# Analysis of serum peptidome profiles of non-metastatic and metastatic feline mammary carcinoma using liquid chromatography-tandem mass spectrometry

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## Abstract

**Background**  Feline mammary carcinoma (FMC) is a common aggressive and highly metastatic cancer affecting female cats. Early detection is essential for preventing local and distant metastasis, thereby improving overall survival rates. While acquiring molecular data before surgery offers significant potential benefits, the current protein biomarkers for monitoring disease progression in non-metastatic FMC (NmFMC) and metastatic FMC (mFMC) are limited. The objective of this study was to investigate the serum peptidome profiles of NmFMC and mFMC using liquid chromatography-tandem mass spectrometry. A cross-sectional study was conducted to compare serum peptidome profiles in 13 NmFMC, 23 mFMC and 18 healthy cats. The liquid chromatography-tandem mass spectrometry analysis was performed on non-trypsinized samples.

**Results**  Out of a total of 8284 expressed proteins observed, several proteins were found to be associated with human breast cancer. In NmFMC, distinctive protein expressions encompassed double-stranded RNA-binding protein Staufen homolog 2 (STAU2), associated with cell proliferation, along with bromodomain adjacent to zinc finger domain 2A (BAZ2A) and gamma-aminobutyric acid type A receptor subunit epsilon (GABRE), identified as potential treatment targets. Paradoxically, positive prognostic markers emerged, such as complement C1q like 3 (C1QL3) and erythrocyte membrane protein band 4.1 (EPB41 or 4.1R). Within the mFMC group, overexpressed proteins associated with poor prognosis were exhibited, including B-cell lymphoma 6 transcription repressor (BCL6), thioredoxin reductase 3 (TXNRD3) and ceruloplasmin (CP). Meanwhile, the presence of POU class 5 homeobox (POU5F1 or OCT4) and laminin subunit alpha 1 (LAMA1), reported as metastatic biomarkers, was noted.

**Conclusion**  The presence of both pro- and anti-proliferative proteins was observed, potentially indicating a distinctive characteristic of NmFMC. Conversely, proteins associated with poor prognosis and metastasis were noted in the mFMC group.

**Keywords**  Feline mammary gland carcinoma, LC–MS/MS, Metastasis, Serum peptidomics

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## Background

Feline mammary tumors rank as the third most frequent tumors, following hematopoietic and skin tumors, constituting approximately 17% of all tumors in female cats [1]. Among these, feline mammary carcinoma (FMC) holds the highest prevalence, contributing to 80% of mammary tumors [2]. The conventional tumor, node and metastasis (TNM)-based staging scheme established by the World Health Organization (WHO) has been developed to assess FMC. Concerning metastasis, this disease is frequently associated with ulceration and demonstrates a propensity for regional or distant metastasis, significantly elevating mortality rates, especially in cases involving lung metastasis [3, 4]. Hence, early diagnosis and the implementation of effective treatment play a pivotal role in preventing both local and distant metastasis, contributing significantly to extended survival times [5]. The standard diagnostic procedure for mammary tumors involves biopsy of affected tissues, followed by a comprehensive histopathological examination. This examination is typically carried out subsequent to mastectomy surgery, serving to confirm the presence of cancer [2]. Additionally, various adjuvant chemotherapy protocols have been employed for FMC treatment involving the use of doxorubicin, either alone or in combination with cyclophosphamide [6, 7]. Acquiring molecular data before surgery could mark a crucial turning point in enhancing our understanding of the disease. A number of tissue molecular markers for FMC, including estrogen receptor (ER), progesterone receptor (PR), feline homologue of HER2 (fHER2), cytokeratin 5/6 (CK5/6) and Ki-67, have been reported to classify FMC subtypes [8]. Regarding serum biomarkers, most markers have been focused on identifying HER2-positive FMC [9–11]. In addition, the identification of therapeutic biomarkers holds paramount importance in facilitating effective communication with pet owners. In human breast cancer, bromodomain adjacent to zinc finger domain 2A (BAZ2A) and gamma-aminobutyric acid type A receptor subunit epsilon (GABRE) have been recognized as potential therapeutic targets [12, 13]. The majority of mammary cancer cases in both cats and humans manifest as malignant glandular epithelial tumors, contrasting with the complex and mixed mammary tumors predominantly observed in dogs [14]. Hence, FMC has been proposed as a potential model for studying human breast cancer when compared to its canine counterpart [15, 16]. Efforts have been undertaken to identify and compare prognostic biomarkers in FMC with their human counterparts, specifically in human triple-negative breast cancer, or considering the effects of the tumor microenvironment [15, 16]. However, with the limited available protein biomarkers for monitoring disease progression in non-metastatic FMC (NmFMC) and metastatic FMC (mFMC), it is difficult to categorize FMCs using the same immunophenotypic and molecular biomarkers established for human breast cancer [17, 18].

The serum peptidome consists of low-molecular-weight peptides that can be actively synthesized or proteolytically cleaved from precursor proteins by endogenous proteases [19]. Serum peptidome profiles serves as potential sources for cancer biomarkers. Serum peptidomics has been used to identify late-stage oral melanoma and late-stage oral squamous cell carcinoma in dogs, as well as sarcomeric gene mutation and hypertrophic cardiomyopathy in cat [20, 21]. Moreover, profiles of tumor-suppressive peptide biomarkers for ovarian and breast cancers were identified in humans [22, 23]. Numerous previous studies have employed proteomics approaches to investigate diseases in cat, including the mucosal proteome in cats with inflammatory bowel disease and alimentary small cell lymphoma, as well as the serum proteome in cats with chronic enteropathies [24, 25]. In addition, potential serum biomarkers were discovered using proteomics in canine mammary tumors and canine lymphoma [26–28].

In a previous study, the serum proteome of feline NmFMC was analyzed compared with healthy controls. However, no comparative omics studies of FMC with and without metastasis have been conducted [29]. In this study, serum samples were also utilized to provide molecular information on NmFMC and mFMC, as serum can be easily obtained in routine clinical practice. The aim of this study was to investigate potential peptidome-based serum biomarker profiles for NmFMC and mFMC using liquid chromatography-tandem mass spectrometry (LC–MS/MS).

## Results

### Sample description data

Among the 36 female cats included in the study, 89% were domestic shorthair (32/36), followed by 8% Persian (3/36) and 3% Khao Manee (1/36). The average age of the cats was 10.6 years. Of these, 64% were neutered (23/36), 28% were intact (10/36) and 8% had an unknown status (3/36). Regarding the metastatic status of FMC, 36% were categorized as NmFMC (13/36), while 64% were classified as mFMC (23/36) (Table 1).

