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Candidate circulating microRNA biomarkers in dogs with chronic pancreatitis

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17

18   ABBREVIATIONS : *C. elegans*, *Caenorhabditis elegans*; cDNA, complementary deoxyribonucleic

19   acid; CPM, Counts per million reads mapped; DNA, deoxyribonucleic acid; HfSA, Hospital for

20   Small Animals; FC, Log_2 Fold Change; FDR, False discovery rate; IPA; Ingenuity Pathway

21   Analysis; IQR: Inter-quartile range; M; million; miR, microRNA; qPCR, quantitative polymerase

22   chain reaction; TMM, Trimmed mean of M values; UMI, unique molecular identifier; R(D)SVS,

23   Royal(Dick)School of Veterinary Studies; RNA, ribonucleic acid.

24

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28

29 WHERE THE WORK WAS DONE: This work was undertaken when all authors worked solely at

30 the University of Edinburgh.

31

33

34 CONFLICTS OF INTEREST: RJM is currently employed by Idexx Laboratories Inc and holds

35 stock/stock options with Idexx Laboratories, Inc. AG now works at Zoetis and holds stock/stock

36 options with Zoetis.

37

38 OFF-LABEL ANTIMICROBIAL DECLARATION: Authors declare no off-label use of
39 antimicrobials

40

46 INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL

47 DECLARATION: Ethical approval for use of biobanked samples for this study was approved by

48 the University of Edinburgh Veterinary Ethics Committee (Approval number: 117.22).

49   HUMAN ETHICS APPROVAL DECLARATION: Authors declare human ethics approval was not

50   needed for this study.

51   ABSTRACT

52   **Background:** Pancreatitis is an important cause of disease and death in dogs. Available

53   circulating biomarkers are not sufficiently sensitive and specific for a definitive diagnosis.

54   **Hypothesis:** Circulating microRNAs would be differentially expressed in dogs with chronic

55   pancreatitis and could have potential as diagnostic biomarkers.

56   **Animals:** Healthy controls (n=19) and dogs with naturally occurring pancreatitis (n=17).

57   **Methods:** A retrospective case-control study. Dogs with pancreatitis were included if they

58   satisfied diagnostic criteria for pancreatitis as adjudicated by three experts. MicroRNA was

59   extracted from stored serum samples and sequenced. Reads were mapped to mature microRNA

60   sequences in the dog, mouse and human genomes. Differentially-expressed microRNAs were

61   identified and the potential mechanistic relevance explored using Qiagen Ingenuity Pathway

62   Analysis (IPA).

63   **Results:** Reads mapping to 196 mature microRNA sequences were detected. Eight circulating

64   microRNAs were significantly differentially expressed in dogs with pancreatitis (≥2-fold change

65   and false discovery rate <0.05). Four of these mapped to the canine genome (cfa-miR-221, cfa-

66   miR-222, cfa-miR-23a and cfa-miR-205). Three mapped to the murine genome (mmu-miR-484,

67   mmu-miR-6240, mmu-miR-101a-3p) and one to the human genome (hsa-miR-1290). Expression

68   in dogs with pancreatitis was higher for seven microRNAs and lower for mmu-miR-101a-3p.

69   Qiagen IPA demonstrated a number of the differently expressed microRNAs are involved in a

70   common pancreatic inflammatory pathway.

71   **Conclusions:** The significantly differentially expressed microRNAs represent promising

72   candidates for further validation as diagnostic biomarkers for canine pancreatitis.

73

74   Pancreatitis in dogs is a highly debilitating and painful disease. It is estimated that over 30% of

75   dogs will develop some degree of pancreatitis in their lifetime, with prevalence of chronic

76   pancreatitis as high as 34% (51/151) in one study of dogs at post-mortem. [1,2] Chronic pancreatitis

77   in dogs is associated with refractory pain and reduced quality of life, precipitating progressive life-

78   limiting impairment of pancreatic function. [3–6]   There are currently few substantive

79   pathophysiological studies in dogs with naturally occurring pancreatitis, so disease insight is often

80   extrapolated from humans and animal models. [7–10]

81

82   Ante-mortem diagnosis of pancreatitis in dogs is challenging, relying heavily on clinical history

83   and a combination of diagnostic tests, all of which have limitations. [3,11] Histopathology, is generally

84   considered the reference diagnostic standard, but is imperfect. [11–13] Improvements are reported

85   in evaluating the pancreas through non-invasive imaging techniques such as ultrasonography

86   and computed tomography, but a number of factors, including operator experience, can highly

87   influence their diagnostic utility. [4,14–19]

88

89   All currently available circulating biomarkers for pancreatitis diagnosis have sub-optimal testing

90   accuracy depending on severity of disease, highlighting the need for better biomarker candidates.

91   [11,14,20–24] The most frequently used assay is canine pancreas-specific lipase immunoreactivity

92   (cPL), available as both a quantitative (Spec cPL™) assay and a semi-quantitative point-of-care

93   (SNAP®) cPL™ test. [11,14,21] Spec cPL™ is generally considered the most sensitive and specific

94   test for pancreatitis in dogs, but requires analysis in a reference laboratory. [21,24] In one study,

95   dogs with concurrent acute and chronic pancreatitis, Spec cPL™ sensitivity was moderate (71%)

96   and specificity was variable depending on the cPL™ cut-off used (86 – 100%).[24] In eight dogs

97   with chronic pancreatitis 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester

98   (DGGR) lipase specificity was 100%, but sensitivity was only 57%.[25,26] MicroRNAs are emerging

99   as sensitive and specific markers of pancreatitis in humans. [27–29] We hypothesized that small

100   RNA-sequencing of serum from dogs with pancreatitis and undertaking post-sequencing analysis

101   would lead to identification of targeted microRNAs that could be further interrogated as diagnostic

102   biomarkers of naturally occurring chronic pancreatitis in dogs.

