IDENTIFICATION OF POTENTIAL PLASMA PROTEIN BIOMARKERS FOR FELINE PANCREATIC CARCINOMA BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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By

RINA NABETA

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Head of the Department of Veterinary Pathology
52 Campus Drive
University of Saskatchewan
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OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

Abstract

In both humans and cats, pancreatic carcinoma is an aggressive cancer with a grave prognosis. Proteomics techniques have successfully identified several blood-based biomarkers of human pancreatic neoplasia. Thus, this study aims to investigate whether similar biomarkers can be identified in the plasma of cats with pancreatic carcinoma (FePAC) by using liquid chromatography tandem mass spectrometry (LC-MS/MS). To facilitate evaluation of the low abundance plasma proteome, a human-based immunodepletion device (MARS-2) was first validated for use with feline plasma. Marked reduction and/or complete removal of albumin and immunoglobulins was confirmed by analysis of electrophoretograms and mass spectral data. Subsequently, plasma collected from 9 cats with p, 10 cats with symptomatic pancreatitis, and 10 healthy control cats was immunodepleted and subjected to LC-MS/MS. Thirty-seven plasma proteins were found to be differentially expressed ($p<0.05$ in one-way ANOVA, Fold-change>2 in fold change analysis). Among these proteins, ETS variant transcription factor 4 ($p<0.05$) was overexpressed, while gelsolin ($p<0.01$), tryptophan 2,3-dioxygenase ($p<0.05$), serpin family F member 1 ($p<0.01$), apolipoprotein A-IV ($p<0.01$), and phosphatidylinositol-glycan-specific phospholipase D ($p<0.05$) were down-regulated in cats with FePAC. Further studies on these potential biomarkers are needed to investigate their diagnostic value.

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1. Introduction

Feline pancreatic carcinoma (FePAC) is one of the most aggressive cancers in cats, with a reported median survival time of 7 days and a 1-year survival rate of 0 % without treatment.[1,2] In recent studies, surgical intervention alone or surgical resection coupled with chemotherapy in cats with localized FePAC improved the outcome drastically, reaching over 1 year of post-surgical survival time in successful cases.[2,3,4] Considering that FePAC is known to metastasize rapidly,[1,2] early detection, while the disease is still localized, is key to improving prognosis. Nevertheless, distinguishing FePAC from other diseases of the pancreas, namely pancreatitis, can be very challenging. Currently available less-invasive diagnostic methods such as routine blood work and imaging do not distinguish between FePAC and pancreatitis since findings often overlap.[1,3,5] Histopathological examination is highly accurate in the ante mortem diagnosis of FePAC but is rarely performed as it requires a relatively expensive, invasive, and risky biopsy procedure. Due to these drawbacks, the definitive diagnosis of FePAC is often made at late stages of the disease, such that the aggressive malignant tumor has already metastasized.[1,3] Discovery of non-invasive and cost-effective biomarkers could significantly improve prognosis in these patients by detecting FePAC at an earlier resectable stage. Given that blood collection is a routine, simple, and inexpensive procedure, blood-based biomarkers would be an ideal solution.

In human medicine, proteomics have been widely employed in the search for pancreatic specific biomarkers in a variety of body fluids, including blood.[6,7] Among several proteomics techniques currently available, liquid chromatography-tandem mass spectrometry (LC-MS/MS) yields high throughput, highly sensitive, and more specific proteomics analysis. Thus, LC-MS/MS has become a mainstay for protein biomarker discovery[8] and has successfully identified

potential biomarkers for pancreatic cancer in humans, such as gelsolin and apolipoprotein A-IV.[9,10,11,12] One of the key steps for successful blood-based biomarker discovery is the use of immunodepletion to overcome the major challenge in blood proteomics, which is the complexity and dynamic range of the abundance of plasma proteins that can reach over 10 orders of magnitude.[7] Since disease-specific biomarkers are often relatively low-abundance proteins (LAPs), the presence of high-abundance proteins (HAPs) may interfere with analysis for biomarker discovery. There are only a few types of proteins that account for in the HAP category, with albumin and immunoglobulins representing the majority of HAPs. Specific immunodepletion devices capture most or all of the targeted HAPs by immunoaffinity via immobilized antibodies bound to the resin of the column. Filtering of the few protein types that make up the HAPs would remove over 90% of the total protein abundance, allowing for a markedly increased chance for LAPs to be detected by proteomics techniques.[13,14,15] Multiple affinity removal system (MARS) is one of the earliest commercially available multi-component immunoaffinity depletion/enrichment columns, and it is commonly used for immunodepletion in proteomics studies in human medicine.[16,17]

Proteomics in feline pancreatic cancer has been little explored to date. A single study in 2015 used simple proteomics methods on feline plasma samples and identified 3 proteins (apolipoprotein A1, apolipoprotein A1 precursor, and alpha-1-acid glycoprotein) that were differentially expressed among cats with FePAC, cats with pancreatitis, and healthy control cats.[18] However, these proteins were HAPs and the changes in levels of expression were not specific for FePAC. The study highlighted the difficulty of assessing the blood proteome. Currently, no commercial products for immunodepletion are validated for use in feline plasma and only sparse studies on the application of human-based devices in veterinary species have

been published.[14,15,19] Given the high sequence homology of albumin, the major HAP in plasma, between humans and cats, the application of human-based immunodepletion products to feline plasma seems promising.

Thus, our goal was to evaluate the low-abundance plasma proteome of cats with FePAC in comparison to cats with pancreatitis and healthy controls using LC-MS/MS. To improve the focus on LAPs, plasma samples were immunodepleted after validation of a commercial immunodepletion device. We hypothesized that cats with FePAC have unique plasma proteomes of low abundance and that differentially expressed plasma proteins may act as candidate biomarkers of FePAC. Discovery of biomarker candidates has the potential for development of a novel diagnostic assay, which would in turn greatly improve the prognosis of cats with FePAC.