### Serum peptidomics profile results

Both peptides degraded from proteins and endogenous peptides were subject to analysis. However, it was observed that only peptides degraded from proteins exhibited differential expression. Out of a total of 8284 detected proteins, 14 were exclusively expressed in NmFMC, 23 in mFMC and 9 in the controls, as

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**Table 1** Samples description data: breed, age, neuter status, clinical stage of cancer

| Sample ID | Metastasis | Clinical stage | Age (years) | Breed | Neuter status | Metastatic site |
|---|---|---|---|---|---|---|
| 1 | Nm[a] | I | 8 | DSH[c] | + | - |
| 2 | Nm | I | 7 | DSH | + | - |
| 3 | Nm | II | 8 | DSH | - | - |
| 4 | Nm | II | 10 | DSH | N/D[d] | - |
| 5 | Nm | II | 3 | Persia | + | - |
| 6 | Nm | II | 15 | DSH | + | - |
| 7 | Nm | II | 5 | DSH | N/D | - |
| 8 | Nm | II | 8 | DSH | N/D | - |
| 9 | Nm | III | 17 | DSH | - | - |
| 10 | Nm | III | 10 | DSH | - | - |
| 11 | Nm | III | 7 | DSH | + | - |
| 12 | Nm | III | 7 | DSH | + | - |
| 13 | Nm | III | 11 | DSH | + | - |
| 14 | m[b] | III | 12 | DSH | + | Lymph node |
| 15 | m | III | 9 | DSH | - | Lymph node |
| 16 | m | III | 10 | DSH | + | Lymph node |
| 17 | m | III | 13 | DSH | + | Lymph node |
| 18 | m | III | 11 | DSH | - | Lymph node |
| 19 | m | III | 15 | DSH | + | Lymph node |
| 20 | m | III | 15 | DSH | - | Lymph node |
| 21 | m | III | 8 | DSH | + | Lymph node |
| 22 | m | III | 9 | Persia | - | Lymph node |
| 23 | m | III | 16 | DSH | - | Lymph node |
| 24 | m | III | 14 | DSH | + | Lymph node |
| 25 | m | III | 10 | DSH | + | Lymph node |
| 26 | m | III | 10 | DSH | + | Lymph node |
| 27 | m | III | 15 | DSH | + | Lymph node |
| 28 | m | III | 13 | DSH | + | Lymph node |
| 29 | m | III | N/D | DSH | + | Lymph node |
| 30 | m | III | 2 | DSH | - | Lymph node |
| 31 | m | III | 16 | Persia | - | Lymph node |
| 32 | m | III | 17 | DSH | + | Lymph node |
| 33 | m | IV | 5 | Khaomanee | + | Lung |
| 34 | m | IV | 13 | DSH | + | Lung |
| 35 | m | IV | 12 | DSH | + | Lung |
| 36 | m | IV | 10 | DSH | + | Lung |

[a] *Nm* non-metastasis

[b] *m* metastasis

[c] *DSH* domestic shorthair

[d] *N/D* Not determined

illustrated in the Venn diagram (Tables 2, 3 and 4; Fig. 1). Proteins uniquely observed in NmFMC included the double-stranded RNA-binding protein Staufen homolog 2 (STAU2), WW domain binding protein 11 (WBP11), proline and serine-rich coiled-coil 1 (PSRC1), complement C1q like 3 (C1QL3), fibroblast growth factor 14 (FGF14), BAZ2A and GABRE. Proteins solely identified in mFMC included B-cell lymphoma 6 transcription repressor (BCL6), thioredoxin reductase 3 (TXNRD3), ceruloplasmin (CP), baculoviral IAP repeat-containing 6 (BIRC6), POU class 5 homeobox 1 (POU5F1, also known as OCT4), laminin subunit alpha 1 (LAMA1), listerin E3 ubiquitin protein ligase 1 (LTN1), 1,4-alpha-glucan branching enzyme 1 (GBE1), calcium voltage-gated channel subunit alpha1 E (CACNA1E) and pleckstrin homology domain-containing S1 (PLEKHS1).

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**Table 2** Nominated proteins uniquely found in non-metastasis of feline mammary carcinoma based on molecular function by UniProtKB/Swiss-Prot

| Database | Protein names | Peptides | Average protein expression ± SD | Molecular function |
|---|---|---|---|---|
| A0A5F5XG56 | WW domain binding protein 11 (WBP11) | ELTPLQAMMLRMAGQEIPEEGR | 3.35 ± 6.37 | RNA processing |
| M3WM94 | Proline and serine rich coiled-coil 1 | LPVPSAIPRPASRMPLTSR | 2.32 ± 5.67 | Cytoplasm, cytoskeleton |
| A0A2I2V4V0 | Bromodomain adjacent to zinc finger domain 2A (BAZ2A) | EEVAKGK | 1.42 ± 5.13 | DNA binding, metal ion binding |
| M3WMX9 | Cysteine-rich PDZ-binding protein (CRIPT) | FSTCRICK | 1.30 ± 4.69 | microtubule binding, PDZ domain binding, protein-containing complex binding |
| M3W4A9 | Gamma-aminobutyric acid type A receptor subunit epsilon (GABRE) | HPDIHARALMPPR | 1.25 ± 4.49 | GABA-A receptor activity, GABA-gated chloride ion channel activity, inhibitory extracellular ligand-gated ion channel activity, neurotransmitter receptor activity, transmitter-gated ion channel activity involved in regulation of postsynaptic membrane potential |
| A0A5F5XTH3 | Family with sequence similarity 120B (FAM120B) | APGTAGQAKDSTGGIR | 1.23 ± 4.43 | |
| A0A5F5XHP5 | Uncharacterized protein | AVGAN | 1.20 ± 4.32 | |
| A0A5F5Y0W5 | Cell cycle progression 1 (CCPG1) | DQNVKQETDGK | 1.20 ± 4.32 | positive regulation of cell cycle, positive regulation of cell population proliferation, positive regulation of transcription by RNA polymerase II, regulation of Rho guanyl-nucleotide exchange factor activity |
| M3X401 | Olfactory receptor (LOC101088169) | AFSTCASHFLVVSLFYGSVMVMYVSPG-SRSHPGTQK | 1.19 ± 4.30 | G protein-coupled receptor activity, olfactory receptor activity |
| A0A2I2UAL5 | Double-stranded RNA-binding protein Staufen homolog 2 (STAU2) | AQQAVANK | 1.19 ± 4.30 | double-stranded RNA binding |
| A0A337S2I6 | Formin homology 2 domain containing 3 (FHOD3) | FNSGDLGR | 1.18 ± 4.25 | actin filament binding |
| A0A5F5Y181 | Fibroblast growth factor (FGF14) | AAAIASGLIR | 1.15 ± 4.14 | growth factor activity |
| A0A2I2UTW8 | von Willebrand factor A domain-containing protein 9 (INTS14) | PTVVVMDVSLSMTRPVSIEGSEEYQRK | 1.11 ± 3.99 | integrator complex activity |
| A0A337STI2 | Complement C1q like 3 (C1QL3) | FTCSIPGIYFFTYHVLMRGGDGTSM-WADLCK | 1.04 ± 3.76 | identical protein binding |

Furthermore, 42 proteins in NmFMC and mFMC exhibited at least twofold differential expression when compared with each other ($p < 0.01$) (Tables 5 and 6). Proteins significantly expressed in NmFMC compared with mFMC included coagulation factor XIII A chain (F13A1), centromere protein F (CENPF), pyruvate dehydrogenase phosphatase catalytic subunit 2 (PDP2), erythrocyte membrane protein band 4.1 (EPB41 or 4.1R), sorting nexin 10 (SNX10), galactosidase beta 1-like 2 (GLB1L2) and trafficking kinesin protein 2 (TRAK2). On the other hand, proteins highly expressed in mFMC compared with NmFMC included WD repeat domain 1 (WDR1), adenylate cyclase 10 (ADCY10) and activity-dependent neuroprotector homeobox (ADNP) (Supplementary Fig. 1).

