# Differences in plasma and peritoneal fluid proteomes identifies potential biomarkers associated with survival following strangulating small intestinal disease.

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**Ethical considerations**: Institutional ethical committee approval was obtained for this study (VREC219/a). All samples were collected with informed owner consent under the University of Liverpool Philip Leverhulme Equine Hospital generic research information and consent forms (RETH000689). Sample collection from clinical cases involved no additional procedures to those performed during routine clinical investigation.

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Summary

- **Background:** Strangulating small intestinal disease (SSID) carries a poor prognosis for survival in comparison to other types of colic, particularly if resection is required. Identification of markers which aid early diagnosis may prevent the need for resection, assist with more accurate prognostication, and/or support the decision on whether surgical intervention is likely to be successful, would be of significant welfare benefit.
- **Objectives:** To apply an unbiased methodology to investigate the plasma and peritoneal fluid (PF) proteomes in horses diagnosed with SSID requiring resection, to identify novel biomarkers which may be of diagnostic or prognostic value.
- **Study Design:** Prospective clinical research study.
- **Methods:** Plasma and PF from horses presented with acute abdominal signs consistent with SSID were collected at initial clinical examination. Samples from eight horses diagnosed with SSID at surgery in which resection of affected bowel was performed, and four control horses euthanased for orthopaedic conditions were submitted for liquid chromatography tandem mass spectrometry. Protein expression profiles were determined using label-free quantification. Data were analysed using analysis of variance to identify differentially expressed proteins between control and all SSID horses and SSID horses which survived to hospital discharge and those which did not. Significance was assumed at $p<0.05$
- **Results:** A greater number of proteins were identified in PF than plasma of both SSID cases and controls, with 123 PF and 13 plasma proteins significantly differentially expressed (DE) between cases and controls ($p<0.05$, ≥2-fold-change). Twelve PF proteins ($p<0.036$) and four plasma proteins ($p<0.05$) were significantly DE between SSID horses which survived and those which did not.
- **Main Limitations:** Major limitations are the low number of samples analysed, variation in duration and severity of SSID and only short-term outcome was considered.
- **Conclusions:** Changes in PF proteome may provide a sensitive indicator of small intestinal strangulation and provide biomarkers relevant to prognosis.

## Introduction

Colic due to small intestinal obstruction is associated with a significantly lower survival rate than caecal or large intestinal obstruction [1, 2]. Likelihood of survival is further decreased with strangulating rather than simple obstruction and if resection following ischaemic insult is required [3, 4]. The greatest mortality occurs in the first 7-10 days post-operatively, frequently as a result of recurrent colic, post-operative ileus and cardiovascular instability consistent with endotoxaemia [5, 6, 7]. There are also significant welfare and financial concerns associated with subjecting a horse to major abdominal surgery and these concerns are particularly relevant when the prospects for short term survival may be poor.

Total protein (TP) concentration of plasma and peritoneal fluid (PF) is routinely measured during clinical investigation to determine the presence of dehydration and protein losing transudative or exudative enteropathies but is non-specific for primary aetiology. Low plasma TP [7, 8] and elevated PF protein concentration [3] have been associated with non-survival, but these are not consistent findings [4, 9]. Peritoneal fluid is an ultrafiltrate of plasma which is produced normally to facilitate separation between parietal and visceral peritoneal surfaces of abdominal viscera [10]. Sanguinous or serosanguinous appearance of PF has been associated with increased incidence of post-operative complications and reduced survival [9] but must be differentiated from iatrogenic contamination due to needle perforation of cutaneous or serosal vessels, or splenic penetration. Qualitative analysis of protein content of these fluids may provide more specific information on the disease process and severity than quantitative assessment. Mass spectrometry-based label-free quantitative proteomics offers a highly automated, reproducible and accurate means of analysing complex mixtures such as biological fluids. Direct comparison between different samples can be made, allowing investigation of relative protein composition and abundances, without the need for expensive, time-consuming and complex labelling techniques, making it well suited to clinical biomarker discovery which typically requires higher sample throughput [11].

The primary aim of this study was to compare the plasma and PF proteomes from control horses without gastrointestinal disease with horses requiring intestinal resection due to strangulating small intestinal disease (SSID), and between those horses which subsequently survived to hospital discharge and those which did not. We hypothesised that PF would be a more sensitive marker of SSID, producing more significant changes than those occurring in the plasma and that it may be possible to identify protein biomarkers associated with survival or non-survival. Additionally, we hypothesised that a proteomic approach would identify proteins associated with biochemical pathways triggered or disrupted as a consequence of SSID which may provide therapeutic targets to improve post-operative survival.

## Materials and Methods

### *Sample collection*

Following institutional ethical committee approval VREC219/a, blood and peritoneal fluid were collected during initial clinical examination from horses presented to the University of Liverpool Philip Leverhulme Equine Hospital with colic signs consistent with SSID. Following clipping and aseptic preparation of the ventral abdomen immediately caudal to the xiphisternum, a 21gauge 2-inch needle[a] was introduced slowly through the abdominal wall, to penetrate the coelomic cavity. Peritoneal fluid was collected by free flow into sterile plain blood collection tubes[b]. If blood contamination or enterocentesis occurred, the needle was withdrawn, and a second needle introduced. These horses were then excluded from the study. Blood was collected from the jugular vein by direct percutaneous venepuncture and placed into lithium heparin tubes[b]. Samples were immediately centrifuged at 1500 rcf for four minutes to sediment cellular component, the supernatant was harvested and frozen in 1mL aliquots at -80$^{o}$C. Samples submitted for subsequent analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) and label-free quantification were from 3 cohorts: 1) horses with SSID which underwent resection of small intestine and subsequently survived to be discharged from the hospital (survivors) (n=4); 2) horses with SSID which underwent resection of small intestine, recovered from surgery but were subsequently euthanased as a consequence of the

disease process (non-survivors) (n=4); 3) horses of similar age, euthanased for chronic orthopaedic conditions with no history or signs of gastrointestinal or peritoneal disease (controls) (n=4). Demographic information, with clinical and outcome details of SSID horses included in the study are provided in Table 1.