2. Material and Methods

2.1 Study Population and Sample Collection

The university animal care committee approved animal use and informed consent was obtained from all owners prior to study participation. The study population consisted of 29 client-owned cats in 3 groups: (1) 9 cats with FePAC, (2) 10 cats with pancreatitis, and (3) 10 healthy control cats (Table 2.1). The diagnosis of FePAC was confirmed with either histological or cytological examination by board-certified veterinary pathologists. Cats with a pancreatic mass but lacking pathological evaluation were excluded. Cats with pancreatitis had supportive clinical signs, fair to good therapeutic response, and one or more of the following ancillary test results: a Spec fPL value of ≥ 3.6 μg/L, positive ultrasound findings (e.g. hypoechoic pancreas, hyperechoic mesentery, abdominal effusion),[20] or histopathological confirmation from a surgical biopsy. Healthy control cats had no evidence of diseases based on clinical history, complete physical examination, and routine blood work including a complete blood count, serum biochemistry panel, and urinalysis.

Whole blood was collected at the time of diagnosis and placed immediately into a sodium citrated tube (BD Vacutainer® sodium citrate tubes, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Following centrifugation for 10 minutes at 2000xg, the plasma fraction was harvested into an Eppendorf tube. A protease inhibitor that is compatible with LC-MS/MS analysis (MS-SAFE Protease and Phosphatase inhibitor, Sigma-Aldrich, St. Louis, MO, USA) was added. The samples were immediately stored and kept at –80°C until use.

2.2 Immunodepletion

To remove HAPs, immunodepletion was performed using the MARS-2 kit (Multi Affinity Removal Spin Cartridge HSA/IgG, Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instruction. The cartridge has immobilized antibodies that capture albumin and immunoglobulins and allow LAPs to flow through. Each plasma sample (50 µL) was diluted with 150 µL Buffer A followed by filtration of the diluted sample through the preconditioned spin cartridge. After two cycles of washing the cartridge with Buffer A, the flow through (depleted plasma containing LAPs) was collected, followed by elution of the bound fraction (HAPs) with Buffer B. Both the flow through and bound fractions were subjected to SDS-PAGE to confirm protein immunodepletion.

2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed for two samples to visually and semi-quantitatively analyze proteomes in plasma, and the flow through and bound fractions. Samples were diluted 1:1 with 2x Laemmli Sample Buffer (BioRad, Hercules, CA, USA), containing 2-mercaptoethanol, and heated at 90°C for 5 minutes on a shaker. The samples and a protein standard (Precision Plus Protein™ Dual Xtra Prestained Protein Standards, BioRad, Hercules, CA, USA) were loaded onto a 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad, Hercules, CA, USA). Stacking of the gel was conducted at 80V for 10 minutes followed by running the gel at 100V for approximately 50 minutes to resolve the proteins. After washing the gel with distilled water (5 minutes x3) on a shaker, the gel was stained with Bio-Safe Coomassie Stain (BioRad, Hercules, CA, USA) overnight before destaining with distilled water (15 minutes x3) on a shaker.

2.4 Protein Concentration Determination

Immunodepleted samples were concentrated using 1K molecular weight cut-off centrifugal devices (Microsep Advance with 1K Omega, Pall Corporation, New York, NY, USA) for 30 minutes at 4500xg and 4°C. Protein concentration was determined, in duplicate, using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol. By comparing the determined concentration of three pairs of plasma and flow through samples (i.e. pre- and post- immunodepletion), the average protein recovery rate was calculated.[15]

2.5 Sample Preparation and Trypsin Digestion

For LC-MS/MS analysis, sample processing followed by trypsin digestion were performed as described before.[21] Briefly, flow through samples were diluted in 100 mM ammonium bicarbonate buffer (ABC). Proteins were denatured using trifluoroethanol (Fisher Scientific, Fair Lawn, NJ, USA), reduced with 10 mM dithiothreitol (MP Biomedicals, Solon, OH, USA), and alkylated with 55 mM iodoacetamide (Fisher Scientific, Fair Lawn, NJ, USA). Samples were then dried using a speedvac vacuum concentrator (Labconco, Kansas City, MO, USA) and treated with cold acetone to remove salts and other contaminants. The samples were then centrifuged at 18000xg for 30 minutes, twice, and the protein pellets were re-suspended in 100 mM ABC for digestion. Proteins were digested in a buffer-containing trypsin (Pierce Trypsin Protease MS-Grade, Thermo Scientific, Waltham, MA, USA) solution (50 ng/µL trypsin in 1 mM HCl/100 mM ABC) at 37°C overnight on a shaker. To minimize incomplete tryptic proteolysis, a second digestion was performed the following day (37°C; 2 hours). Digested samples were completely dried out using the speedvac.

2.6 Liquid Chromatography and Tandem Mass Spectrometry

Digested samples were reconstituted in MS grade water:10% acetonitrile (ACN):formic acid (FA) (97:3:0.1 v/v; Fisher Scientific, Hampton, NH, SA) followed by vortexing. The resulting solutions were centrifuged at 18000xg for 10 min at 4ºC and desalted on a ZipTip C18 column (Merck Millipore, Burlington, MA, USA). LC-MS/MS analysis was performed in triplicate on a nanospray LC-MS/MS system, which consisted of an Agilent 6550 iFunnel quadrupole time-of-flight mass spectrometer equipped with an Agilent 1260 series LC instrument and a Chip Cube LC-MS interface (Agilent Technologies, Santa Clara, CA, USA). Chromatographic peptide separation was accomplished using a high-capacity high performance LC-Chip II: G4240-62030 Polaris-HR-Chip_3C18 column. Plasma peptides were first concentrated by an enrichment column and then separated by a separation column using a linear gradient solvent system, comprising of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN). The linear gradient was 3–25% solvent B for 105 minutes, 25–40% solvent B for 15 minutes, 40–90% solvent B for 5 minutes and finally held at 90% solvent B for 5 minutes, at a flow rate of 0.3 µL/min. Separated peptides were then subjected to MS/MS analysis. Initially, positive-ion electrospray MS data were obtained, and spectral results were collected over a mass range of 250–1700 mass/charge (m/z) at a scan rate of 8 spectra/sec. Following a survey scan of the precursor ions, the top 20 most intense precursor ions for each MS scan were selected for MS/MS analysis with active exclusion for 0.25 min. MS/MS data were collected over a range of 100–1700 m/z and a set isolation width of 1.3 atomic mass units.