In addition, 280 proteins in NmFMC, 616 proteins in mFMC and 170 proteins commonly found in both NmFMC and mFMC were differently expressed compared with the controls ($p < 0.01$). Among these, insulin receptor (INSR), SR-related CTD associated factor 1 (SCAF1) and pyruvate dehydrogenase kinase 1 (PDK1) were differentially expressed in NmFMC compared with controls, whereas ligand-dependent nuclear receptor corepressor (LCOR) was differentially expressed in mFMC compared with controls. In addition, endoglin (ENG), checkpoint kinase 1 (CHEK1), epidermal growth factor receptor (EGFR) and DEAH-box helicase 32 (putative) (DHX32) were significantly expressed in both NmFMC and mFMC compared with controls (Supplementary Tables 1 − 3). Moreover, regarding the relationship with chemotherapy drugs, either doxorubicin or cyclophosphamide, associations were observed for some proteins found exclusively in the NmFMC group, including GABRE and BAZ2A, and in the mFMC group, including BCL6, TXNRD3, BIRC6 and GBE1 (Fig. 2) [30].

**Table 3** Nominated proteins uniquely found in metastasis of feline mammary carcinoma based on molecular function by UniProtKB/Swiss-Prot

| Database | Protein names | Peptides | Average protein expression ±SD | Molecular function |
|---|---|---|---|---|
| M3W9Z2 | Laminin subunit alpha 1 (LAMA1) | LAGALDSGLGSVR | 3.14 ± 7.04 | extracellular matrix structural constituent, glycosphingolipid binding, protein C-terminus binding, signaling receptor binding |
| A0A5F5XM44 | Voltage-dependent R-type calcium channel subunit alpha (CACNA1E) | IHYTEMYEMLTLMSPPLGLGK | 3.02 ± 5.91 | calcium ion binding, voltage-gated calcium channel activity |
| A0A2I2UP49 | FRAS1 related extracellular matrix 2 (FREM2) | GASTLRTLATGHLGFMITSK | 2.66 ± 5.94 | basement membrane, integral component of membrane, cell adhesion, cell communication, embryonic digit morphogenesis development, morphogenesis of an epithelium |
| A0A5F5XYI6 | Cyclic AMP-responsive element-binding protein 3-like protein 2 (CREB3L2) | EYMDSLEKK | 2.52 ± 5.63 | cAMP response element binding, DNA-binding transcription activator activity, RNA polymerase II-specific |
| A0A337SWR8 | Folliculin interacting protein 1 (FNIP1) | CSSDANMLGEMMFGSVAMSYK | 2.41 ± 6.35 | lysosomal membrane |
| A0A2I2UKP7 | Interleukin 31 (IL31) | IILELRPMSKGLLQDYVSK | 2.15 ± 5.67 | cytokine activity, cytokine receptor binding |
| D3U64 | POU domain protein (POU5F1) | FEALQLSFK | 2.03 ± 5.38 | chromatin DNA binding, cytokine binding, DNA-binding transcription activator activity, RNA polymerase II-specific, DNA-binding transcription factor activity, RNA polymerase II-specific, RNA polymerase II cis-regulatory region sequence-specific DNA binding, RNA polymerase II intronic transcription regulatory region sequence-specific DNA binding, transcription factor binding, ubiquitin protein ligase binding |
| A0A5F5Y685 | Retinol dehydrogenase 11 (RDH11) | LTLSGPVLACRLSLVIPQACR | 2.03 ± 5.37 | aldehyde dehydrogenase (NADP+) activity |
| M3VVR7 | Thioredoxin-disulfide reductase (TXNRD3) | LLGLIEGNR | 1.80 ± 4.77 | electron transfer activity, flavin adenine dinucleotide binding, protein disulfide oxidoreductase activity, thioredoxin-disulfide reductase activity |
| M3WID6 | Glutamate receptor (GRIN2B) | TAKNMANLSGVNGSPQSALDFIR | 1.78 ± 5.9 | glutamate-gated calcium ion channel activity, glycine binding, ligand-gated ion channel activity, NMDA glutamate receptor activity, signaling receptor activity |
| A0A337SR21 | 1,4-alpha-glucan branching enzyme (GBE1) | GIQLHKMIR | 1.44 ± 4.83 | 1,4-alpha-glucan branching enzyme activity, cation binding, hydrolase activity, hydrolyzing O-glycosyl compounds |
| M3WY11 | Pleckstrin homology domain containing S1 (PLEKHS1) | GQHQRTGESHAR | 1.41 ± 4.74 | actin binding, G protein-coupled glutamate receptor binding, synaptic receptor adaptor activity |
| M3WXJ0 | Homer scaffold protein 2 (HOMER2) | TDIEESK | 1.38 ± 4.57 | cysteine-type endopeptidase inhibitor activity, ubiquitin conjugating enzyme activity |
| M3W539 | Baculoviral IAP repeat containing 6 (BIRC6) | AGKIFSQMNNIMSK | 1.30 ± 4.29 | acetylcholine receptor binding |
| A0A5F5XXV1 | UBX domain protein 2A (UBXN2A) | FNISHR | 1.28 ± 4.24 | nucleic acid binding, protein-lysine N-methyltransferase activity |
| A0A5F5XK47 | Uncharacterized protein | ELPSPQSHPDTALLGQSPGAR | 1.25 ± 4.29 | ribosomal large subunit binding, ubiquitin protein ligase activity, zinc ion binding |
| A0A337S289 | EEF1A lysine methyltransferase 1 (EC 2.1.1.-) (EEF1AKMT1 N6AMT2) | EDFSIYIFEYDK | 0.80 ± 3.84 | |
| A0A2I2UHD6 | E3 ubiquitin-protein ligase listerin (LTN1) | VKDAAEGGSGSEEAGGR | 0.71 ± 3.40 | |