*Sample preparation*

Five milligrams of plasma and PF from each horse was bound to 10 µL of ProteoMiner™ Beads[c] to compress the dynamic range of proteins. Beads were washed with phosphate buffered saline prior to binding and mixed with plasma or PF for 2 hours at room temperature, then washed with 25 mM ammonium bicarbonate (AmBic). One hundred and fifty microliters of 25 mM AmBic and 10 µL of 0.05% Rapigest™ [d] was added to the beads and shaken at 550 rpm for 10 min at 80°C. Samples were reduced by the addition of 10 µL of 60 mM dithioreitol and incubated at 60°C for 10 minutes, then alkylated by the addition of 10 µL of 180 mM iodoacetamide and incubated at room temperature for 30 minutes in the dark. Proteomic grade trypsin[e] was reconstituted in 25 mM acetic acid to a concentration of 0.2 µg $\mu L^{-1}$ and 10 µL added to the samples to achieve an enzyme:protein ratio approximately 1:50 w/w. Following overnight incubation on a rotating mixer at 37°C, digestion was terminated and Rapigest™ [d] removed by acidification with 1 µL trifluoroacetic acid and incubation at 37°C for 45 min, then centrifugation at 15,000 rcf for 30 min. To check for complete digestion each sample was analysed pre- and post-acidification by SDS-PAGE.

*LC-MS/MS*

For LC-MS/MS analysis, a 2 µL injection was analysed using an Ultimate 3000 RSLC™ nano system[f] coupled to a QExactive™ mass spectrometer[f]. Samples were loaded onto the trapping column PepMap100, C18, 300 µm x 5 mm[f] using partial loop injection, for seven minutes at a flow rate of 4 µL $min^{-1}$ with 0.1% (v/v) FA. Samples were then resolved on the analytical column Easy-Spray C18 75 µm x 500 mm 2 µm column[f], using a gradient of 97% A (0.1% formic acid), 3% B (99.9% ACN 0.1% formic acid), to 60% A, 40% B, over 90 minutes, at a flow rate of 300 nL $min^{-1}$. A spray

voltage of 1.7 kV was used with a capillary temperature of 280°C. Technical replicates were not run and blanks were not used between runs due to the very low (typically < 0.01%) carry-over of the system.

The data-dependent (DDA) program used for data acquisition consisted of a 70,000 resolution full-scan MS scan (AGC set to 1e6 ions with a maximum fill time of 250 ms) with the 10 most abundant peaks selected for MS/MS using a 17,000 resolution scan (AGC set to 5e4 ions with a maximum fill time of 250 ms) with an ion selection window of 3 m/z and a normalised collision energy of 30. To avoid repeated selection of peptides for MS/MS the program used a 30 second dynamic exclusion window.

*Data analysis*

Label free relative quantification was performed with Progenesis QI™ version 2[d], with plasma and peritoneal fluid analyses handled independently. The Progenesis QI workflow creates a virtual aggregate run comprising all data from individual samples, allowing features to be cross identified from other samples. This overcomes the stochastic sampling limitations of DDA when the sample is too complex for the duty cycle of the mass spectrometer. Respective abundance values of all proteins identified in all samples are reported, rather than qualitative lists of protein identifications for each sample. Samples were aligned according to retention time using a combination of manual and automatic alignment. Default peak picking parameters were applied and features with charges from 1+ to 4+ featuring three or more isotope peaks were retained. A Mascot Generic File[g] created by Progenesis QI™ was searched against the Uniprot reference proteome for horse (downloaded April 2015, 22,963 entries). A fixed carbamidomethyl modification for cysteine and variable oxidation modification for methionine were specified. A precursor mass tolerance of 10 ppm and a fragment ion mass tolerance of 0.01 Da were applied. To maximise the number of proteins identified for subsequent quantification, protein identification threshold was set at >1 unique peptide. The results

were then filtered to obtain a peptide false discovery rate (FDR) of 1%. The protein identification list was then re-imported into Progenesis QI™ for analysis of differential expression. Analysis of variance (ANOVA) of the mean normalised abundance of each protein was performed across the experimental groups, using the groupings 'Controls', 'All SSID horses', 'SSID survivors' and 'SSID non-survivors', with the level of significance set at $p<0.05$.Those proteins with a $\geq 2.0$ fold-change (FC) between comparator groups were reported. Due to the large number (123) of PF proteins returned as significant between Controls and All SSID horses using the stringency criteria $p<0.05$, $\geq 2$ fold change, >1 peptide, this result was further adjusted for FDR $q<0.05$.