2.7 Data analysis

For protein identification, obtained raw tandem mass spectral data were searched against mammalian species, instead of cats (*Felis catus*), in the UniProtKB Swiss-Prot database considering the incomplete annotation of protein sequences in cats[13], using Spectrum Mill

(Agilent Technologies, Santa Clara, CA, USA) as the database search engine. Search parameters were set as follows: a fragment mass error of 50 parts per million (ppm), a parent mass error of 20 ppm, trypsin cleavage specificity, and carbamidomethyl as a fixed modification of cysteine. Trypsin specific digestion was applied and a maximum of 2 missed cleavages were allowed. In addition, four stages of database search in variable modification mode were carried out with different sets of variable modifications, as described by Nair et al.[21] Spectrum Mill validation was performed at peptide and protein levels (1% false discovery rate, FDR). For comparison of relative abundance of proteins, label-free quantification utilising spectral intensities was applied. The Mass Profiler Professional (MPP, version 15.0, Agilent, Santa Clara, CA, USA) software was used for statistical analysis using one-way ANOVA and fold change (FC) analysis, and for bioinformatics analysis using principal component analysis (PCA). A cut-off value of $p<0.05$ and the Benjamini-Hochberg FDR set at <1% were used to determine statistically significant results. Also, FC values in spectral intensities were considered to classify proteins as up- or down-regulated. Differentially expressed proteins were defined at both $p<0.05$ in one-way ANOVA and FC>2.

In order to link the identified differentially expressed proteins to various biological pathways, gene ontology (GO) analysis by PANTHER classification system (http://www.pantherdb.org/) and functional enrichment analysis utilising protein-protein interaction networks by STRING (https://string-db.org/) were conducted. Considering incomplete GO annotations and mapped pathways in cats, equivalent human proteins were entered. The human counterparts of the determined differentially expressed proteins used for pathway analysis are listed in Table 2.2.

3. Results

3.1 Evaluation of the Efficacy of the MARS-2 device

SDS-PAGE revealed a marked reduction of protein bands that are consistent with both albumin and IgG in the flow through fractions as compared to plasma samples (Figure 3.1).

The mean protein concentrations of three pairs of plasma samples before and after immunodepletion were 59.3 g/L and 3.7 g/L, respectively, resulting in the protein recovery rate of 23.7% on average (Table 3.1). Thus, approximately 76.3% of total protein was depleted by the treatment with the MARS-2 kit.

In the immunodepleted fraction, almost all albumin was removed and no or little albumin was detected by LC-MS/MS analysis (Table 3.1). Moreover, immunodepletion resulted in a marked increase in the overall number of identified proteins by LC-MS/MS. On average, 555 proteins were identified in immunodepleted plasma compared to 202 proteins in untreated plasma, an increase of 274% (Figure 3.2). IgG peptides were poorly identified by LC-MS/MS on both pre- and post-treatment plasma samples.

3.2 Biomarker Discovery by LC-MS/MS

Differential analyses employing one-way ANOVA and FC analysis identified 37 differentially expressed proteins ($p<0.05$ and $FC>2$) (Table 2.2). PCA demonstrated that individual immunodepleted plasma proteomes within each diseased group (FePAC and pancreatitis) loosely clustered while those within healthy control group closely clustered together (Figure 3.3A). When PCA was performed by group, proteomics profiles from different groups were distinct from one another (Figure 3.3B). Panther GO analysis was used to classify the

differentially expressed proteins by molecular function or biological process. The results are shown in Table 3.2.

Six proteins were differentially expressed in cats with FePAC compared to cats with pancreatitis and healthy control cats with little overlap in relative abundance between the groups (Figure 3.4). These proteins include ETS variant transcription factor 4 (ETV4; $p<0.05$), gelsolin ($p<0.01$), tryptophan 2,3-dioxygenase (TDO-2; $p<0.05$), serpin family F member 1 (serpin F1; $p<0.01$), apolipoprotein A-IV ($p<0.01$), and phosphatidylinositol-glycan-specific phospholipase D (GPLD; $p<0.05$). While ETV4 was overexpressed, other identified proteins were downregulated in cats with FePAC compared to cats with pancreatitis and healthy controls. String analysis revealed that 4 of these proteins (ETV4, gelsolin, serpin F1, and TDO-2) are involved in pathways known to lead to the development of cancer, such as cellular proliferation, apoptosis, angiogenesis, and metabolism.

Interestingly, 6 proteins were found to be differentially expressed only in cats with pancreatitis: alpha smooth muscle actin ($p<0.01$), obg-like ATPase 1 ($p<0.05$), plexin A3 ($p<0.01$), probable G-protein coupled receptor 153 ($p<0.05$), retina-specific copper amine oxidase ($p<0.01$), and TDO-2 ($p<0.05$). A further 5 proteins were found to be differentially expressed in both FePAC and pancreatitis compared to healthy controls: alpha-2-HS-glycoprotein ($P<0.01$), alpha-2-macroglobulin ($p<0.01$), culin-4B ($p<0.05$), dystonin ($p<0.05$), and serum paraoxonase lactonase 3 ($p<0.05$).

4. Discussion

Our study demonstrated the efficacy of the MARS-2 kit for immunodepletion of feline plasma, followed by the successful identification of differentially expressed proteins between cats with FePAC, cats with pancreatitis, and healthy control cats via tandem mass spectrometric analysis of immunodepleted plasma. These identified proteins may act as potential biomarkers for FePAC.