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**Table 3** (continued)

| Database | Protein names | Peptides | Average protein expression ± SD | Molecular function |
|---|---|---|---|---|
| M3VZY5 | Ceruloplasmin (CP) | VESIQCFQNTEAGSPTIMLLSLK | 0.70 ± 3.37 | ATP binding, hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides, nucleic acid binding, nucleosome-dependent ATPase activity, zinc ion binding |
| A0A291NGU8 | IgH variable region (Fragment) | YWGQGALVTVSSASPK | 0.70 ± 3.37 | |
| A0A337SN80 | BCL6 transcription repressor (BCL6) | DEFLNSRMLMPQDIMAYR | 0.68 ± 3.26 | DNA-binding transcription repressor activity, RNA polymerase II-specific, RNA polymerase II cis-regulatory region sequence-specific DNA binding |
| A0A337SQW1 | Family with sequence similarity 189 member B (FAM189B) | MPSPSDSSR | 0.65 ± 3.12 | WW domain binding |
| M3W513 | Polypeptide N-acetylgalactosaminyl-transferase (GALNT8) | IFLGVIGSLDGGMLVYGGENVELSLR | 0.54 ± 2.6 | carbohydrate binding, polypeptide N-acetylgalactosaminyltransferase activity |

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**Table 4** Nominated proteins uniquely found in normal controls based on molecular function by UniProtKB/Swiss-Prot

| Database | Protein names | Peptides | Average protein expression ± SD | Molecular function |
|---|---|---|---|---|
| A0A337RXV1 | Zinc finger protein 26 (ZNF84) | THTGEKPHGCIQCGK | 2.60 ± 5.78 | DNA-binding transcription factor activity, RNA polymerase II-specific, metal ion binding, RNA polymerase II cis-regulatory region sequence-specific DNA binding |
| A0A337SAK8 | Olfactory receptor (LOC101089999) | IPSAEGKQK | 2.55 ± 5.70 | G protein-coupled receptor activity, olfactory receptor activity |
| A0A5F5Y4V9 | Transcription elongation factor A2 (TCEA2) | LLDASDAK | 1.82 ± 5.14 | nucleic acid binding, zinc ion binding |
| M3VZ25 | Solute carrier family 27 member 3 (SLC27A3) | GHKVR | 1.80 ± 5.09 | long-chain fatty acid transporter activity, nucleotide binding |
| A0A337SXC3 | Rap guanine nucleotide exchange factor 4 (RAPGEF4) | LGSGEGLIIVKMSSGGEK | 1.80 ± 5.09 | guanyl-nucleotide exchange factor activity |
| A0A5F5Y2R7 | Scavenger receptor cysteine-rich domain-containing protein SCART1-like (SCART1) | GTEPTIRNCR | 1.69 ± 4.76 | scavenger receptor activity |
| M3VV17 | Tubulin alpha chain (TUBAL3) | VGINSQPPTVTPGGDLAK | 0.91 ± 3.74 | GTPase activity, GTP binding, structural constituent of cytoskeleton |
| A0A5F5Y6C1 | Histone methyltransferase SMYD2 (SMYD2) | HYPLYSLNVASMWLKLGR | 0.89 ± 3.66 | histone-lysine N-methyltransferase activity |
| A0A2I2U173 | Serine/threonine-protein phosphatase 4 regulatory subunit 1-like (LOC101092674) | SNFPGVLADYLTPIVVRYLTDPNNQVR | 0.78 ± 3.23 | |

However, no associations with chemotherapy drugs were exhibited in nine proteins uniquely expressed in a control group.

## Discussion

The present study sheds light on the differential protein expression observed in NmFMC and mFMC at the peptidome level. Notably, both pro- (e.g., STAU2, BAZ2A and GABRE) and anti-proliferative proteins (e.g., C1QL3 and EPB41) were identified in NmFMC, while proteins associated with poor prognosis (e.g., BCL6, TXNRD3 and CP) and metastasis (e.g., POU5F1 and LAMA1) were prominent in the mFMC group. The upregulation of STAU2, observed in T and B cells in human breast cancer patients may promote tumor growth through the RNA transport process of various inflammatory cytokine molecules suggesting its potential as a novel diagnostic biomarker for human breast cancer screening [31]. Similarly, WBP11 has been linked to the activation of the fibroblast growth factor receptor (FGFR)-Wingless/Integrated (Wnt)-β-catenin pathway in human gastric cancer [32]. Additionally, PSRC1 implicated in cancer cell proliferation and was downregulated by the tumor suppressor p53 in human hepatocellular carcinoma [33]. Moreover, several candidates identified in the NmFMC group, including BAZ2A, GABRE, INSR, SCAF1, PDK1 and PDP2, have been proposed as potential therapeutic targets. BAZ2A and GABRE, uniquely expressed in

NmFMC, exhibited relationship with chemotherapy drugs (Fig. 2A), suggesting their significance in treatment response [11, 12]. In human triple-negative breast cancer, inhibition of BAZ2A has been demonstrated to induce apoptosis, while BAZ2A has also been implicated in regulating hypermethylation, contributing to advanced tumor stages and recurrence in prostate cancer [34]. GABRE activation has the potential to sensitize cancer cells to radiation, chemotherapeutic agents and immune checkpoint inhibitors [12].

Remarkably, proteins prominently expressed in NmFMC compared with other groups, such as F13A1, CENPF, INSR, SCAF1 and PDK1, have been implicated in human breast cancer, further supporting the potential use of FMC as a model for studying human breast cancer. For instance, F13A1 was prominently expressed in human estrogen receptor-negative breast cancer, while targeting CENPF resulted in tumor growth inhibition in human breast cancer [35, 36]. Differential splicing of INSR occurs more commonly in human breast cancer than in non-tumor breast tissues, and *SCAF1* has been proposed as a cancer prognostic biomarker [37, 38]. Furthermore, PDK1 plays a role in the growth and survival of human breast cancer cells [39, 40]. Paradoxically, a group of proteins, including C1QL3, EPB41, SNX10, FGF14, GLB1L2 and TRAK2, have been reported as good prognostic markers or tumor suppressors in the NmFMC group. Notably, complement C1q was previously shown
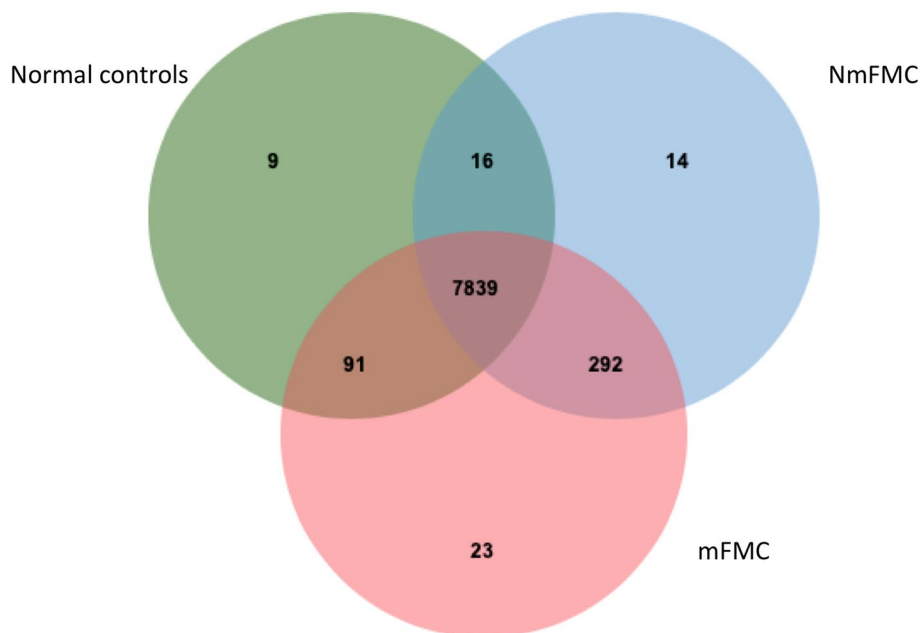
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**Fig. 1** Venn diagram of proteins differentially expressed in NmFMC and mFMC and normal controls