## Results

### *Protein identification*

A greater number of proteins were reported in PF than plasma in all cohorts, based on identification using >1 unique peptide. Numbers [mean±SD (range)] of plasma and PF proteins respectively were: control 274.5±5.9 (268-282) and 330.5±23.4 (303-350); survivors 298.5±22.5 (284 -332) and 335.2±7.8 (325-343); and non-survivors 292.5±4.7 (286-296) and 549±501.2 (122-1268). These differences were not statistically significant between groups ($p=0.08$ for plasma; $p=0.5$ for PF). Details for individual horses are given in Table 1.

### *Control versus all SSID horses*

Thirteen plasma proteins and 123 PF proteins were significantly differentially expressed (DE) between control and all SSID horses ($p<0.05$, $\geq 2$ fold change, >1 peptide). The number of significant PF proteins reduced to 45 when adjusted for false discovery rate ($q<0.05$). Four of these proteins were also significantly DE in plasma. Table S1 (Supplementary Information) lists these proteins and their distribution between fluid types, with UniProt accessions and number of unique peptides used for identification.

### *SSID survivors versus non-survivors*

Four plasma proteins and 12 PF proteins were DE between SSID horses which survived to hospital discharge and those which did not ($p<0.05$). None of these proteins were common to both fluids. Two proteins in both plasma (monocyte differentiation antigen CD14 precursor peptide and plasminogen) and PF (basic transcription factor 3 and proteasome subunit alpha 3) showed increased expression associated with non-survival, with the remainder significantly reduced. All 12 PF proteins were also significantly DE between control and all SSID horses, with direction of change consistent between controls and SSID and survival and non-survival, suggesting a continuum of expression change. Magnitude and direction of change of these 16 proteins is given in Table 2. Identification details of these proteins are given in Table S1 and normalised relative abundances are given in Table S2 (Supplementary Information).

## Discussion

The pathological changes occurring in SSID are complex and rapidly progressive, resulting from both obstruction to the gut lumen and partial or complete vascular occlusion. Small intestinal mucosa shows ultrastructural changes within minutes of ischaemic insult, becoming necrotic within 2-4 hours. Degenerative changes continue following restoration of perfusion and motility [12], complicating accurate prognostic assessment. Previous studies have attempted to identify pre-, intra- and post-operative physiological, surgical and biochemical factors [4, 8, 9, 13] which can inform likelihood of post-operative survival. This is the first study to use an unbiased approach to describe changes in plasma and PF proteomes and we report distinct differences in both biofluids between horses suffering from SSID and controls, and between horses with SSID which survived to hospital discharge and those which did not. Of the 45 PF proteins DE between controls and all SSID horses, 12 were also DE between survivors and non-survivors, suggesting a continuum of expression levels of these markers with increasing disease severity. Fewer plasma proteins were DE overall, and interestingly none of those associated with non-survival were significant at the control versus all SSID level, or common to significant PF proteins. The most clinically relevant outcome from this study is

identification of 16 proteins DE between the surviving and non-surviving cohorts and these proteins are discussed below.

*Plasma proteins*

Two of the four DE plasma proteins are associated with haemostatic balance. Beta tubulin 1 class VI is a cytoskeletal protein found only in platelets and mature megakaryocytes [14]. Upregulation in survivors indicates primary haemostatic activity in response to vascular degeneration. Plasmin is the predominant fibrinolytic enzyme found in the circulation and extracellular matrix and increased expression of its zymogen in non-survivors may reflect dysregulation of the pro-/anti-coagulation balance, predisposing to a more thrombogenic state.

The two other significant plasma proteins are mainly associated with cell survival and inflammation. Heat shock proteins (HSP) are typically expressed in the cytosol, nucleus or organelles, with HSP family A member 5 restricted to the endoplasmic reticulumin [15]. These proteins mediate intracellular trafficking and processing of both constitutively expressed proteins involved in cellular homeostasis and stress induced proteins associated with cell survival, consistent with our finding of highest expression in survivors.

Progressive loss of mucosal barrier function facilitates translocation of luminal Gram negative bacteria into the peritoneal cavity, from where they can be rapidly absorbed into the systemic circulation. Macrophage surface antigen CD14 acts in concert with the TLR4 receptor to co-ordinate and modulate the host immune response to bacterial lipopolysaccharide (LPS) [16]. Increased expression of its precursor peptide, demonstrated in our non-survival cohort, likely represents a response to increased systemic LPS challenge.

*Peritoneal fluid proteins*

A greater proportion of significant peritoneal fluid proteins were associated with inflammation. As a primary interface between the host and its external environment, the intestinal mucosa possesses both

innate and adaptive immune regulatory function, comprised of both discrete lymphoid aggregates and diffuse populations of leukocytes distributed throughout the mucosa [17].

The exopeptidase carboxypeptidase E (CPE) is produced by specialised entero-endocrine cells (EEC) and processes peptide hormones such substance P, somatostatin and vasoactive intestinal peptide to their active forms. Neuropeptides are expressed in the intestinal mucosa by EEC and peptidergic neurones closely associated with mucosal lymphoid tissue and mast cells and exhibit both pro- and anti-inflammatory effects, depending on specific peptide and sub-population of leukocyte [17]. The importance of CPE to maintenance of intestinal homeostasis has been demonstrated using *cpe-/-* mice, where loss of enzyme expression produced increased baseline mucosal levels of IL-6 and greater response to chemically induced colitis, an effect reversible by administration of recombinant enteric neuropeptides [18]. Reduced levels of CPE in the non-surviving cohort would suggest dysregulated cytokine expression in these animals, likely resulting in greater intestinal inflammation.

