venn diagram illustrating the overlap of differentially expressed proteins across four pairwise comparisons: oral melanoma (OM) versus control (CTRL), OM versus benign oral tumours (BN), oral squamous cell carcinoma (OSCC) versus CTRL and OSCC versus BN. The central intersection highlights 17 proteins consistently altered in all comparisons, suggesting their potential as shared biomarkers of malignancy.

CTRL and OM versus BN groups, while 45 were shared between the OSCC versus CTRL and OSCC versus BN comparisons (Table S4). A total of 104 proteins were subjected to further statistical analysis using the Kruskal–Wallis test with post hoc comparisons to confirm differential expression among groups (Table S5). A hierarchical heatmap demonstrated clear clustering of proteins based on expression levels, particularly distinguishing oral cancers (OM and OSCC) from non-oral cancer groups (BN and CTRL; Figure 3).

These findings highlight the potential of serum proteomic profiling to define molecular signatures associated with canine oral cancers, supporting improved diagnostic classification and insights into tumour biology. Among the 104 candidate proteins, four were consistently upregulated: CCAAT-binding factor domain-containing protein (CEBPZ), phosphodiesterase 4D (PDE4D), decaprenyl diphosphate synthase subunit 1 (PDSS1) and synaptotagmin-like 2 (SYTL2). Contrary, 13 proteins were significantly downregulated across all comparisons: ornithine decarboxylase antizyme 3 (OAZ3), Rho-GAP domain–containing protein (RhoGAP), centriolar coiled–coil protein 110 (CCP110), non-specific serine/threonine protein kinase 8 (NEK8), receptor–type tyrosine–protein phosphatase F (PTPRF), protein phosphatase 1 regulatory subunit 9B (PPP1R9B), kinase non-catalytic C-lobe domain containing 1 (KNDC1), ASH1-like histone lysine methyltransferase (ASH1L), interleukin 23 receptor (IL23R), UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1), eukaryotic translation initiation factor 2A (EIF2A), peptidase M12B domain-containing protein (Peptidase M12B) and Kringle containing transmembrane protein 2 (KREMEN2; Table 2). To gain further insight into cancer-related molecular mechanisms and drug interactions of these biomarker candidates, protein–chemotherapy drug interaction networks were constructed based on differentially altered proteins in OM and OSCC using STITCH 5.0, and associated pathways were analysed via the KEGG database ($p < 0.05$).

3.3 | Protein–Protein and Protein–Chemotherapy Drug Interaction Analysis

Protein–protein and protein–chemotherapy drug interactions were constructed using STITCH 5.0, with edge confidence scores indicating the strength of predicted functional associations. The network analysis revealed that the upregulated protein PDE4D, along with downregulated proteins OAZ3, PTPRF, IL23R, CCP110 and NEK8 in OM and OSCC, interact with previously identified biomarkers such as PTPN5, PTPN1, BRCA2, WBP2, P2RY1 and PSME4. These proteins also showed associations with commonly used chemotherapy drugs including doxorubicin, cisplatin and cyclophosphamide (Figure 4). Functional enrichment analysis using KEGG pathway maps identified key pathways involving these proteins, including the adherens junction (AJ) pathway, insulin signalling and insulin resistance (FDR < 0.05), underscoring the involvement of candidate proteins in critical signalling cascades and drug response mechanisms associated with canine oral malignancies (Table S6). All supplementary data, including protein lists and enrichment details, are available as Supporting Information.