to be associated with extended disease-free survival in basal-like breast cancer and improved overall survival in HER2-positive breast cancer in humans [41]. Elevated levels of EPB41 expression have been correlated with prolong survival in human breast cancer patients [42]. Moreover, FGF14 and SNX10 have demonstrated tumor suppressive properties in colorectal cancer [43, 44]. Conversely, decreased expression of GLB1L2 and TRAK2 has been documented in prostate cancer and osteosarcoma, respectively [23, 45]. Hence, these proteins have the potential to serve as good prognostic biomarkers for FMC, especially NmFMC. The coexistence of both pro- and anti-proliferative proteins, acting as tumor promoters and suppressors, respectively, presents a distinctive characteristic of NmFMC. A comprehensive examination of their protein expression, with a particular focus on its correlation with survival outcomes, necessitates further investigation in a larger patient cohort.

In the mFMC group, there was marked protein expression of BCL6, TXNRD3, CP and BIRC6, which have been linked with poor prognosis in human breast cancer [46–50]. BCL6, identified as a master transcription factor for regulating follicular helper cell proliferation, has been demonstrated to inhibit apoptosis, thereby promoting tumor invasion, migration and growth. BCL6 expression also promotes tumor angiogenesis and is associated with human breast cancer progression and poor prognosis [47]. Moreover, BCL6 inhibitors have shown potent effects against these tumor types [47, 51]. TXNRD3 is

involved in oxidative stress and has been associated with poor prognosis in various cancers [48]. CP is a plasma protein for copper binding and is associated with various immune pathways and inflammatory responses related to the tumor microenvironment. In invasive human breast cancer, low levels of this protein were correlated with low tumor immune cell infiltration status and better prognosis [49]. BIRC6 has demonstrated overexpression in triple-negative human breast cancer cells and tissues, positively correlated with epidermal growth factor receptor (EGFR), and associated with poor patient survival time [50].

Proteins significantly upregulated in mFMC compared to NmFMC included WDR1, which is associated with cell motility. Overexpression of this protein correlated with shorter distant metastasis-free survival, especially in basal-like tumors of human breast cancer [52]. Notably, unique metastatic biomarkers found in mFMC, such as POU5F1 (OCT4) and LAMA1, have been identified in human breast cancer [53, 54]. POU5F1 has been reported as a biomarker in both undifferentiated cells and several cancer cells, suggesting shared characteristics between these cell types. A previous study identified POU5F1 as a potential candidate for predicting metastasis in human breast cancer [53]. LAMA1 has been shown to mediate cell attachment, migration and tissue organization. In metastatic human breast tumors, overexpression of fibronectin and LAMA1 proteins were exhibited in mice, promoting the degradation processes of extracellular

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**Table 5** Overexpressed proteins with at least twofold differences of non-metastasis (NmFMC) compared with metastasis of feline mammary carcinoma (mFMC) and controls

| Database | Protein names | Peptides | NmFMC | | mFMC | | Controls | | Molecular function |
|---|---|---|---|---|---|---|---|---|---|
| | | | Median | IQR[d] | Median | IQR | Median | IQR | |
| A0A5F5XY49 | Family with sequence similarity 246 member B (FAM246B) | SVYGASEALR | 17.53 | 2.33 | 15.50 | 2.73 | 17.71 | 3.15 | |
| A0A5F5XNK1 | Katanin p60 ATPase-containing subunit a-like 2 (KATNAL2) | LLKPLSAFIGMNSEMR | 16.16 | 0.92 | 14.12 | 15.9 | 13.78 | 16.40 | ATP binding, isomerase activity, microtubule binding, microtubule-severing ATPase activity |
| M3W5A2 | Trafficking kinesin protein 2 (TRAK2) | TPNAQENGR | 17.37 | 1.58 | 15.78 | 2.89 | 17.48 | 2.70 | GABA receptor binding, myosin binding, signaling receptor binding |
| M3W5L0 | Pyruvate dehyrogenase phosphatase catalytic subunit 2 (PDP2) | EALMYSFQR | 15.36 | 3.01 | 13.42 | 2.19 | 14.91 | 4.25 | pyruvate dehydrogenase phosphatase activity, metal ion binding, protein serine/threonine phosphatase activity |
| A0A5F5XU84 | Centromere protein F (CENPF) | AATQMLEELK | 16.57 | 4.46 | 0.00 | 15.36 | 7.92 | 18.10 | dynein complex binding, microtubule binding, protein homodimerization activity, transcription factor binding |
| M3W4Q9 | Intraflagellar transport 22 (IFT22) | SSASGRAPADR | 16.85 | 1.80 | 14.89 | 15.71 | 14.56 | 15.72 | |
| M3WSU5 | Methyl-CPG binding domain protein 5 (MBD5) | MFLSVSLQK | 15.03 | 0.81 | 15.12 | 0.79 | 15.34 | 0.84 | |
| M3VYG2 | Cub and sushi multiple domains 2 (CSMD2) | ARMCDAHLR | 15.07 | 0.75 | 13.85 | 14.93 | 15.45 | 2.16 | membrane integral component |
| A0A337S9A6 | Insulin receptor substrate 2 (IRS2) | AGAPK | 15.00 | 1.03 | 14.68 | 0.78 | 14.74 | 0.91 | insulin receptor binding, phosphatidylinositol 3-kinase binding, protein kinase binding |
| A0A5F5XXD4 | Zinc finger protein 324 (ZNF324) | MATAALTDR | 17.55 | 1.33 | 0.00 | 15.36 | 17.58 | 14.34 | metal ion binding |
| A0A337S8X8 | Uncharacterized protein | GPGMDVSGPK | 14.88 | 1.05 | 13.93 | 1.37 | 14.66 | 1.38 | |
| A0A5F5XL47 | Rwd domain-containing protein 3 (RWDD3) | IILILLQGDR | 16.94 | 2.88 | 0.00 | 14.94 | 0.00 | 19.14 | positive regulation of protein SUMOylation |
| A0A5F5XCD6 | Traf3 interacting protein 1 (TRAF3IP1) | AELAELEQLIRDQQDK | 14.66 | 1.12 | 13.98 | 2.33 | 14.38 | 15.07 | microtubule binding |
| A0A337S420 | Band 4.1 (erythrocyte membrane protein band 4.1) (protein 4.1) (EPB41) | LTSTDTIPK | 15.49 | 1.42 | 13.78 | 2.04 | 15.05 | 2.17 | actin binding, calmodulin binding, structural molecule activity |
| A0A5F5Y758 | Translocase of inner mitochondrial membrane domain containing 1 (TIMMDC1) | VFAAGAVAADSENQK | 15.42 | 16.84 | 0.00 | 13.43 | 14.22 | 16.74 | |
| A0A337SRT0 | Neuronal-specific septin-3 (SEPTIN3) | SPGPAGPGSVGQK | 14.87 | 18.78 | 0.00 | 0.00 | 6.83 | 16.47 | GTP binding |
| A0A5F5XNQ6 | Pparg related coactivator 1 (PPRC1) | WGQSPPPQQR | 14.71 | 19.67 | 0.00 | 0.00 | 0.00 | 16.96 | RNA binding, transcription coregulator activity, transcription factor binding |
| M3WL91 | Collagen type v alpha 2 chain (COL5A2) | GDPGSHGRVGDR | 14.12 | 14.54 | 0.00 | 10.24 | 0.00 | 13.76 | extracellular matrix structural constituent, metal ion binding |
| M3WJD9 | Beta-galactosidase-1-like protein 3 (GLB1L2) | NAEDVEDTVSK | 13.38 | 17.65 | 0.00 | 0.00 | 0.00 | 14.57 | beta-galactosidase activity |
| A0A5F5XWE8 | Coagulation factor XIII A chain (F13A1) | EVGGDGIR | 15.69 | 16.35 | 0.00 | 0.00 | 0.00 | 0.00 | metal ion binding, protein-glutamine gamma-glutamyl-transferase activity |
| A0A337SJJ4 | Sorting nexin 10 (SNX10) | EEFVSVWVR | 0.00 | 14.47 | 0.00 | 0.00 | 0.00 | 0.00 | 1-phosphatidylinositol binding, ATPase binding |