Circulating levels of retinol binding protein 4 (RBP4) correlate highly with insulin resistance, metabolic syndrome and cardiovascular disease in humans [19]. Present in glandular and smooth muscle cells of the small intestine, RBP4 is the sole retinol transport molecule in the blood and demonstrates indirect inhibition of insulin signalling through induction of pro-inflammatory cytokines via the JNK and TLR4 pathways [19]. Activation of NADPH oxidase and NF-κB has also been demonstrated in human vascular endothelial cells, inducing production of pro-inflammatory molecules involved in leukocyte recruitment and adherence [20]. Reduced expression with SSID and particularly in non-survivors is therefore interesting and contrary to expectations. However, RBP4 levels also correlate with dietary intake of retinol, whilst intense exercise and the acute phase protein TNFα both downregulate RBP4 expression [21]. Reduced expression may therefore reflect greater duration or severity of intestinal dysfunction and/or increased levels of pain-associated physical activity, whilst still being compatible with a systemic inflammatory response.

Leucine rich repeat neuronal 4 (LRRN4) is a validated and highly expressed marker of primary mesothelial cells [22]. The mesothelial cell monolayer comprising the visceral and parietal

peritoneum is highly reactive, capable of recognising microbial pathogen surface antigens following loss of intestinal barrier function and serosal leakage. Free floating mesenchymal cells become recruited to areas of tissue damage and under these conditions, mesothelial-mesenchymal transition (MMT) can occur, cells assuming a more fibroblast-like phenotype [23]. This increases the likelihood of serosal fibrosis and adhesion formation, predisposing to persistent dysmotility and recurrence of colic signs, major causes of death in the early post-operative period [4, 7]. Lowest LRRN4 levels in non-survivors may indicate greater MMT in this cohort, secondary to more extensive disease.

Effective mucosal barrier function relies on mechanical as well as immunological integrity [24]. Increased trans- and intra-mural tension from intraluminal fluid sequestration and intramural oedema and haemorrhage, compound the risk of disruption to tissue architecture already compromised by hypoxic necrosis of serosal mesothelium and mucosal epithelium. Several of our significant PF proteins exhibit important extracellular matrix (ECM) associated functions, often in conjunction with inflammatory or other properties.

Serpin family A member 1 is a serine proteinase inhibitor which, whilst primarily regulating the proteolytic activity of neutrophil elastase, also has activity against plasmin, thrombin and plasminogen activator, as well as functioning as an acute-phase protein with anti-inflammatory and immunomodulatory properties [25, 26]. Reduced expression in non-survivors would likely increase proteolytic activity in the ECM and contribute to inflammation and changes in coagulation.

Secretion of galectin 3 binding protein stimulates IL-6 expression in stromal cells [27] and its associated protein Galectin-3 is widely distributed in epithelial cells, dendritic cells, mast cells, neutrophils, monocytes/macrophages and lymphocytes, with reduced expression associated with reduced intracellular adhesion in human intestinal epithelial cells [28]. *Gal3-/-* mice exhibit obtunded peritoneal macrophage and lymphocyte infiltration and increased sensitivity of peritoneal macrophages to apoptotic stimuli [29]. Lowest expression in non-survivors may, therefore, indicate disruption to the inflammatory response as well as reduced mechanical integrity of the intestinal epithelial barrier.

A Disintegrin and Metalloprotease-like Decysin 1 (ADAMDEC1) possesses atypical metalloprotease activity, functioning independently of normal intrinsic inhibitory mechanisms regulating metalloproteases [30]. Interestingly, and consistent with our findings, higher expression appears to be protective. Down regulation is associated with Crohn's disease in human patients [31] and a role in intestinal immunity and inflammation has been shown using *Adamdec1-/-* mice. When exposed to oral *Citrobacter* or *Salmonella* challenge, knock-out mice demonstrated increased susceptibility to colitis, weight loss and mortality, associated with increased translocation of bacteria to the systemic circulation [31].

Peptidase inhibitor 16 (PI16) is an extracellular protease and potent inhibitor of MMP-2 highly expressed by endothelial cells subjected to normal and elevated shear stress. Profound downregulation by low or oscillatory flow states and pro-inflammatory cytokines increases susceptibility to proteolytic sub-endothelial matrix degradation and endothelial detachment [32]. Reduced expression would, therefore, be expected in isolated tissues with compromised perfusion and from circulating inflammatory cytokines and a disturbed systemic circulation, conditions consistent with SSID. Lower PI16 expression identified in non-survivors is compatible with greater compromise to vascular and intestinal wall integrity in these individuals.

The enzyme β-1, 4-glucuronyltransferase 1 initiates the process of glycosylation of the α-subunit of the transmembrane protein dystroglycan, acting as a priming enzyme for other glycosyltransferases to complete the complex post-translational modifications necessary to produce the functional molecule [33]. Dystroglycan is a ubiquitously expressed cell adhesion molecule with crucial roles in basement membrane assembly [34]. Extensively studied due to its role in muscular dystrophies, dystroglycan has also been shown to play an important role in the regulation of interactions between intestinal epithelial cells and the extracellular matrix [35]. Reduced expression associated with poor outcome may indicate a reduced ability to maintain intestinal epithelial barrier function.