4 | Discussion

In this study, GeLC–MS/MS was employed to identify novel serum biomarker candidates associated with canine oral tumours. PLS–DA analysis successfully classified samples into four distinct groups: OM, OSCC, benign lesions (BN) and healthy and periodontitis cases (CTRL). A Venn diagram analysis revealed 17 overlapping proteins across four pairwise comparisons (OM vs. CTRL, OM vs. BN, OSCC vs. CTRL and OSCC vs. BN). This consistent differential expression across various comparisons suggests these proteins may be central to distinguishing malignant from benign and healthy conditions. Importantly, these proteins provided insight into molecular differences in tumour progression and were consistent with previous findings from analyses of canine oral tumour saliva and tissue (Pisamai et al. 2018; Ploypetch et al. 2020). Among the identified proteins, PDE4D was markedly upregulated in both OM and OSCC, whereas OAZ3, CCP110, NEK8, PTPRF and IL23R were consistently downregulated. These proteins are known for their roles in key cellular processes such as cell cycle regulation, DNA repair and signal transduction and their altered expression likely contributes to the pathogenesis of canine oral cancers. Functional enrichment analysis (using ShinyGo 0.80) identified insulin signalling, insulin resistance and AJs as the most significantly enriched KEGG pathways involving these proteins.

The insulin signalling pathway regulates metabolism, proliferation and survival, and its dysregulation is often observed in cancer progression (Le et al. 2023). Similarly, insulin resistance has been associated with an increased cancer risk by promoting