denote a significant difference in the same row at $p < 0.01$

[d] *IQR* interquartile range

**Table 6** Overexpressed proteins with at least twofold differences of metastasis (mFMC) compared with non-metastasis of feline mammary carcinoma (NmFMC) and controls

| Database | Protein names | Peptides | mFMC | | NmFMC | | Controls | |
|---|---|---|---|---|---|---|---|---|
| | | | Median | IQR[d] | Median | IQR | Median | IQR |
| A0A2I2UY44 | Phosphofurin acidic cluster sorting protein 1 | DLNSVVIAVK | 17.35 | 2.31 | 15.25 | 2.19 | 15.91 | 3.52 |
| M3WWL3 | Cytochrome P450 2F5 (CYP2F1) | DLIARSVR | 15.16 | 1.25 | 14.26 | 1.82 | 0.00 | 15.53 |
| A0A5F5XI19 | ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (ST3GAL6) | GGGSSLMEGDAK | 16.92 | 2.88 | 13.93 | 15.48 | 14.20 | 16.18 |
| A0A5F5Y4G3 | L-2-hydroxyglutarate dehydrogenase (L2HGDH) | AQALDRDGNLIEDFVFDGGVGDIGNR | 15.84 | 1.64 | 13.89 | 5.62 | 15.27 | 2.28 |
| M3W363 | ADAM metallopeptidase with thrombospondin type 1 motif 3 (ADAMTS3) | GTFTRTPR | 14.38 | 1.17 | 13.37 | 13.74 | 14.56 | 2.41 |
| A0A337S4J8 | Secretoglobin family 3A member 1 (SCGB3A1) | SLLGSLMYLG | 14.60 | 1.36 | 13.75 | 14.06 | 14.64 | 11.77 |
| M3WEW2 | WD repeat domain 1 (WDR1) | YTNLTLR | 14.94 | 0.81 | 13.94 | 14.78 | 14.58 | 1.30 |
| A0A2I2V2S6 | Follistatin like 5 (FSTL5) | GNNCK | 14.98 | 0.74 | 14.25 | 14.31 | 13.98 | 15.04 |
| M3XE38 | Activity dependent neuroprotector homeobox (ADNP) | DCEKYKPGVLLGFNMK | 13.46 | 1.13 | 12.25 | 1.50 | 13.67 | 1.79 |
| M3WFB1 | Membrane bound transcription factor peptidase, site 1 (MBTPS1 SLC38A8) | RVLWDQYHNLR | 13.98 | 1.39 | 10.17 | 12.63 | 0.00 | 0.00 |
| M3WLU1 | Adenylate cyclase 10 (ADCY10) | ISFHQNFYTIQIFMATVLGLNTCKHYK | 13.09 | 1.52 | 11.30 | 12.29 | 12.23 | 13.23 |
| A0A2I2UCH4 | Transmembrane protein 255B (TMEM255B) | MQPPVPGPLALLDNTEGFARR | 13.94 | 4.41 | 0.00 | 12.00 | 0.00 | 9.27 |
| A0A337SDK0 | Rho GTPase activating protein 21 (ARHGAP21) | GNEAYSGNAR | 14.06 | 1.83 | 0.00 | 14.43 | 11.56 | 14.65 |
| M3WW40 | Pre-mRNA processing factor 39 (PRPF39) | RHGNMEEAEHLLQDAIK | 14.19 | 9.17 | 0.00 | 0.00 | 14.94 | 13.49 |
| M3WS25 | Caspase-14-like (LOC101084312) | DGERVSLEDIFEMFNNK | 14.03 | 2.93 | 11.92 | 13.01 | 12.02 | 14.87 |
| M3WU27 | Coiled-coil domain containing 73 (CCDC73) | EKEIEGLK | 14.20 | 1.71 | 0.00 | 12.63 | 14.26 | 11.64 |
| A0A337SU95 | Testis associated actin remodelling kinase 2 (TESK2) | VREIPPFR | 14.15 | 10.69 | 0.00 | 0.00 | 0.00 | 14.40 |
| M3W938 | Ganglioside induced differentiation associated protein 1 like 1 (GDAP1L1) | RHLANATTDLMK | 12.88 | 1.84 | 0.00 | 12.25 | 10.31 | 13.69 |
| M3W3G6 | [Heparan sulfate]-glucosamine N-sulfotransferase (EC 2.8.2.8) (NDST3) | EGTRMNTNDVK | 12.55 | 2.52 | 0.00 | 11.36 | 0.00 | 7.77 |
| M3WJL7 | Methyltransferase like 3 (METTL3) | LSAMMGAVAEK | 13.09 | 9.51 | 0.00 | 0.00 | 0.00 | 8.86 |
| M3WUK1 | Uroplakin 2 (UPK2) | KMESIGLGMAR | 15.46 | 16.73 | 0.00 | 0.00 | 0.00 | 17.60 |

denote a significant difference in the same row at $p < 0.01$

[d] *IQR* interquartile range

matrix proteins in cancer metastasis [54]. Moreover, several proteins prominently observed in mFMC in this study have been reported as potential poor prognostic markers in various other cancers. These proteins include LTN1 (ovarian cancer), GBE1, CACNA1E and ADCY10 (lung cancers), as well as PLEKHS1 and ADNP (bladder cancer) [55–59]. Additionally, another group of proteins notably found in both NmFMC and mFMC compared to the controls ($p < 0.01$) consisted of ENG, CHEK1, EGFR and DHX32. All of these proteins have been associated with poor or unfavorable prognosis in human breast cancer [60–63]. Inhibition of ENG has been shown to prevent tumor angiogenesis and metastatic spread in human breast cancer [60]. High expression of CHEK1 in

(See figure on next page.)