Amine oxidase enzymes catalyse the oxidative deamination of primary amines, with the production of ammonia and hydrogen peroxide. Primary amine oxidase is found in serum, smooth muscle, adipose

tissue and endothelium of mammals [36], whilst diamine oxidase is expressed in high concentrations in the intestinal mucosa [37]. Plasma diamine oxidase levels increase during gut development, becoming static at maturity, and have been shown to alter reciprocally with experimentally induced small intestinal mucosal damage in the adult rat [37], compatible with lowest expression in our non-surviving SSID cohort.

Tenascins are ECM glycoproteins generally exhibiting limited expression in healthy adult tissues, but early upregulation at sites of injury and inflammation. Tenascins contain a fibrinogen-like globe (FBG) domain, conserved between all family members which has been shown to drive inflammation by TLR4 receptor activation, inducing NF-κB and IL-6, IL-8 and TNF synthesis [38]. Interestingly, tenascin-X shows an almost opposite pattern of activity and expression to other family members, being more highly constitutively expressed, particularly in connective tissue and the muscularis mucosae of the digestive tract, where it functions to regulate cell-matrix interactions and expression and assembly of fibrillar collagens and elastic fibres. Tenascin-X induces little or no NF-κB and cytokine activation, with weak or absent expression in early tissue injury, only becoming upregulated during resolution of injury, coincident with extracellular matrix assembly and maturation [38, 39]. Lowest expression in non-survivors is, therefore, consistent with ongoing inflammation and failure to resolve intestinal wall degradation.

The two PF proteins highest in the non-survivor cohort were basic transcription factor 3 like 4 (BTF3) and proteasome subunit alpha, type 3 (PSMA3). The general RNA polymerase II BTF3 is involved in cell cycle regulation and apoptosis, with increased expression demonstrated in a range of human carcinomas, promoting transcription and protein synthesis and reducing apoptotic activity [40]. PSMA3 forms the key substrate recognition element of the cells core protein degradation unit the 20S proteasome. Normally constitutively synthesised, increased and modified production is induced by TNF-α, IFN-γ or lipopolysaccharides [41]. Principally intracellular constituents, increases in both these proteins may indicate increases in both the anabolic and catabolic functions of cellular metabolism, or reflect leakage of intracellular content from ischaemic tissue.

Several limitations to this study must be recognised. Although ages and sexes were similar between survivors, non-survivors and controls, this represents a clinical cohort and it was, therefore, not possible to match these exactly. Typically for discovery proteomics, a relatively small number of samples was investigated, generating a comprehensive identification of proteins, but at the expense of sensitivity. A targeted approach, based on the proteins of interest identified would allow a greater sample throughput, increasing sensitivity, and this work is currently ongoing, as is validation of our findings in both dependent and independent cohorts. There is also the opportunity to extend this work to other types of colic for comparison. Although our findings suggest a continuum of expression levels of some PF proteins between controls, survivors and non-survivors, inclusion of horses with non-strangulating small intestinal disease would be a useful addition to further explore the clinical utility of developing our approach. Biological fluids are complex mixtures and highly abundant proteins can obscure changes in those expressed at lower concentrations. We utilised Proteominer™ beads in the initial stages of sample processing to address this complication. This technology selectively reduces the concentration of highly abundant proteins by their combination with peptide ligands anchored in a solid phase, reducing the dynamic range of protein concentrations in the sample, obtunding this masking effect. It is, however, possible that some residual effect remained. To maximise throughput of biological replicates, we did not run technical replicates or blanks between samples. We have previously identified minimal technical variation and sample carry-over with our workflow, but future work should incorporate these elements.

In conclusion, we have demonstrated a greater abundance of DE proteins in PF than plasma in response to SSID and between survivors and non-survivors. The proteins we have identified associated with non-survival to hospital discharge principally coordinate immunomodulation, cell adhesion and matrix integrity, haemostatic balance and cell cycle regulation, indicating the range of pathophysiological sequelae of SSID. Further analysis of these proteins and their associated pathways may identify potential targets for post-operative therapeutic intervention to aid in securing a successful outcome. Validating the utility of these proteins as biomarkers of prognosis also offers the potential to develop a stable-side test which may aid in clinical decision making.

## Manufacturers Details

a BD Microlance 3, Becton Dickinson Co Ltd., Drogheda, Ireland

b BD Vacutainer, Becton Dickinson Co Ltd., Plymouth, UK

c Bio-Rad Laboratories Ltd., Watford, UK

d Waters Ltd., Manchester, UK

e Promega UK Ltd., Southampton, UK

f Thermo Scientific, Hemel Hempstead, UK

g Matrix Science, London, UK

## References

1. Phillips, T.J. and Walmsley, J.P. (1993) Retrospective analysis of the results of 151 exploratory laparotomies in horses with gastrointestinal disease. *Equine Vet J* **25**, 427-431.

2. Christophersen, M.T., Dupont, N., Berg-Sørensen, K.S., Konnerup, C., Pihl, T.H. and Andersen, P.H. (2014) Short-term survival and mortality rates in a retrospective study of colic in 1588 Danish horses. *Acta Vet Scand* **56**, 20.

3. Allen, D., White, N.A. and Tyler, D.E. (1986) Factors for prognostic use in equine obstructive small intestinal disease. *J Am Vet Med Assoc* **189**, 777-780.

4. Mair, T.S. and Smith, L.J. (2005) Survival and complication rates in 300 horses undergoing surgical treatment of colic. Part 1: Short-term survival following a single laparotomy. *Equine Vet J* **37**, 296-302.