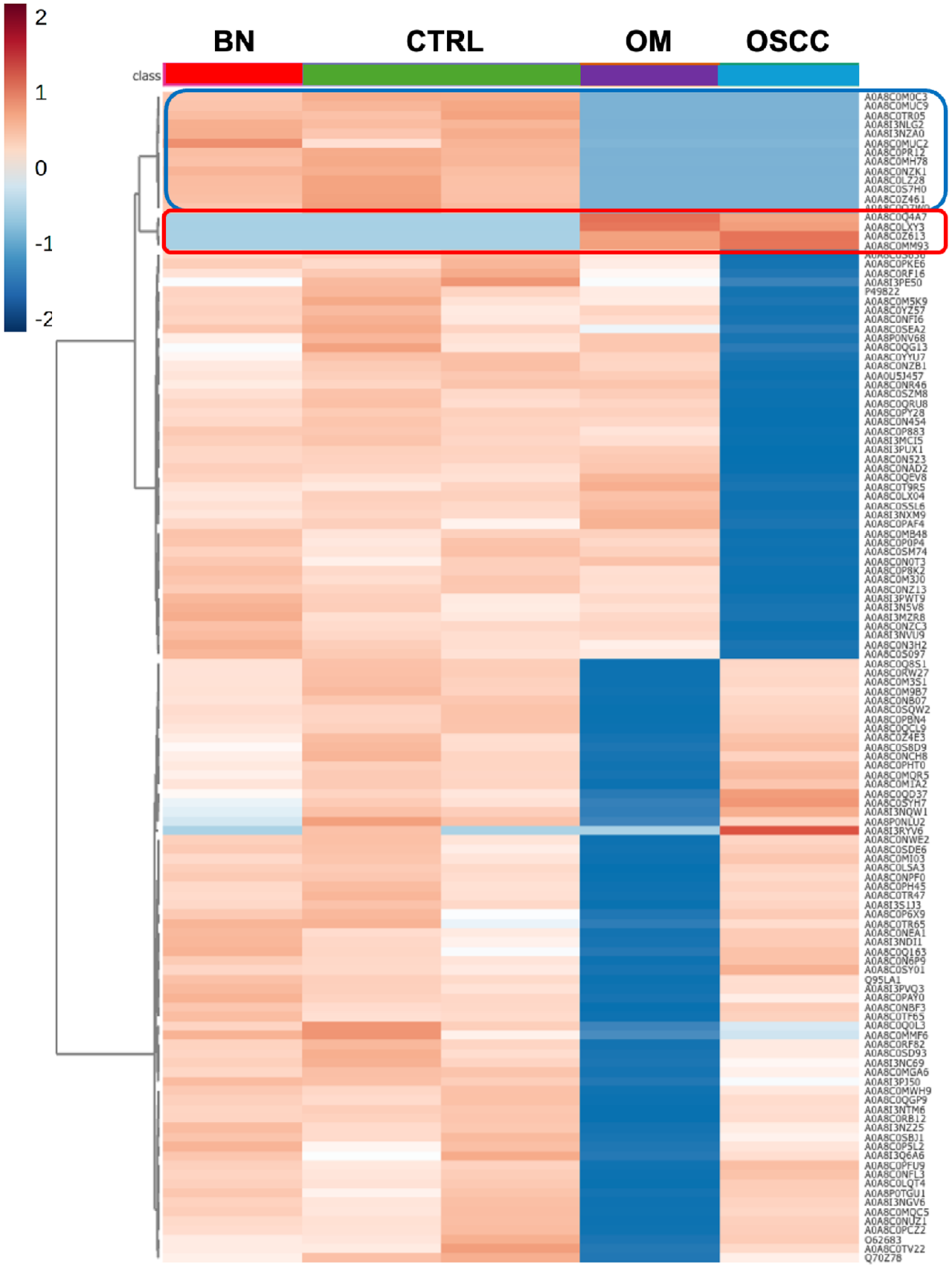

FIGURE 3 | Heatmap of differentially expressed proteins across four groups: benign oral tumours (BN), control (CTRL), oral melanoma (OM) and oral squamous cell carcinoma (OSCC). Each row represents a protein, and each column corresponds to an individual sample. Protein expression levels are colour-coded on a gradient from blue (downregulated in OM and OSCC relative to BN and CTRL) to red (upregulated in OM and OSCC relative to BN and CTRL).

TABLE 2 | Significant differences in protein alterations of LOM vs. CTRL, LOM vs. BN, OSCC vs. CTRL and OSCC vs. BN groups based on biological process involvement, cellular component and molecular function.

Protein IDs	Protein names (gene names)	Pepide sequence	-log 10(p)	FDR	Gene Ontology (biological process)	Gene Ontology (cellular component)	Gene Ontology (molecular function)
A0A8C0PR12	ASH1-like histone lysine methyltransferase (ASH1L)	DKLDIFR	2.3946	0.00847	flagellated sperm motility [GO:0030317]; inflammatory response [GO:0006954]; MAPK cascade [GO:0000165]	Golgi apparatus [GO:0005794]; nucleoplasm [GO:0005654]	chromatin binding [GO:0003682]; DNA binding [GO:0003677]
A0A8C0LZ28	Kringle containing transmembrane protein 2 (KREMEN2)	AFDGSR	1.9414	0.014	Wnt signalling pathway [GO:0016055]	Plasma membrane [GO:0005886]	N/A
A0A8C0MUC9	Ornithine decarboxylase antizyme 3 (OAZ3)	ESLTATLEYVEEK	2.3871	0.00847	Viral translational frameshifting [GO:0075523]	N/A	Ornithine decarboxylase inhibitor activity [GO:0008073]