The MARS-2 kit successfully reduced a marked amount of the targeted HAPs, albumin and likely IgG, from feline plasma. Feline IgG is currently poorly annotated in the protein database; thus, IgG depletion could not be confirmed using mass spectrometry. However, SDS-PAGE supported substantial reduction of feline IgG by the MARS-2 kit by a decrease in IgG band intensity (Figure 3.1). There is a lack of veterinary species-specific immunodepleting devices and only a few studies have investigated the use of human-specific immunodepleting devices for veterinary use. One study found that a human multiple affinity removal spin cartridge (MARS–Hu14) was successful in depleting HAPs from canine cerebral spinal fluid, particularly albumin.[14] This product employs a similar principle to the MARS-2 kit used in this study and immobilizes anti-human antibodies that target the top 14 HAPs on the column. The high efficacy can be explained by the high sequence homology (80.1%) of albumin, the most abundant HAP, between humans and dogs.[14,15] Previous research has also demonstrated successful immunodepletion of targeted HAPs from canine and bovine biological fluids using the human MARS-6 device.[19,22] Immunodepletion of feline plasma samples has been attempted previously, however researchers found that none of the 4 human-based HAP immunodepletion devices evaluated were efficacious.[15] These results were surprising, given that feline and human albumin also share a high degree of sequence homology (82.4%).[15] In contrast, our study demonstrated

successful removal of albumin and likely IgG from feline plasma, which may reflect an increased antibody affinity for feline albumin in the MARS-2 kit compared to the previously tested devices. To the authors' best knowledge, this is the first published study validating the use of a human immunoaffinity device in feline plasma prior to proteomics analysis.

LC-MS/MS identified 6 proteins (ETV4, gelsolin, serpin F1, TDO-2, apolipoprotein A-IV, GPLD) that were significantly differentially expressed in the plasma of cats with FePAC compared to cats with pancreatitis and healthy controls, of which 3 proteins (gelsolin, serpin F1, apolipoprotein A-IV) have been found to be aberrantly expressed in blood from humans with pancreatic cancers.[12,23] These proteins may be potential biomarkers of FePAC.

ETV4 was overexpressed in cats with FePAC in our study. ETV4, also known as Adenovirus E1A enhancer-binding protein (E1A-F), is a member of the E-twenty-six transforming-specific (ETS) family of oncogenic transcription factors.[24,25] The ETS transcription factors are highly evolutionarily-conserved proteins with as high as 94.8% sequence homology between human and feline ETV4 proteins as determined by BLAST searching (https://www.uniprot.org/blast/). ETV4, like other ETS members, plays an important role in physiological tissue development and pathological cancer development and progression. In pancreatic development, ETV4 expression was found to be segregated in pancreatic progenitor cells and probably involved in mesenchymal-to-epithelial signaling.[26] ETV4 overexpression has been reported in several human cancers and implicated in tumor cell proliferation, invasion, and metastasis collaborating with key cellular signaling.[27,28,29,30] In human pancreatic cancer cells, aberrant ETV4 expression promoted cell-cycle progression by regulating Cyclin D1 transcription.[30] Interestingly, studies have shown that more than 90% of human pancreatic cancers harbor an activating mutation of KRAS, one of the upstream signaling

molecules/proteins of ETV4, with lesser frequency of mutations in other well-known cancer genes such as TP53, SMAD4, and p16/CDKN2A.[31,32,33] Activating KRAS mutation is one of the earliest mutations noted in human pancreatic cancers and is considered a crucial event for the initiation of cancer,[32] setting the stage for tumor progression to more advanced and aggressive pancreatic cancers through additional events such as acinar-to-ductal reprogramming and genomic/chromosomal instabilities.[34,35,36,37] Similar KRAS mutations were found in 2 of the 3 cases in FePAC in one study.[38] Since ETV4 is involved in several key signaling pathways in cancer such as Ras/MAPK, PI3K/Ras, Wnt/β-catenin, and MET[27,28,29,39] and knockdown or silencing of ETV4 expression could reverse its cancer-promoting effects,[28,29,30] ETV4 is considered as a potentially promising therapeutic target.[40] Given the similarities between feline and human pancreatic tumours, ETV4 may act as both a potential diagnostic biomarker and therapeutic target in cats.

Decreased expression of gelsolin was found in cats with FePAC in our study. Gelsolin is an actin-modulating protein closely associated with caspase-3 with both anti- and pro- apoptotic functions depending on the specific cell type, tissue, and pathological condition.[41,42] Thus, it may act as either a tumor-promoting or a tumor-suppressing molecule.[41,43,44] In humans, gelsolin tissue expression is downregulated in 71% of pancreatic cancer, likely mediated by the ubiquitin-proteasome pathway rather than by transcriptional regulation.[45] Furthermore, gelsolin protein expression in serum was found to trend downwards up to 3 years before the diagnosis of pancreatic cancer.[12] Gelsolin down-regulation has been implicated in altered cytoskeletal reorganization and cell motility and reduced anti-tumor activities such as delayed cell cycle, inhibited tumor growth, and promoting apoptosis.[42,44,45,46] One study suggested gelsolin may play

a tumor suppressive role in ras proto-oncogene activated tumors .[47] The role of gelsolin in feline pancreatic cancer is currently unknown.

In our study, TDO-2 expression was unique in that levels trended lower in cats with FePAC but higher in cats with pancreatitis compared to healthy controls. This differs from the other candidate proteins, which tended in the same direction for both FePAC and pancreatitis. Because of this distinct feature, TDO-2 might be a promising candidate biomarker especially useful to differentiate between FePAC and pancreatitis. TDO-2 is a functional ortholog of indoleamine-2,3-dioxygenase 1 and is a key player of the kynurenine (Kyn) pathway that is important for tryptophan metabolism.[48,49] TDO-2 is expressed by many cancers including hepatocarcinomas, glioblastomas, and renal carcinomas.[50] In cancer cells, TDO-2 expression is associated with response to replication stress and DNA damage, immunosuppression, and resistance to immunotherapy.[51,52,53] Downregulation of TDO-2 in FePAC could be associated with diminished responses to DNA damage[51] while upregulation in pancreatitis might reflect pancreatic infiltration of FOXP3 positive regulatory T cells.[52,53]