377 5. Freeman, D.E., Hammock, P., Baker, G.J., Goetz, T., Foreman, J.H., Schaeffer, D.J., Richter, R.A.,
378 Inoue, O. and Magid, J.H. (2000) Short and long term survival and prevalence of postoperative ileus
379 after small intestinal surgery in the horse. *Equine Vet J* **S32**, 42-51.

380 6. Proudman, C.J., Smith, J.E., Edwards, G.B. and French, N.P. (2002) Long term survival of equine
381 surgical colic cases. Part 1: Patterns of mortality and morbidity. *Equine Vet J* **34**, 432-437.

382 7. Morton, A.J. and Blikslager, A.T. (2002) Surgical and postoperative factors influencing short-term
383 survival of horses following small intestinal resection: 92 cases (1994-2001). *Equine Vet J* **34**, 450-
384 454.

385 8. Proudman, C.J., Edwards, G.B., Barnes, J. and French, N.P. (2005) Factors affecting long-term
386 survival of horses recovering from surgery of the small intestine. *Equine Vet J* **37**, 360-365.

387 9. Mair, T.S. and Smith, L.J. (2005) Survival and complication rates in 300 horses undergoing
388 surgical treatment of colic. Part 3: Long-term complications and survival. *Equine Vet J* **37**, 310-314.

389 10. Tarn, A.C. and Lapworth, R. (2010) Biochemical analysis of ascitic (peritoneal) fluid: what
390 should we measure? *Ann Clin Biochem* **47**, 397-407.

391 11. Zhu, W., Smith, J.W. and Huang, C-M. (2010) Mass spectrometry-based label-free quantitative
392 proteomics. *J Biomed Biotech* 2010, article ID 840518. doi: 10.1155/2010/840518.

393 12. White, N.A., Moore, J.N. and Trim, C.M. (1980) Mucosal alterations in experimentally induced
394 small intestinal strangulation obstruction in ponies. *Am J Vet Res* **41**, 193-198.

395 13. McCarthy, R.N. and Hutchins, D.R. (1988) Survival rates and post-operative complications after
396 equine colic surgery. *Aust Vet J* **65**, 40-43.

397 14. Yang, H., Ganguly, A. and Cabral, F. (2011) Megakaryocyte lineage-specific class VI β-tubulin
398 suppresses microtubule dynamics, fragments microtubules and blocks cell division. *Cytoskeleton* **68**,
399 175-187.

400 15. Daugaard, M., Rohde, M. and Jäättelä, M. (2007) The heat shock protein 70 family: highly
401 homologous proteins with overlapping and distinct functions. *FEBS Letters* **581**, 3702-3710.

402 16. Gangloff, S.C., Zähringer, U., Blondin, C., Guenounou, M., Silver, J. and Goyert, S.M. (2005)
403 Influence of CD14 on ligand interactions between lipopolysaccharide and its receptor complex. *J*
404 *Immunol* **175**, 3940-3945.

405 17. Stead, R.H., Bienenstock, J. and Stanisz, A.M. (1987) Neuropeptide regulation of mucosal
406 immunity. *Immunol Rev* **100**, 333-359.

407 18. Bär, F., Föh, B., Pagel, R., Schröder, T., Schlichting, H., Hirose, M., Lemcke, S., Klinger, A.,
408 König, P., Karsten, C.M., Büning, J., Lehnert, H., Fellerman, K., Ibrahim, S.M. and Sina, C. (2014)
409 Carboxypeptidase E modulates intestinal immune homeostasis and protects against experimental
410 colitis in mice. *Plos ONE* **9**, e102347. Doi:10.1371/journal.pone.0102347.

411 19. Norseen, J., Hosooka, T., Hammarstedt, A., Yore, M.M., Kant, S., Aryal, P., Kiernan, U.A.,
412 Phillips, D.A., Maruyama, H., Kraus, B.J., Usheva, A., Davis, R.J., Smith, U. and Kahn, B.B. (2012)
413 Retinol-Binding Protein 4 inhibits insulin signaling in adipocytes by inducing proinflammatory
414 cytokines in macrophages through a c-Jun N-terminal Kinase- and Toll-Like Receptor 4-dependent
415 and retinol-independent mechanism. *Mol Cell Biol* **32**, 2010-2019.

416 20. Farjo, K.M., Farjo, R.A., Halsey, S., Moiseyev, G. and Ma, J.X. (2012) Retinol-Binding Protein 4
417 induces inflammation in human endothelial cells by an NADPH oxidase- and Nuclear Factor Kappa
418 B-dependent and retinol-independent mechanism. *Mol Cell Biol* **32**, 5103-5115.

419 21. Kotnik, P., Fischer-Posovszky, P. and Wabitsch, M. (2011) RBP4: a controversial adipokine. *Eur*
420 *J Enodocrinol* **165**, 703-711.

421 22. Kanamori-Katayama, M., Kaiho, A., Ishizu, Y., Okamura-Oho, Y., Hino, O., Abe, M., Kishimoto,
422 T., Sekihara, H., Nakamura, Y., Suzuki, H., Forrest, A.R.R. and Hayashizaki, Y. (2011) LRRN4 and

423 UPK3B Are Markers of Primary Mesothelial Cells. *PLoS ONE* **6**, e25391.
424 doi:10.1371/journal.pone.0025391.

425 23. Mutsaers, S.E., Prele, C.M-A., Pengelly, S. and Herrick, S.E. (2016) Mesothelial cells and
426 peritoneal homeostasis. *Fertility and Sterility* **106**, 1018-1024.