A0A8C0NZK1	Centriolar coiled–coil protein 110 (CCP110)	AAEMGMPNKK	1.9414	0.014	Centriole replication [GO:0007099]; regulation of cytokinesis [GO:0032465]	Centriole [GO:0005814]	N/A
A0A8C0Z613	CCAAT-binding factor domain-containing protein (CEBPZ)	KMLDPGLMMCSK	2.5368	0.00847	Positive regulation of transcription by RNA polymerase II [GO:0045944]	Nucleus [GO:0005634]	Transcription coactivator activity [GO:0003713]
A0A8C0Z461	Non-specific serine/threonine protein kinase (NEK8)	SLVPGSTGSR	1.9414	0.014	N/A	Cytoplasm [GO:0005737]	ATP binding [GO:0005524]; protein serine/threonine kinase activity [GO:0004674]
A0A8C0LXY3	Phosphodiesterase (PDE4D)	MEAEGSSVPAR	2.5368	0.00847	cAMP catabolic process [GO:0006198]; negative regulation of adenylate cyclase-activating G protein-coupled receptor signaling pathway [GO:0106072]	Calcium channel complex [GO:0034704]; cytosol [GO:0005829]; plasma membrane [GO:0005886]	3',5'-cyclic-AMP phosphodiesterase activity [GO:0004115]; ATPase binding [GO:0051117]; cAMP binding [GO:0030552]
A0A8C0Q4A7	Decaprenyl diphosphate synthase subunit 1 (PDSS1)	AVLAGDLILS AASMALAR	2.5368	0.00847	Isoprenoid biosynthetic process [GO:0008299]; ubiquinone biosynthetic process [GO:0006744]	Heterotetrameric polyprenyl diphosphate synthase complex [GO:0032478]	All-trans-decaprenyl-diphosphate synthase activity [GO:0097269]; all-trans-nonaprenyl-diphosphate synthase (geranyl-diphosphate specific) activity [GO:0052923]