Decreased plasma expression of serpin F1 was noted in cats with FePAC. Serpin F1, also known as pigment epithelium-derived factor (PEDF) encoded by SERPINF1 gene in humans, has been known to have anti-tumor activities.[23,54,55] The pancreas is one of the organs where PEDF is highly expressed.[54] In one study, serum PDEF concentration was found to be decreased in human patients with pancreatic adenocarcinoma compared to healthy controls when evaluated by ELISA.[23] Reduced PDEF tissue expression in human pancreatic carcinoma has also been confirmed by quantitative real-time PCR analysis and immunohistochemistry.[55,56] Additionally, several studies suggest that PDEF may act as a potent inhibitor of tumoral neoangiogenesis, tumor growth, autophagy, adiposity, and tumor invasion in human pancreatic cancer cells.[23,55,57]

Loss of PDEF expression has been associated with acquisition of a metastatic phenotype of human pancreatic cancer.[55] PEDF is also an important negative regulator of adiposity and loss of expression may lead to changes of lipid metabolism in the tumour microenvironment associated with tumour invasion of the pancreas.[55,58] Interestingly, apolipoprotein A-IV, an important lipid-binding protein, was also found to be downregulated in cats with FePAC in our study. A similar decrease in apolipoprotein A-IV has been reported in human pancreatic cancer,[12] and suggests that alterations in lipid metabolism may be an important factor in pancreatic tumorigenesis in both species.

Overall, this study highlights the many molecular similarities between human and feline malignant pancreatic cancer; indeed, cats with FePAC may be an excellent naturally-occurring animal model for human pancreatic disease. Furthermore, these similarities would suggest that therapeutic strategies for human pancreatic cancer may also be effective in cats. However, this study had several limitations. Firstly, as this is a pilot study, the sample size is small. As most cases of FePAC are diagnosed post-mortem, fresh plasma samples are not always available, further limiting sample size. Given the relative rarity of FePAC and difficulty in ante-mortem diagnosis, this sample size was deemed adequate for initial investigation. In addition, the control group consisted of relatively younger individuals than the other study groups, and age-related differences in protein expression may potentially be a confounding factor. Secondly, the feline protein database is not as robust as in humans or other well-studied laboratory animals, and it is possible that poorly annotated proteins were missed in our study. Five of the 6 potential biomarker proteins were initially identified using the mammalian UniProtKB Swiss-Prot database, however we confirmed high homologies of these identified proteins with their feline specific counterparts (> 80.9% for the potential biomarkers; > 72% for all the differentially

expressed proteins) as listed in Table 2.2. Due to the very limited availability of feline-specific antibodies validated for western blot and enzyme-linked immunosorbent assay, partial verification of the identified proteins was performed using strict mass spectrometry database search parameters. These parameters included manual validation of peptides and proteins at 1% FDR, at least 2 tryptic peptides present in every protein identified, peptide score greater than 5, and SPI (Scored Peak Intensity) greater than 60%. Lastly, since we focused on only FePAC and pancreatitis in this study, it remains unclear if the identified potential biomarkers can differentiate FePAC from other pancreatic diseases. Further studies with larger sample size including other pancreatic diseases and absolute quantitative methodology (e.g., enzyme-linked immunosorbent assay) are needed to validate our findings.

Although it was not the primary focus of the current study, differentially expressed plasma proteins were identified in cats with pancreatitis (actin, alpha smooth muscle, obg-like ATPase 1, plexin A3, probable G-protein coupled receptor 153, retina-specific copper amine oxidase, and TDO-2). Further exploration into their potential utility as diagnostic biomarkers of feline pancreatitis is warranted.

5. Conclusion

In conclusion, the MARS-2 device effectively removed albumin and likely IgG from feline plasma and is recommended to improve the discovery of low abundance, disease-specific biomarkers in shotgun proteomics of feline plasma. In addition, ETV4, gelsolin, serpin F1, TDO-2, apolipoprotein A-IV, and GPLD were differentially expressed in cats with FePAC compared to cats with pancreatitis and healthy cats and may act as novel plasma biomarkers for FePAC. Further studies are warranted to investigate their diagnostic potential.

Table 2.1. Signalment of Study Population.

Study Group	Age (year)	Sex	Breed
FePAC	3	FS	DSH
FePAC	7	FS	DSH
FePAC	11	MN	Maine Coon
FePAC	11	MN	Maine Coon cross
FePAC	11	MN	DSH
FePAC	14	MN	DLH
FePAC	15	FS	DMH
FePAC	16	MN	DSH
FePAC	18	FS	DSH
Pancreatitis	2	MN	DSH
Pancreatitis	9	MN	DSH
Pancreatitis	11	MN	DLH
Pancreatitis	13	FS	DSH
Pancreatitis	14	FS	DSH
Pancreatitis	14	FS	DSH
Pancreatitis	14	MN	DLH
Pancreatitis	16	MN	DSH
Pancreatitis	16	MN	DSH
Pancreatitis	18	FS	DLH
Control	1	MN	DSH
Control	1.5	FS	DSH
Control	2	MN	DSH
Control	3	FS	DLH
Control	3	MN	DMH
Control	4	FS	DSH
Control	6	FS	Manx
Control	6	MN	DSH
Control	7	FS	DSH
Control	9	MN	DSH

Footnote

FePAC: feline pancreatic carcinoma, FS: female spayed, MN: male neutered, DSH: domestic short hair, DLH: domestic long hair, DMH: domestic medium hair.

Table 2.2. Differentially expressed proteins between cats with FePAC, cats with pancreatitis, and control cats.