**Fig. 2** Involvement of serum proteins in FMC and chemotherapy drugs, doxorubicin and cyclophosphamide, in networks of protein – chemotherapy drug interactions. **A** Serum proteins in NmFMC include bromodomain adjacent to zinc finger domain 2A (BAZ2A) and gamma-aminobutyric acid type A receptor subunit epsilon (GABRE). **B** Serum proteins in mFMC include POU domain protein (POU5F1), cyclic AMP-responsive element-binding protein 3-like protein 2 (CREB3L2), BCL6 transcription repressor (BCL6), baculoviral IAP repeat containing 6 (BIRC6), FRAS1 related extracellular matrix 2 (FREM2), 1,4-alpha-glucan branching enzyme (GBE1), glutamate receptor (GRIN2B), thioredoxin-disulfide reductase (TXNRD3), ceruloplasmin (CP) and retinol dehydrogenase 11 (RDH11)

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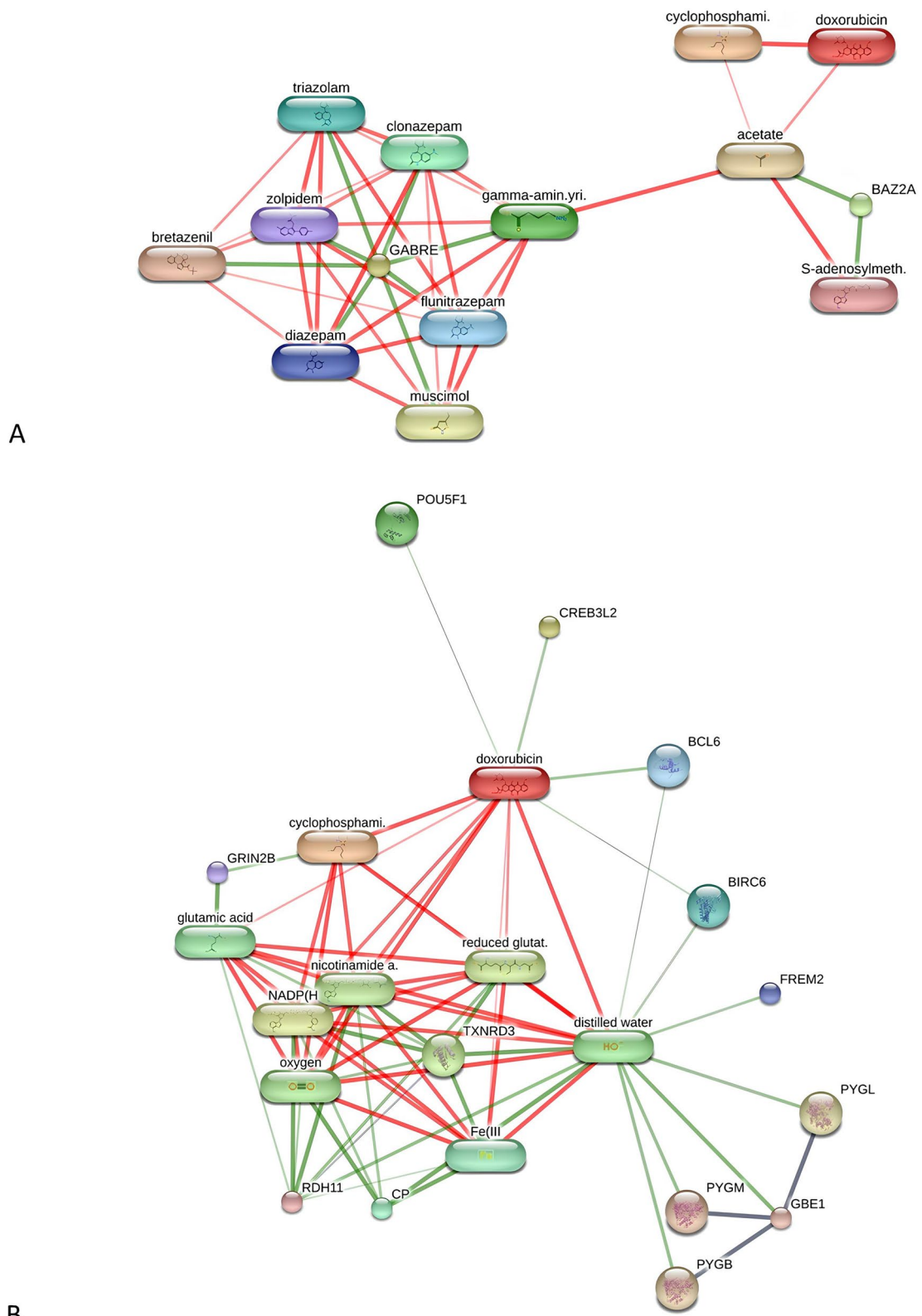


A



B

**Fig. 2**  (See legend on previous page.)

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Nigerian human breast cancer patients is associated with an aggressive phenotype and poor prognosis [61]. EGFR has been linked to the pathogenesis and progression of human breast cancer [62–64]. DHX32 expression has been associated with a poor prognosis in human breast cancer patients [63]. Several proteins found in the present study, including EGFR, BIRC6 and FGF, are associated with EGF. The functions of these proteins and their association with novel FMC diagnostic and/or prognostic biomarkers should be further investigated. The limitations of the present study include a restricted population size, the absence of tissue proteomics profiles and a lack of long-term follow-up data due to infrequent return visits by most cat patients after surgery. Further research involving a larger population and a comparison with tissue proteomics profiles is necessary to investigate the precise roles of these candidates.

## Conclusion

Serum peptidomics revealed potential candidates that were either uniquely or highly expressed in NmFMC and mFMC. In NmFMC, diagnostic candidates with paradoxical characteristics were observed, displaying either the promotion or suppression of cell proliferation, highlighting the distinctive nature of this type of cancer. Meanwhile, potential poor prognostic and metastatic candidates were identified in mFMC. The relationship of proteins in NmFMC or mFMC with chemotherapy drugs was observed. The discovery of similar protein candidates in both FMC and human breast cancer supports the potential utility of FMC as a model for studying mechanisms and identifying therapeutic targets in human breast cancer.