(Continues)

TABLE 2 | (Continued)

Protein IDs	Protein names (gene names)	Pepide sequence	-log 10(p)	FDR	Gene Ontology (biological process)	Gene Ontology (cellular component)	Gene Ontology (molecular function)
A0A8C0M0C3	Peptidase M12B domain-containing protein (Peptidase M12B)	LLPSS	2.3871	0.00847	Extracellular matrix organisation [GO:0030198]; proteolysis [GO:0006508]	Extracellular region [GO:0005576]	Metal ion binding [GO:0046872]; metalloendopeptidase activity [GO:0004222]
A0A8C0MH78	Rho-GAP domain-containing protein (RhoGAP)	LSGCTAK	2.3871	0.0084704	Signal transduction [GO:0007165]	N/A	N/A
A0A8C0MM93	Synaptotagmin-like 2 (SYTL2)	SSELSR	2.5368	0.00847	Intracellular protein transport [GO:0006886]	Membrane [GO:0016020]	Small GTPase binding [GO:0031267]
A0A8C0MUC2	Eukaryotic translation initiation factor 2A (EIF2A)	INVINVTNK	2.3871	0.00847	N/A	N/A	Translation initiation factor activity [GO:0003743]
A0A8C0Q7W0	Interleukin 23 receptor (IL23R)	ILLLIPK	2.3871	0.00847	Negative regulation of interleukin-10 production [GO:0032693]; positive regulation of interleukin-12 production [GO:0032735]; positive regulation of T-helper 1 type immune response [GO:0002827]	Cell surface [GO:0009986]; interleukin-23 receptor complex [GO:0072536]	Interleukin-12 receptor binding [GO:0005143]; interleukin-23 binding [GO:0042019]; interleukin-23 receptor activity [GO:0042020]
A0A8C0S7H0	UDP-N-acetylglucosamine pyrophosphorylase 1 (UAP1)	GARPSRGR	1.9414	0.014	N/A	N/A	Uridylyltransferase activity [GO:0070569]
A0A8C0TR05	Kinase non-catalytic C-lobe domain containing 1 (KNDC1)	TLLLDMAR	1.9414	0.014	Small GTPase-mediated signal transduction [GO:0007264]	N/A	Guanyl-nucleotide exchange factor activity [GO:0005085]
A0A8I3NLG2	Protein phosphatase 1 regulatory subunit 9B (PPP1R9B)	EMMEQR	2.3871	0.00847	Actin filament organisation [GO:0007015]; calcium-mediated signaling [GO:0019722]; cell migration [GO:0016477]	Cortical actin cytoskeleton [GO:0030864]; filopodium [GO:0030175]; lamellipodium [GO:0030027]; nucleoplasm [GO:0005654]	N/A
A0A8I3NZA0	Receptor-type tyrosine-protein phosphatase F (PTPRF)	TFALHKSGSSEK	1.9414	0.014	Cell migration [GO:0016477]; peptidyl-tyrosine dephosphorylation [GO:0035335]	Membrane [GO:0016020]; neuron projection [GO:0043005]; neuronal cell body [GO:0043025]	Cell adhesion molecule binding [GO:0050839]; protein tyrosine phosphatase activity [GO:0004725]

Abbreviations: BN, benign tumours; CTRL, control; N/A, not available; OM, oral melanoma; OSCC, oral squamous cell carcinoma.