Protein Name	Accession Number	Species	Peptides	Feline Accession Number	Blast Score	Identity	Human Accession Number	p-value	P vs CNT	FePAC vs CNT	FePAC vs P
Actin, alpha skeletal muscle	P68138	BOVIN	24	M3XC93	1,975	100	P68133	<0.01	-16.864992	-9.368687	7.496306
Afamin	G3MYZ3	BOVIN	25	A0A2I2U1Z3	2,783	86.2	P43652	<0.05		-1.1510677	
Alpha-2-HS-glycoprotein	P12763	BOVIN	15	A0A5F5XVZ8	1,109	72.1	P02765	<0.01	-1.3988627	-1.6472092	
Alpha-2-macroglobulin	P01023	HUMAN	87	A0A5F5Y1U9	6,154	80.4	P01023	<0.01	-1.1701049	-2.4385605	-1.2684555
Antithrombin-III	P32262	SHEEP	47	M3WLL8	2,171	90.5	P01008	<0.01		-1.2290363	-1.1722164
Apolipoprotein A-IV	**M3W955**	**FELCA**	**67**				**P06727**	**<0.01**		**-2.1958313**	**2.5749626**
Attractin	Q9WU60	MOUSE	36	M3W7M4	7,331	93.3	O75882	<0.01	-4.1417274	-15.561871	-11.420143
Beta,beta-carotene 9',10'-oxygenase	Q9BYV7	HUMAN	19	M3W5I3	2,558	81.3	Q9BYV7	<0.05	-5.3938503	-12.272613	-6.8787622
Beta-2-microglobulin	Q5MGS7	FELCA	6				P61769	<0.01	-16.431286	-9.481145	6.950142
Ceruloplasmin	P00450	HUMAN	56	M3VZY7	4444	78	P00450	<0.05			1.055048
Clusterin	P10909	HUMAN	19	M3WKP2	1808	77	P10909	<0.05		-1.0526733	
Coagulation factor V	P12259	HUMAN	42	M3W922	8,507	73.8	P12259	<0.01	1.2268294	-10.118454	-10.407904
Complement C3	Q2UVX4	BOVIN	107	A0A5F5XM97	5,834	69.7	P01024	<0.05		-1.1308498	
Complement C4-A	P0C0L4	HUMAN	90	A0A5F5XFU6	7,583	84.9	P0C0L4	<0.01	13.173285	12.907164	
Complement C5	P01031	HUMAN	58	M3VV58	7,389	83.2	P01031	<0.05		-1.6847361	-1.0383453
CUB domain-containing protein 1	Q5U462	MOUSE	16	M3X665	3,504	79.4	Q9H5V8	<0.05		-1.1132488	-1.0346165
Cullin-4B	A2A432	MOUSE	25	A0A2I2U2R4	4,388	97.7	Q13620	<0.05	9.72421	11.683286	1.9590759
Dystonin	Q91ZU6	MOUSE	83	A0A337SS21	29,654	79.3	Q03001	<0.05	-14.450918	-5.399578	9.05134
ETS translocation variant 4	**P28322**	**MOUSE**	**6**	**M3WCC6**	**2,410**	**91.6**	**P43268**	**<0.05**	**3.2091727**	**15.689953**	**12.48078**
Fibrinogen beta chain	Q8K0E8	MOUSE	29	M3WII3	2,124	81.9	P02675	<0.01	-12.577568	-23.241371	-10.663803
Gelsolin	**Q3SX14**	**BOVIN**	**62**	**A0A5F5XIG6**	**3,773**	**97.5**	**P06396**	**<0.01**	**-13.373325**	**-23.693678**	**-10.320353**
Glutathione peroxidase 3	P46412	MOUSE	15	A0A2I2V5E5	1,105	91.2	P22352	<0.01		-1.5812588	1.608635
Haptoglobin	P00738	HUMAN	46	A0A5F5XGD0	1,402	81.2	P00738	<0.01	-13.34193	1.1305217	14.472452
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	HUMAN	43	M3WN59	4,019	84.6	P19827	<0.05		-1.6036777	-1.5493221
Interleukin-1 receptor accessory protein	Q9NPH3	HUMAN	21	M3WNU0	2,164	78.3	Q9NPH3	<0.05	-3.701624	-13.771996	-10.070372
Lactotransferrin	P02788	HUMAN	42	M3WKU0	2,829	73.2	P02788	<0.01		-1.8605976	
Obg-like ATPase 1	Q2HJ33	BOVIN	1	A0A337SKM4	1,649	88	Q9NTK5	<0.05	12.922482	3.5281854	-9.394296
Phosphatidylinositol-glycan-specific phospholipase	**P80108**	**HUMAN**	**20**	**M3WEG4**	**3,779**	**80.9**	**P80108**	**<0.05**	**-2.0831642**	**-10.566344**	**-8.48318**
Pigment epithelium-derived factor	**P97298**	**MOUSE**	**24**	**M3W5N1**	**1,873**	**87**	**P36955**	**<0.01**		**-11.541511**	**-10.817403**
Plasminogen	P12545	MACMU	48	M3X3T9	3871	82.5	P06867	<0.01		-1.5404549	
Plexin-A3	P51805	HUMAN	17	A0A5F5Y6Q2	9,367	94.7	P51805	<0.01	23.007515	1.9541477	-21.053368
Probable G-protein coupled receptor 153	Q8K0Z9	MOUSE	5	M3W5P7	2,714	84.2	Q6NV75	<0.05	12.750242	5.667575	-7.0826674
Prothrombin	Q19AZ8	PIG	38	M3WSI8	2,895	83.4	P00734	<0.01		-1.5178871	1.5679913
Retina-specific copper amine oxidase	Q812C9	MOUSE	11	A0A2I2U0U5	3,349	82.4	O75106	<0.01	-19.352238	-13.968805	5.3834324
Serum paraoxonase/arylesterase 1	P27170	RABIT	10	A0A2I2UU74	1,620	84.2	P27169	<0.05		-1.5522995	
Serum paraoxonase/lactonase 3	Q9BGN0	RABIT	11	M3W9B9	1,496	80.2	Q15166	<0.05	-6.4269104	-12.697208	-6.2702985
Tryptophan 2,3-dioxygenase	**P48776**	**MOUSE**	**9**	**M3X838**	**1,937**	**89.9**	**P48775**	**<0.05**	**1.0822392**	**-9.809718**	**-10.891957**

Footnote

Peptide: the number of peptides per protein detected by LC-MS/MS. FELCA: cat, MACMU: rhesus macaque. P-values were obtained from one-way ANOVA after the Benjamini-Hochberg procedure and fold-change values in comparison between the three study groups were obtained from fold-change analysis. Negative value indicates down-regulation while positive value indicates overexpression. FePAC: feline pancreatic carcinoma, P: pancreatitis, CNT: control. Potential biomarkers identified in this study are highlighted in blue.