## Materials and methods
### Animals

A cross-sectional study was conducted involving 13 cats diagnosed with spontaneous NmFMC, 23 with spontaneous mFMC and 18 healthy cats. Initially, patients were staged according to the TNM system: stage I (tumor diameter < 2 cm), stage II (tumor diameter 2 to 3 cm), stage III (tumor diameter < 3 cm with lymph node metastasis or tumor diameter > 3 cm) or stage IV (any tumor size with lymph node or distant metastasis). Staging was confirmed by histopathology, indicating the presence of FMC [5]. The patients were categorized into 13 samples with NmFMC, characterized by the absence of lymph node or distant metastases, and 23 samples with mFMC, demonstrating lymph node and/or distant metastases (Table 1). Thoracic radiographs, including ventrodorsal and lateral views, were examined to identify distant metastases. Whole blood samples from a control group were collected from 18 healthy cats visiting the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, with no history or clinical signs of mammary disease. The study was conducted following the ethical guidelines required by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand (approval number 1831091) and written informed consents were obtained from all cat owners.

### Sample collection and preparation

Whole blood samples were collected once from the cephalic or saphenous veins of both patients before surgery and from a control group. After collection, samples were centrifuged at $3000 \times g$ for 15 min at 4 ℃ to obtain serum. The serum was then mixed with Halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 ℃ until analysis.

### LC–MS/MS analysis and data processing

Total protein concentrations were assessed using the colorimetric Pierce Modified Lowry's assay (Thermo Fisher Scientific, Waltham, MA, USA), based on the reduction of Folin-Ciocalteu reagent by Tyr and Trp residues in proteins under alkaline conditions. Protein samples at 0.1 µg/µL in 0.1% formic acid were processed using Nanosep Centrifugal Devices with a 10 K Omega membrane (Pall Corporation, Port Washington, NY, USA) to remove proteins larger than 10 kDa. Peptide separation was performed using A 75 µm diameter × 5 cm length Acclaim PepMap nanocolumn (Thermo Fisher Scientific). The nanoLC system was connected to electrospray ionization MS in positive ion mode and quadrupole ion-trap MS (Bruker Daltonics, Billerica, MA, USA). Peptides were eluted with a 4–70% linear gradient of eluent B (80% acetonitrile in water containing 0.1% formic acid) at a flow rate of 0.3 µL/min for 20 min. Regeneration and equilibration were carried out with 90% and 4% eluent B, respectively, for 40 min per run. A scan range of 400–1500 m/z, 3 averages, and up to 5 precursor ions selected from the MS scan at 200–2800 m/z were used for peptide fragment mass spectra analysis in data-dependent AutoMS mode. The LC–MS/MS results were converted into an mzXML file using CompassXport software (Bruker Daltonics). Protein quantification was performed based on peptide intensity using DeCyder MS Differential Analysis software (GE Healthcare, Chicago, IL, USA). PepDetect in MS mode facilitated automated peptide detection, charge state assignments, and assessment of peptide ion signal intensities. Proteins were identified based on one or more peptides with a MASCOT score corresponding to $p < 0.05$ (Matrix Science, Boston, MA, USA) and were annotated using the NCBI *Felis catus*

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database. The false discovery rate (FDR) was analyzed using Metaboanalyst 5.0 software, and low confidence identifications were removed [65]. Protein sequences and molecular functions were annotated using Uni-ProtKB/Swiss-Prot entries (http://www.uniprot.org/). The relationship between sample groups was visualized using a jVenn diagram [66]. The association between candidate proteins and chemotherapy drugs was analyzed using Stitch version 5.0 [67]. The hierarchical abundance of nominated proteins in each group was represented using Morpheus heatmap (https://software.broadinstitute.org/morpheus).

### Statistical analysis

Differential protein expression in controls, NmFMC and mFMC was analyzed using the R package. Normality testing was conducted using the Shapiro–Wilk test, and statistical significance was determined using the Mann–Whitney U test in R, with a significant level set at $p < 0.05$.

### Abbreviations

| | |
|---|---|
| ADCY10 | Adenylate cyclase 10 |
| ADNP | Activity-dependent neuroprotector homeobox |
| BAZ2A | Bromodomain adjacent to zinc finger domain 2A |
| BCL6 | B-cell lymphoma 6 transcription repressor |
| BIRC6 | Baculoviral IAP repeat containing 6 |
| CACNA1E | Calcium voltage-gated channel subunit alpha1 E |
| CENPF | Centromere protein F |
| CHEK1 | Checkpoint kinase 1 |
| CP | Ceruloplasmin |
| CU-ACUC | Chulalongkorn University Animal Care and Use Committee |
| C1QL3 | Complement C1q like 3 |
| DHX32 | DEAH-box helicase 32 |
| EGFR | Epidermal growth factor receptor |
| ENG | Endoglin |
| EPB41 | Erythrocyte membrane protein band 4.1 |
| FGF14 | Fibroblast growth factor 14 |
| FMC | Feline mammary carcinoma |
| GABRE | Gamma-aminobutyric acid type A receptor subunit epsilon |
| GBE1 | 1,4-Alpha-glucan branching enzyme 1 |
| GLB1L2 | Galactosidase beta 1-like 2 |
| INSR | Insulin receptor |
| LAMA1 | Laminin subunit alpha 1 |
| LC | Liquid chromatography |
| LCOR | Ligand-dependent nuclear receptor corepressor |
| LTN1 | Listerin E3 ubiquitin protein ligase 1 |
| MS | Mass spectrometry |
| mFMC | Metastatic feline mammary carcinoms |
| NmFMC | Non-metastatic feline mammary carcinoma |
| PDK1 | Pyruvate dehydrogenase kinase 1 |
| PDP2 | Pyruvate dehydrogenase phosphatase catalytic subunit 2 |
| PLEKHS1 | Pleckstrin homology domain containing S1 |
| POU5F1 | POU class 5 homeobox 1 |
| PSRC1 | Proline and serine rich coiled-coil 1 |
| SCAF1 | SR-related CTD associated factor 1 |
| SNX10 | Sorting nexin 10 |
| STAU2 | Double-stranded RNA-binding protein Staufen homolog 2 |
| TNM | Tumor, node and metastasis |
| TRAK2 | Trafficking kinesin protein 2 |
| TXNRD3 | Thioredoxin reductase 3 |
| WBP11 | WW domain binding protein 11 |
| WDR1 | WD repeat domain 1 |
| WHO | World Health Organization |

## Supplementary Information

### Acknowledgements

### Authors' contributions
GS and SR designed the study. WP, TT and AR collected samples. WP, NP and WB performed the experiments and analyses. GS, WP and SR analyzed data. GS and WP drafted the manuscript. GS and AR finalized the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials
The datasets generated and/or analysed during the current study are available in the ProteomeXchange repository, PXD035906.

## Declarations

### Ethics approval and consent to participate
All experimental protocols were approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Faculty of Veterinary Science, Chulalongkorn University (Approval number 1831091). All procedures were performed in accordance with the relevant guidelines and regulations. Written informed consents were obtained from all dog owners.

### Consent for publication
Not applicable.

### Competing interests
The authors declare no competing interests.

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