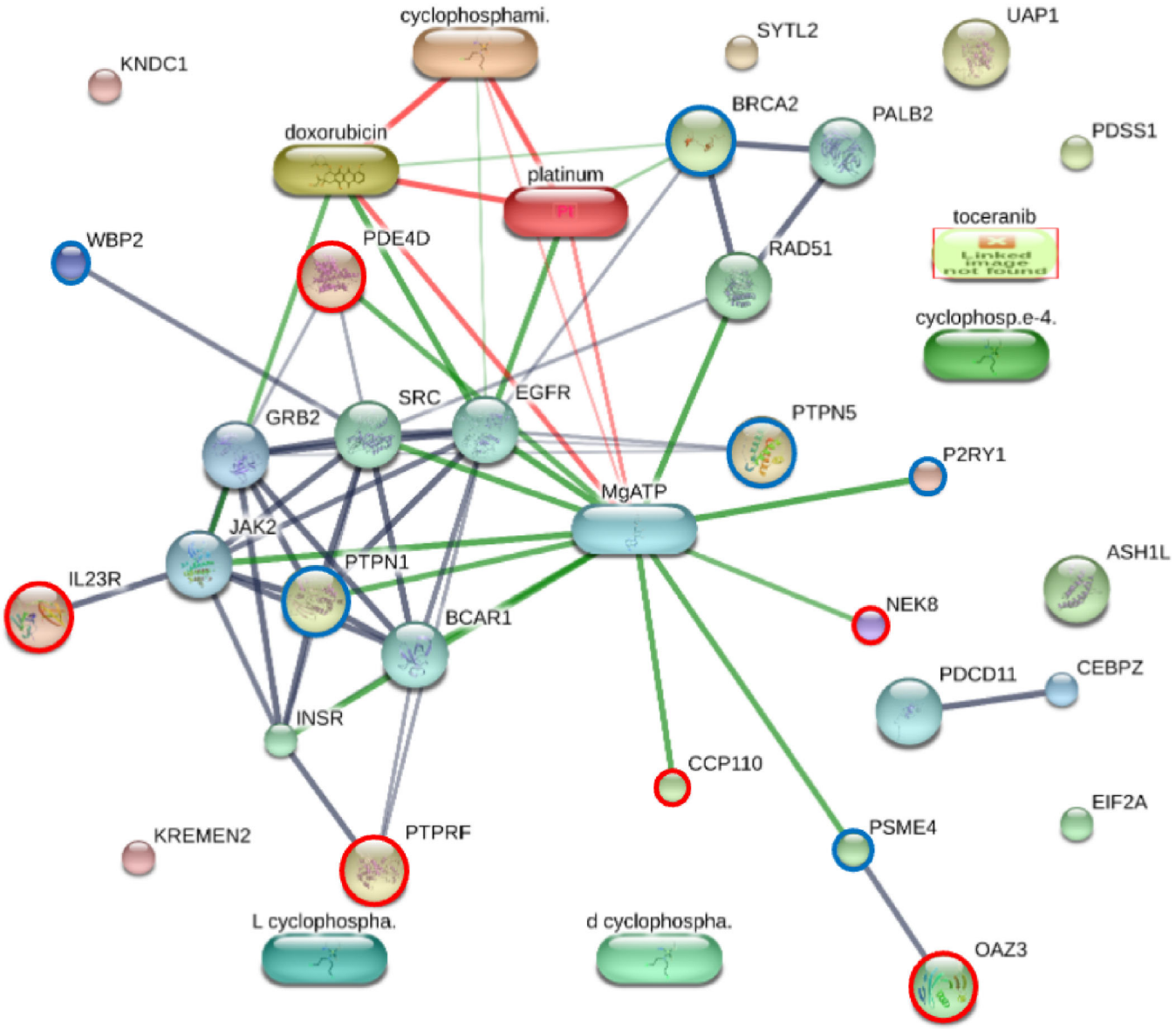

FIGURE 4 | Protein–protein and protein–drug interaction network analysis. The network diagram illustrates interactions among candidate proteins identified in this study (red circles) and proteins previously reported in canine oral cancers using GeLC MS/MS (blue circles). Edges indicate known or predicted associations, including physical binding and functional links. Interactions with chemotherapeutic agents such as doxorubicin, platinum-based drugs (carboplatin) and cyclophosphamide derivatives are also shown. Green and red lines represent positive and negative correlations, respectively, while grey lines indicate known protein–protein associations from curated databases.

DNA damage and carcinogenesis (Arcidiacono et al. 2012). Of particular interest, overexpression of PDE4D has been linked to oncogenesis via interaction with the pro-oncogenic insulin-like growth factor 2 (IGF2). Furthermore, PDE4 upregulation has been reported in glioblastoma, medulloblastoma and lung cancer (Sengupta et al. 2011). Notably, the PDE4D inhibitor has shown promise in reducing tumour proliferation and overcoming resistance in glioblastoma (Sengupta et al. 2011; Pullamsetti et al. 2013; Ramezani et al. 2017). In line with these findings, our study identified upregulated PDE4D expression in OM and OSCC, supporting its potential as a therapeutic target in canine oral cancers. OAZ3 regulates polyamine metabolism, a process essential for cell proliferation and redox balance (Pegg 2009). The observed downregulation of OAZ3 in OM and OSCC may reflect disrupted insulin-mediated regulation. Elevated polyamine levels have been associated with the development of breast cancer and the pathophysiology of insulin resistance (Çelik et al. 2017). Thus, reduced OAZ3 expression in canine oral cancers may represent a novel convergence of metabolic and oncogenic dysregulation. In addition to OAZ3, PTPRF is a known negative regulator of insulin signalling and is typically elevated in insulin-resistant tissues (Goldstein et al. 1998). In the present study, PTPRF was found to be downregulated in the serum of canine OM and OSCC serum samples. This reduction may reflect a loss of negative regulatory control over insulin signalling, potentially facilitating tumour progression. Collectively, the downregulation of PTPRF and OAZ3, along with the upregulation of PDE4D, underscored a common mechanism of insulin signalling dysregulation in the pathogenesis of canine oral malignancies.