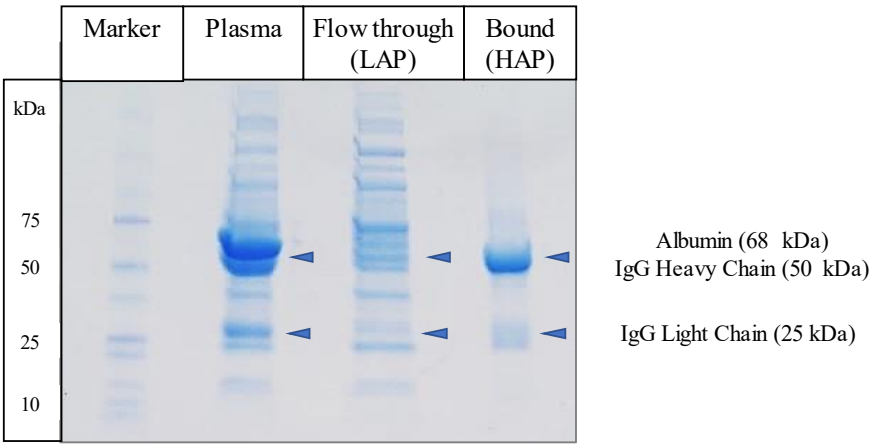
Table 3.1. Efficacy of MARS-2 device.

Sample	Protein Recovery Rate (%)	Spectral Intensity of Albumin ($x10^8$)			Spectral Counts of Albumin		
		Pre-treatment	Post-treatment	Efficiency (%)	Pre-treatment	Post-treatment	Efficiency (%)
1	18.9	224.0	0.0	100.0	460	0.0	100.0
2	28.4	93.4	0.0	100.0	452	8.0	98.2
3	23.6	232.0	0.0	100.0	584	0.0	100.0
Average	23.6	183.1	0.0	100.0	499	2.7	99.5

Table 3.2. Results of Panther GO analysis of 37 differentially expressed proteins in feline pancreatic carcinoma, showing molecular function and biological process analyses.

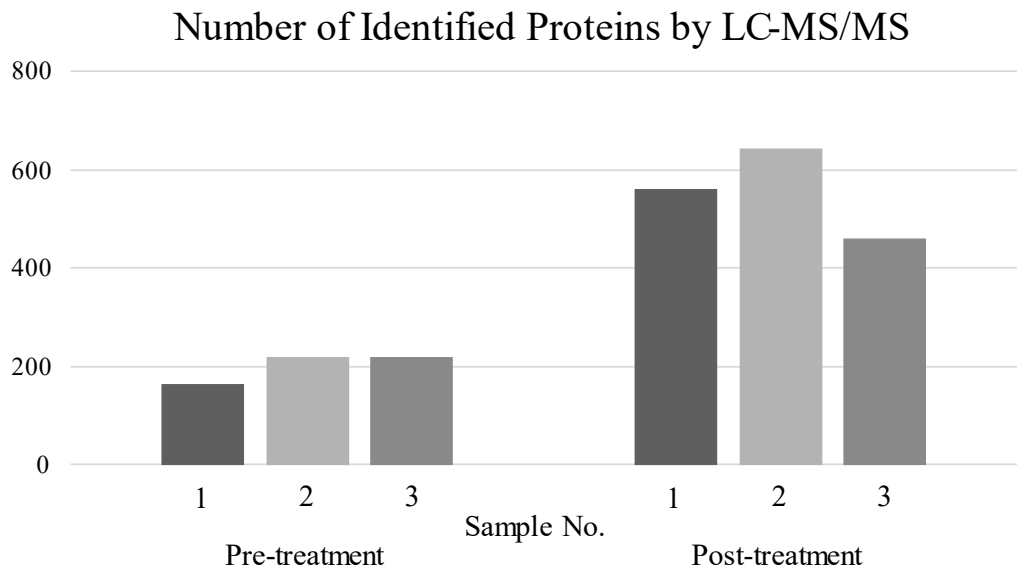
Panther GO analysis	Description	Percentage (%)
Panther GO-Slim molecular function analysis	Catalytic activity	37.8
	Binding	27.0
	Molecular function regulator	13.5
	Molecular transducer activity	2.7
	Structural molecule activity	2.7
Panther GO-Slim biological process analysis	Cellular process	40.5
	Metabolic process	37.8
	Biological regulation	32.4
	Response to stimuli	21.6
	Localization	13.5
	Multicellular organismal process	13.5
	Developmental process	8.1
	Biological adhesion	5.4
	Immune system process	5.4
	Interspecies interaction between organisms	2.7
	Locomotion	2.7
	Signaling	2.7

Figure 3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of plasma, flow through, and bound fraction.



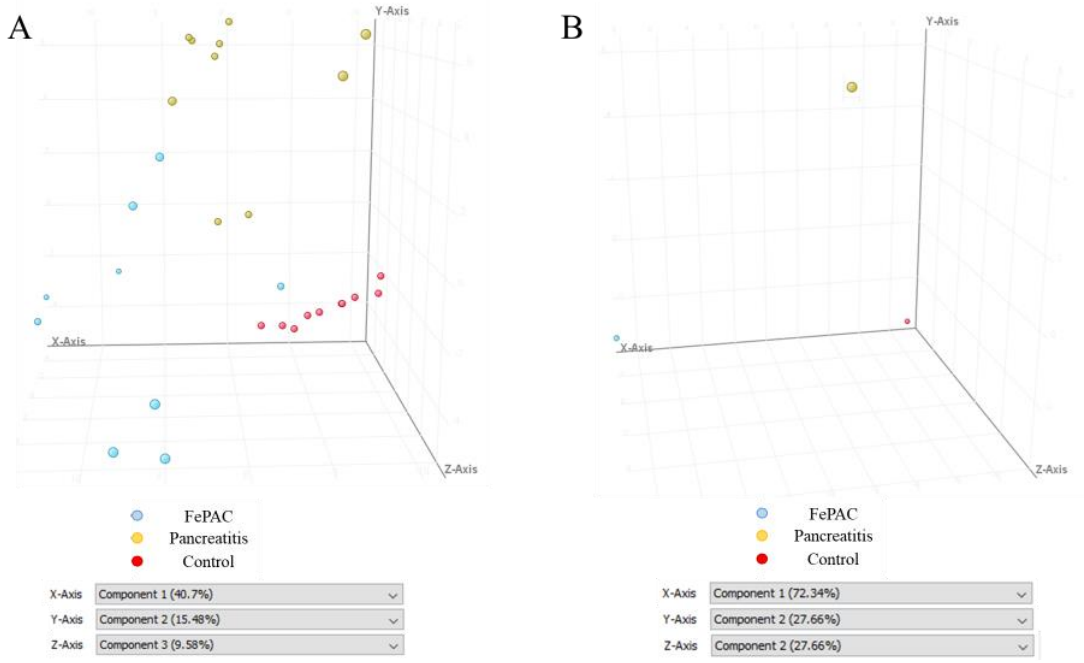
SDS-PAGE revealed a marked reduction of protein bands that are consistent with both albumin and IgG in the flow through fractions as compared to plasma samples.