427 24. Turner, J.R. (2009) Intestinal mucosal barrier function in health and disease. *Nature Reviews*
428 *Immunol* **9**, 799-809.

429 25. Farshchian, M., Kivisaari, A., Ala-aho, R., Riihilä, P., Kallajoki, M., Grénman, R., Peltonen, J.,
430 Pihlajaniemi, T., Heljasvaara, R. and Kähäri, V-M. (2011) Serpin Peptidase Inhibitor Clade A
431 Member 1 (SerpinA1) is a novel biomarker for progression of cutaneous squamous cell carcinoma.
432 *Am J Pathol* **179**, 1110–1119.

433 26. Greene, C.M., Marciniak, S.J., Teckman, J., Ferrarotti, I., Brantly, M.L., Lomas, D.A., Stoller,
434 J.K. and McElvaney, N.G. (2016) α-1 antitrypsin deficiency. *Nature Reviews* **2**, 1-17.

435 27. Fukaya, Y., Shimada, H., Wang, L-C., Zandi, E. and DeClerk, Y.A. (2008) Identification of
436 galectin-3-binding protein as a factor secreted by tumor cells that stimulates interleukin-6 expression
437 in the bone marrow stroma. *J Biol Chem* **283**, 18573–18581.

438 28. Jiang, K., Rankin, C.R., Nava, P., Sumagin, R., Kamekura, R., Stowell, S.R., Feng, M., Parkos,
439 C.A. and Nusrat, A. (2014) Galectin-3 regulates desmoglein-2 and intestinal epithelial intercellular
440 adhesion. *J Biol Chem* **289**, 10510-10517.

441 29. Hsu, D.K., Yang, R-Y., Pan, Z., Yu, L., Salomon, D.R., Fung-Leung, W-P. and Liu, F-T. (2000)
442 Targetted disruption of the Galectin-3 gene results in attenuated inflammatory responses. *Am J Pathol*
443 **156**, 1073-1083.

30. Lund, J., Olsen, O.H., Sørensen, E.S., Stennicke, H.R., Petersen, H.H. and Overgaard, M.T. (2013) ADAMDEC1 is a metzincin metalloprotease with dampened proteolytic activity. *J Biol Chem* **288**, 21367-21375.

31. O'Shea, N.R., Chew, T.S., Dunne, J., Marnane, R., Nedjat-Shokouhi, B., Smith, P.J., Bloom, S.L., Smith, A.M. and Segal, A.W. (2016) Critical role of the disintegrin metalloprotease ADAM-like Decysin-1 [ADAMDEC1] for intestinal immunity and inflammation. *J Crohn's and Colitis* **10**, 1417–1427.

32. Hazell, G.G.J., Peachey, A.M.G., Teasdale, J.E., Sala-Newby, G.B., Angelini, G.D., Newby, A.C. and White, S.J. (2016) PI16 is a shear stress and inflammation-regulated inhibitor of MMP2. *Scientific Reports* **6**, 39553; DOI: 10.1038/srep39553.

33. Praissman, J.L., Live, D.H., Wang, S., Ramiah, A., Chinoy, Z.S., Boons, G-J., Moremen, K. and Wells, L. (2014) B4GAT1 is the priming enzyme for the LARGE-dependent functional glycosylation of α-dystroglycan. *eLife* **3**, e03943. Doi: 10.7554/eLife.03943.

34. Mathew, G., Mitchell, A., Down, J.M., Jacobs, L.A., Hamdy, F.C., Eaton, C., Rosario, D.J., Cross, S.S. and Winder, S.J. (2013) Nuclear targeting of dystroglycan promotes the expression of androgen regulated transcription factors in prostate cancer. *Scientific Reports* **3**, 2792.

35. Driss, A., Charrier, L., Yan, Y., Nduati, V., Sitaraman, S. and Merlin, D. (2006) Dystroglycan receptor is involved in integrin activation in intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* **290**, G1228-G1242.

36. Stolen, C.M., Yegutkin, G.G., Kurkija¨rvi, R., Bono, O., Alitalo, K. and Jalkanen, S. (2004) Origins of Serum Semicarbazide-Sensitive Amine Oxidase. *Circ Res* **95**, 50-57.

37. Luk, G.D., Bayless, T.M. and Baylin, S.B. (1980) Diamine oxidase (histaminase). A circulating marker for rat intestinal mucosal maturation and integrity. *J Clin Invest* **66**, 66-70.

467 38. Zuliani-Alvarez, L., Marzeda, A.M., Deligne, C., Schwenzer, A., McCann, F.E., Marsden, B.D.,
468 Piccinini, A.M. and Midwood, K.S. (2017) Mapping tenascin-C interaction with toll-like receptor 4
469 reveals a new subset of endogenous inflammatory triggers. *Nature Communications* **8,** 1595. DOI:
470 10.1038/s41467-017-01718-7.

471 39. Valcourt, U., Alcaraz, L.B., Exposito, J-Y., Lethias, C. and Bartholin, L. (2015) Tenascin-X:
472 beyond the architectural function. *Cell Adhesion and Migration* **9**, 154-165.

473 40. Liu, Q., Zhou, J-P., Li, B., Huang, Z-C., Dong, H-Y., Li, G-Y., Zhou, K. and Nie, S-L. (2013)
474 Basic transcription factor 3 is involved in gastric cancer development and progression *World J*
475 *Gastroenterol* **19**, 4495-4503.