AJs are essential for maintaining epithelial cohesion, structural organisation and mechanical integrity. In stratified epithelia, they function in coordination with desmosomes to regulate tissue architecture and prevent malignant transformation (Hirai et al. 1989; Samiei et al. 2019; Groeger and Meyle 2019). Our proteomic analysis revealed a significant downregulation of IL23R in both OM and OSCC. While IL23R is commonly associated with immune responses, it has also been implicated in the AJ pathway and cancer progression, particularly through its role in the Wnt/β–catenin signaling pathway, contributing to epithelial–mesenchymal transition (EMT) and metastasis in human oral cancers (Chen et al. 2015). It is important to note that while IL23 signalling can promote SCC development, it has also exhibited a protective role in melanocytic cells by enhancing DNA repair and inhibiting tumour initiation (Langowski et al. 2006; Nasti et al. 2017). Therefore, reduced IL23R expression in canine oral cancers may compromise AJ integrity, disrupt cell adhesion and promote tumour invasion and progression. These findings suggest IL23R as a potential regulator of epithelial stability and immune homeostasis, whose downregulation could facilitate malignant transformation in both OM and OSCC. Targeting AJ-related pathways may represent a novel therapeutic strategy in canine oral tumours. NEK8, a serine/threonine protein kinase, regulates the cell cycle, DNA damage response and primary cilia disassembly (Fry et al. 2012; Panchal and Evan Prince 2023). Previous studies have linked NEK8 dysfunction to impaired cell–cell adhesion through reduced E-cadherin expression and disrupted desmosomal protein organisation (Natoli et al. 2008). Although NEK8 overexpression has been reported in pancreatic, breast and colorectal cancers, implicating it in tumourigenesis and metastasis, its dysregulated expression (downregulation) in our study, observed in OM and OSCC, suggests a potential role in promoting tumour cell detachment, invasion and progression via AJ disruption (Bowers and Boylan 2004; Carter et al. 2010; Choi et al. 2013). This makes NEK8 a promising candidate biomarker. CCP110, a crucial centrosomal protein involved in centrosome duplication and cell cycle control, has been implicated in both ciliopathies and oncogenesis due to its role in maintaining genomic stability (Spektor et al. 2007; Bettencourt-Dias et al. 2011). Its function is regulated by the cyclin E/CDK2 complex and is known to promote centrosome amplification and tumour progression (Oliver and MacDonald 2001; Cai et al. 2007). Disruption of CCP110, potentially together with NEK8, may impair cilia disassembly, centrosome function and epithelial architecture, all contributing to tumour development. Collectively, the altered expression of IL23R, NEK8 and CCP110, observed in canine OM and OSCC, supports their role in centrosome dysfunction, loss of AJ integrity and epithelial disorganisation—hallmarks of oral tumourigenesis. These findings suggest a potential connection of an IL23R–NEK8–CCP110 signalling axis in the pathogenesis of canine oral cancers and highlight a potential novel target for future therapeutic development.

5 | Conclusion

This study highlights the value of GeLC–MS/MS–based proteomics in uncovering key molecular features of canine oral cancers from serum samples. By integrating our previous findings with existing knowledge from tissue and saliva analyses, we identified proteins such as PDE4D, OAZ3 and PTPRF linked to dysregulated insulin signalling. Furthermore, the downregulation of IL23R, NEK8 and CCP110 pointed to disruptions in cell adhesion, ciliary function and genomic stability. These findings collectively suggest that interconnected pathways involving metabolism, cell adhesion and cell cycle control contribute to oral tumourigenesis in dogs. Despite the use of pooled samples and limited cohort size, this integrated approach offers promising biomarker candidates for future validation. Further studies using larger sample sets and targeted assays are critically needed to confirm their clinical relevance.

Author Contributions

Sittiruk Roytrakul: methodology, software, investigation, validation, formal analysis, writing – review and editing, visualisation, supervision. **Janthima Jaresitthikunchai**: formal analysis, data curation, validation. **Narumon Phaonakrop**: formal analysis, data curation, validation. **Sekkarin Ploypetch**: conceptualisation, methodology, data curation, investigation, validation, formal analysis, funding acquisition, project administration, writing – original draft, writing – review and editing, visualisation, resources. **Gunnaporn Suriyaphol**: conceptualisation, methodology, formal analysis, investigation, writing – original draft, writing – review and editing, visualisation, supervision, project administration, validation.

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Ethics Statement

The study protocol was approved by the Mahidol University–Institute Animal Care and Use Committee (MU–IACUC), Thailand (Protocol No. MUVS-2021-04-14). Ethical approval was obtained before the commencement of the study. Informed consent was secured from all participants after explaining the study objectives and procedures. Participant confidentiality was maintained, and appropriate measures were taken to minimise any potential risk or discomfort.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The MS proteomics data have been deposited in the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the jPOSTrepo partner with the data set identifier JPST003196 and PXD053604 (https://repository.jpostdb.org/preview/1696748189668633d9ea532, access key: 5234).

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