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

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23

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.

51

52 **Introduction**

53 Colic due to small intestinal obstruction is associated with a significantly lower survival rate than
54 caecal or large intestinal obstruction [1, 2]. Likelihood of survival is further decreased with
55 strangulating rather than simple obstruction and if resection following ischaemic insult is required [3,
56 4]. The greatest mortality occurs in the first 7-10 days post-operatively, frequently as a result of
57 recurrent colic, post-operative ileus and cardiovascular instability consistent with endotoxaemia [5, 6,
58 7]. There are also significant welfare and financial concerns associated with subjecting a horse to
59 major abdominal surgery and these concerns are particularly relevant when the prospects for short
60 term survival may be poor.

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

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

86 **Materials and Methods**

87 _Sample collection_

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

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

107 *Sample preparation*

108 Five milligrams of plasma and PF from each horse was bound to 10 µL of ProteoMiner™ Beads[c] to
109 compress the dynamic range of proteins. Beads were washed with phosphate buffered saline prior to
110 binding and mixed with plasma or PF for 2 hours at room temperature, then washed with 25 mM
111 ammonium bicarbonate (AmBic). One hundred and fifty microliters of 25 mM AmBic and 10 µL of
112 0.05% Rapigest™ [d] was added to the beads and shaken at 550 rpm for 10 min at 80°C. Samples were
113 reduced by the addition of 10 µL of 60 mM dithioreitol and incubated at 60°C for 10 minutes, then
114 alkylated by the addition of 10 µL of 180 mM iodoacetamide and incubated at room temperature for
115 30 minutes in the dark. Proteomic grade trypsin[e] was reconstituted in 25 mM acetic acid to a
116 concentration of 0.2 µg μL^{-1} and 10 µL added to the samples to achieve an enzyme:protein ratio
117 approximately 1:50 w/w. Following overnight incubation on a rotating mixer at 37°C, digestion
118 was terminated and Rapigest™ [d] removed by acidification with 1 µL trifluoroacetic acid and
119 incubation at 37°C for 45 min, then centrifugation at 15,000 rcf for 30 min. To check for complete
120 digestion each sample was analysed pre- and post-acidification by SDS-PAGE.
121

122 *LC-MS/MS*

123 For LC-MS/MS analysis, a 2 µL injection was analysed using an Ultimate 3000 RSLC™ nano
124 system[f] coupled to a QExactive™ mass spectrometer[f]. Samples were loaded onto the trapping column
125 PepMap100, C18, 300 µm x 5 mm[f] using partial loop injection, for seven minutes at a flow rate of 4
126 µL min^{-1} with 0.1% (v/v) FA. Samples were then resolved on the analytical column Easy-Spray C18
127 75 µm x 500 mm 2 µm column[f], using a gradient of 97% A (0.1% formic acid), 3% B (99.9% ACN
128 0.1% formic acid), to 60% A, 40% B, over 90 minutes, at a flow rate of 300 nL min^{-1}. A spray

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

132

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

139 *Data analysis*

140 Label free relative quantification was performed with Progenesis QI™ version 2[d], with plasma and
141 peritoneal fluid analyses handled independently. The Progenesis QI workflow creates a virtual
142 aggregate run comprising all data from individual samples, allowing features to be cross identified
143 from other samples. This overcomes the stochastic sampling limitations of DDA when the sample is
144 too complex for the duty cycle of the mass spectrometer. Respective abundance values of all proteins
145 identified in all samples are reported, rather than qualitative lists of protein identifications for each
146 sample. Samples were aligned according to retention time using a combination of manual and
147 automatic alignment. Default peak picking parameters were applied and features with charges from
148 1+ to 4+ featuring three or more isotope peaks were retained. A Mascot Generic File[g] created by
149 Progenesis QI™ was searched against the Uniprot reference proteome for horse (downloaded April
150 2015, 22,963 entries). A fixed carbamidomethyl modification for cysteine and variable oxidation
151 modification for methionine were specified. A precursor mass tolerance of 10 ppm and a fragment ion
152 mass tolerance of 0.01 Da were applied. To maximise the number of proteins identified for
153 subsequent quantification, protein identification threshold was set at >1 unique peptide. The results

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

162 **Results**

163 *Protein identification*

164 A greater number of proteins were reported in PF than plasma in all cohorts, based on identification
165 using >1 unique peptide. Numbers [mean±SD (range)] of plasma and PF proteins respectively were:
166 control 274.5±5.9 (268-282) and 330.5±23.4 (303-350); survivors 298.5±22.5 (284 -332) and
167 335.2±7.8 (325-343); and non-survivors 292.5±4.7 (286-296) and 549±501.2 (122-1268). These
168 differences were not statistically significant between groups ($p=0.08$ for plasma; $p=0.5$ for PF).
169 Details for individual horses are given in Table 1.

170 *Control versus all SSID horses*

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

177 *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

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

205 *Plasma proteins*

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

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

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

224 *Peritoneal fluid proteins*

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

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

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

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

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

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

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

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

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

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

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

295 The enzyme β-1, 4-glucuronyltransferase 1 initiates the process of glycosylation of the α-subunit of
296 the transmembrane protein dystroglycan, acting as a priming enzyme for other glycosyltransferases to
297 complete the complex post-translational modifications necessary to produce the functional molecule
298 [33]. Dystroglycan is a ubiquitously expressed cell adhesion molecule with crucial roles in basement
299 membrane assembly [34]. Extensively studied due to its role in muscular dystrophies, dystroglycan
300 has also been shown to play an important role in the regulation of interactions between intestinal
301 epithelial cells and the extracellular matrix [35]. Reduced expression associated with poor outcome
302 may indicate a reduced ability to maintain intestinal epithelial barrier function.

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

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

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

322 The two PF proteins highest in the non-survivor cohort were basic transcription factor 3 like 4 (BTF3)
323 and proteasome subunit alpha, type 3 (PSMA3). The general RNA polymerase II BTF3 is involved in
324 cell cycle regulation and apoptosis, with increased expression demonstrated in a range of human
325 carcinomas, promoting transcription and protein synthesis and reducing apoptotic activity [40].
326 PSMA3 forms the key substrate recognition element of the cells core protein degradation unit the 20S
327 proteasome. Normally constitutively synthesised, increased and modified production is induced by
328 TNF-α, IFN-γ or lipopolysaccharides [41]. Principally intracellular constituents, increases in both
329 these proteins may indicate increases in both the anabolic and catabolic functions of cellular
330 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.

358 **Manufacturers Details**

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

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

361 c Bio-Rad Laboratories Ltd., Watford, UK

362 d Waters Ltd., Manchester, UK

363 e Promega UK Ltd., Southampton, UK

364 f Thermo Scientific, Hemel Hempstead, UK

365 g Matrix Science, London, UK

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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

482 **Table 2. Plasma and peritoneal fluid proteins significantly differentially expressed (>2 fold**
483 **change in normalised abundance) for SSID survival versus non-survival analysis. Data also**
484 **given for control versus SSID cases for peritoneal fluid proteins as the same proteins were**
485 **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.

486