Figure 3.2. The number of identified proteins by LC-MS/MS before and after immunodepletion.



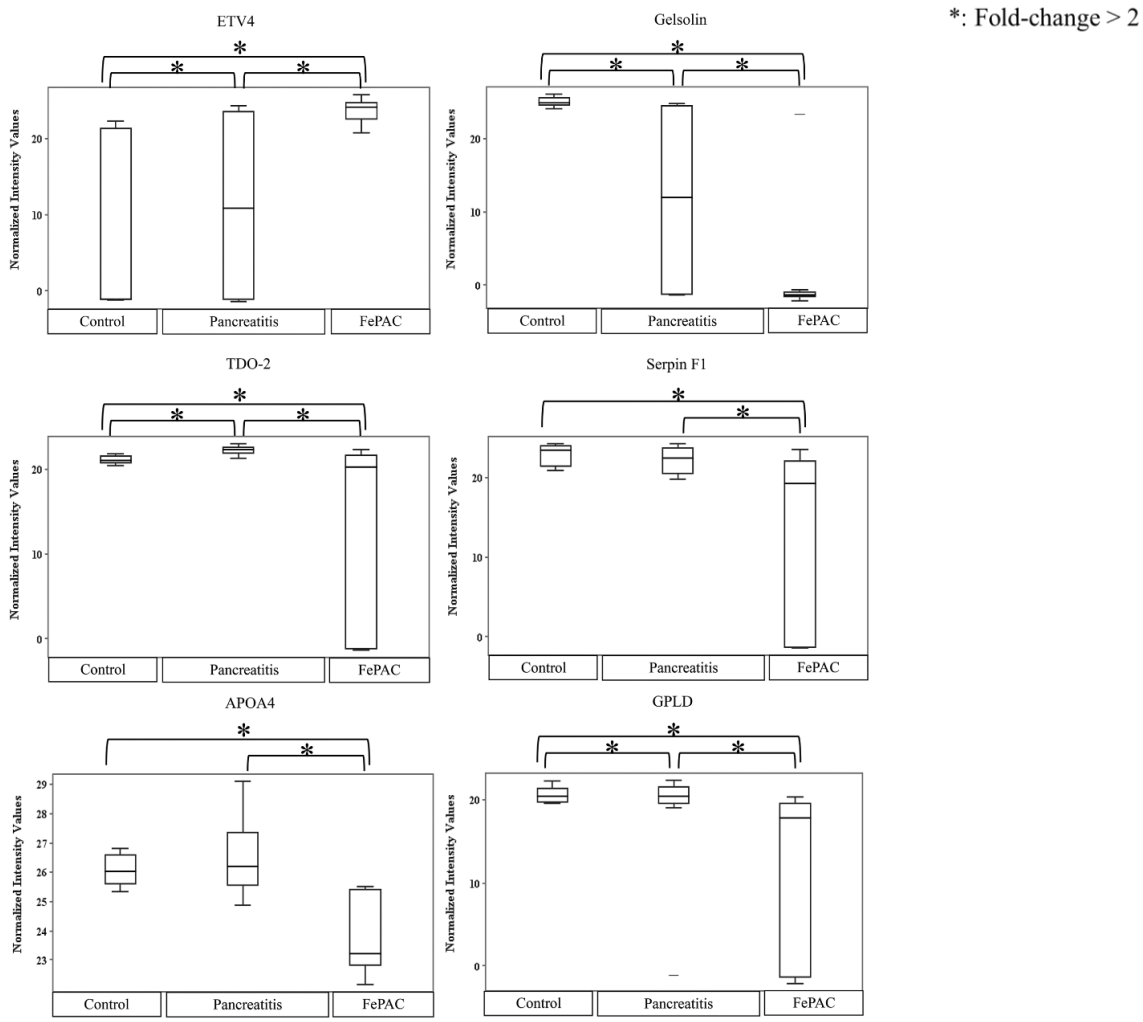
Application of immunodepletion by MARS-2 device resulted in a marked increase in the overall number of identified proteins by LC-MS/MS. On average, 555 proteins were identified in immunodepleted plasma compared to 202 proteins in untreated plasma, an increase of 274%.

Figure 3.3. Principal component analysis (PCA).



(A) PCA by individual samples: Immunodepleted plasma proteomes within each diseased group (FePAC and pancreatitis) loosely clustered while those within healthy control group closely clustered together. (B) PCA by group: Overall proteomics profiles of different groups were distinct from one another. FePAC: feline pancreatic carcinoma.

Figure 3.4. Relative abundance of biomarker candidate proteins.



The box depicts 25-75 percentile with median depicted as a line through a box. The upper and lower whisker represents maximum and minimum values, respectively. ETV4 was significantly overexpressed while the other candidate proteins were significantly down-regulated in the FePAC group compared to the other two groups. ETV4: ETS variant transcription factor 4, TDO-2: tryptophan 2,3-dioxygenase, APOA4: apolipoprotein A-IV, GPLD: phosphatidylinositol-glycan-specific phospholipase D. FePAC: feline pancreatic carcinoma.

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