476 41. Jung, T. and Grune, T. (2013) The proteasome and the degradation of oxidised proteins: Part 1 -
477 structure of proteasomes. *Redox Biology* **1**, 178-182.

478

479 **Table 1. Details of control horses and pathology, surgical procedure and outcome of eight horses**

480 **with strangulating small intestinal disease.**

| | Number of proteins (concentration) | | PF gross appearance | Pathology/surgical procedure | Outcome |
|---|---|---|---|---|---|
| **Horse** | **Plasma** | **PF** | | | |
| 8yr old Dales gelding | 284 (68g/L) | 325 (40g/L) | Haemorrhagic | EFE; 6m resection; jejunoileostomy | Survived to discharge (32 days) |
| 17yr old cob gelding | 289 (69g/L) | 334 (8g/L) | Clear, pale yellow | PL; 2.75m resection; jejunojejunostomy | Survived to discharge (9 days) |
| 13yr old Clydesdale gelding | 289 (68g/L) | 339 (26g/L) | Serosanguinous | PL; 0.75m resection; jejunojejunostomy | Survived to discharge (9 days) |
| 14yr old Welsh Cob mare | 332 (62g/L) | 343 (22g/L) | Clear yellow | PL; 1.5m resection; jejunojejunostomy | Survived to discharge (10 days) |
| 6yr old Warmblood gelding | 292 (62g/L) | 122 (36g/L) | Sanguinous | MR; 3.5m resection; jejunojejunostomy | Euthanased 5 days later due to POR |
| 10yr old Irish Sport Horse mare | 296 (65g/L) | 1268* (58g/L) | Haemorrhagic | EFE; 6.75m resection; jejunojejunostomy | Euthanased 12 days later due to chronic intra-abdominal haemorrhage/shock |
| 19yr old Thoroughbred cross gelding | 296 (68g/L) | 327 (32g/L) | Serosanguinous | Cause of strangulation not identified at surgery; 6.5m resection; jejunoileostomy | Euthanased 4 days later due to POR |
| 17yr old Welsh Cob mare | 286 (65g/L) | 479 (42g/L) | Serosanguinous | PL; 3m resection; jejunojejunostomy | Euthanased 8 days later at relaparotomy; multiple adhesions |
| 12yr old Cob gelding | 272 (68g/L) | 350 (4g/L) | Clear, pale yellow | Control | |
| 18yr old Irish Draft cross gelding | 268 (59g/L) | 350 (2g/L) | Clear, colourless | Control | |
| 17yr old Irish Draft mare | 282 (66g/L) | 303 (1g/L) | Clear, colourless | Control | |
| 9yr old Irish Draft cross gelding | 276 (66g/L) | 319 (2g/L) | Clear, pale yellow | Control | |

PF peritoneal fluid; EFE epiploic foramen entrapment; PL pedunculated lipoma; MR mesenteric rent; POR post-operative reflux;* haemolysed sample.

481

**Table 2. Plasma and peritoneal fluid proteins significantly differentially expressed (>2 fold change in normalised abundance) for SSID survival versus non-survival analysis. Data also given for control versus SSID cases for peritoneal fluid proteins as the same proteins were significantly differentially expressed in both cohorts.**

| **Protein** | **Control versus SSID** | | | **Survival versus non-survival** | | |
|---|---|---|---|---|---|---|
| | **Max fold change** | **ANOVA (p)** | **Highest mean condition** | **Max fold change** | **ANOVA (p)** | **Highest mean condition** |
| **Peritoneal Fluid** | | | | | | |
| Carboxypeptidase E | -8.8 | 0.001 | Control | -5.8 | 0.007 | Survival |
| Retinol binding protein 4 | -3.2 | 0.017 | Control | -4.9 | 0.007 | Survival |
| Peptidase inhibitor 16 | -3.0 | 0.032 | Control | -4.8 | 0.012 | Survival |
| Tenascin-X | -3.1 | 0.007 | Control | -2.8 | 0.016 | Survival |
| ADAM-like decysin 1 | -4.1 | 0.006 | Control | -4.2 | 0.020 | Survival |
| Serpin family A member 1 | -3.2 | 0.001 | Control | -2.2 | 0.020 | Survival |
| Amine oxidase | -3.5 | 0.017 | Control | -3.1 | 0.024 | Survival |
| Beta-1,4-glucuronyltransferase 1 | -2.9 | 0.003 | Control | -2.5 | 0.026 | Survival |
| Leucine rich repeat neuronal 4 | -5.5 | 0.005 | Control | -4.1 | 0.028 | Survival |
| Galectin 3 binding protein | -7.6 | 0.0004 | Control | -4.1 | 0.029 | Survival |
| Basic transcription factor 3 like 4 | 23.8 | 0.030 | SSID | 16.3 | 0.035 | Non-Survival |
| Proteasome subunit alpha, type 3 | 21.1 | 0.032 | SSID | 13.4 | 0.036 | Non-Survival |
| **Plasma** | | | | | | |
| Heat shock protein family A (Hsp70) member 5 | | | | -2.3 | 0.03 | Survival |
| Tubulin beta 1 class VI | | | | -3.9 | 0.05 | Survival |
| Monocyte differentiation antigen CD14 precursor peptide | | | | 3.2 | 0.05 | Non-survival |
| Plasminogen | | | | 2.1 | 0.05 | Non-survival |

SSID – Strangulating Small Intestinal Disease.