103

104   MATERIALS & METHODS

105   A retrospective study, for which all dogs were enrolled for at the Royal (Dick) School of Veterinary

106   Studies, University of Edinburgh, UK. Dogs presented to the Hospital for Small Animals (HfSA)

107   as referral cases or for routine appointments. They were only considered for the study if a blood

108   sample was undertaken as part of their routine clinical investigation and residual serum was

109   stored in the HfSA biobank. Dogs were initially selected (n = 24) by Veterinary nomenclature

110   (VeNOM) retrospective search for a diagnosis of pancreatitis. Dogs allocated a diagnosis of

111   pancreatitis then retrospectively had their pancreatitis grade scored by three blinded HfSA senior

112   boarded medicine clinicians according to the system described by McCord et al. in the absence

113   of a validated chronic pancreatitis score. [21] In brief, dogs were stratified by a likelihood score for

114   pancreatitis of 0 – 4. For this 0: definitely not pancreatitis, 1: probably not pancreatitis, 2; possibly

115   pancreatitis, 3: probably pancreatitis, 4: definitely pancreatitis. Only dogs graded 2 and above

116   were included in the study (n = 17). Three dogs were graded as probably having pancreatitis

117   (grade 3), and 14 dogs for possibly having pancreatitis (grade 2). Each dog was independently

118   scored after reviewing the history, results of routine haematology, serum biochemistry, SNAP®

119   cPL™ test, cPL™ assay, abdominal ultrasound, further diagnostic procedures, including

120   histopathology, final outcome and final diagnosis as available. The panel evaluated the dogs

121 together, but assigned a score independently. Blood samples from healthy dogs exhibiting no

122 signs of systemic disease were included in the study as controls. These samples were already

123 present in the existing biobank. Attempt was made to match control samples as far as possible to

124 dogs with pancreatitis for age, sex, breed and time in storage. This study was approved by the

125 University of Edinburgh Veterinary Ethics Research Committee (Approval number: 117.22).

126 Sample storage and RNA isolation

127 Serum was placed in -20°C within twelve hours of sample collection. The serum was then

128 transferred within 24 hours to long term storage at -80°C.[30,31] Mean blood sample storage time

129 was 1,653 days (range: 0 – 2,144 days) for the control group and 1,641 days (range: 0 – 2,316

130 days) for the pancreatitis group (P = 0.833). MicroRNA was extracted in two batches using a

131 miRNeasy Serum/Plasma kit (Qiagen, The Netherlands) following the manufacturer's guidelines

132 and as per Vliegenthart *et al.* and Oosthuyzen *et al.* 2018. [32,33] Briefly, total RNA was extracted

133 from 50 µL of serum diluted in 150 µL nuclease free water. RNA was extracted using lysis reagent

134 (1000 µL) and chloroform (200 µL). The RNA was purified on a RNeasy miniElute spin column

135 and eluted in 15 µL RNase-free water and stored at −80°C. Extraction efficiency was assessed

136 by adding 6 x 10^9 copies/µl of synthetic *C. elegans* miR-39 spike-in control (Norgen Biotek,

137 Canada) after the addition of lysis reagent.

138

139 Library Preparation

140 Thirty-six sequencing libraries were prepared from microRNA samples using the QIAseq miRNA

141 library kit according to the provided protocol for RNA isolated from serum, 5µL of each RNA

142 sample was used as input to library construction. In this protocol, a unique molecular identifier

143 (UMI) was introduced during cDNA synthesis, to facilitate read counting in the sequencing

144 dataset. Libraries were purified using QIASeq miRNA NGS Beads (QMN beads). Purified libraries

145 were then amplified for 22 cycles of PCR. Amplified libraries were also purified and size selected

146 with QMN beads to enrich for library fragments from microRNA.

147

148 Library Quality Control

149 Libraries were quantified by fluorometry using the Qubit dsDNA HS assay and assessed for

150 quality and fragment size using the Agilent Bioanalyser with the DNA HS Kit. Libraries were

151 purified by electrophoresis on 3% agarose gels, to remove fragments of inappropriate sizes, such

152 as adapter-dimers. The final library was then quantified by fluorometry and assessed on the

153 Agilent Bioanalyser to ensure removal of adapter-dimers.

154

155 Sequencing

156 Sequencing was performed on the NextSeq 2000 platform (Illumina Inc, USA) using NextSeq

157 1000/2000 P2 Reagents (100 Cycles) v3. PhiX Control v3 library (Illumina Inc, USA) was spiked

158 in at a concentration of one percent to enable troubleshooting in the event of run failure.

159 Sequencing was single-end 1x75.

160 Data analysis

161 Data were summarized as median and range for age of study subjects. Sample time in storage

162 analysis was calculated by non-parametric Mann-Whitney U test. Nominal P values equal to or

163 less than .05 were considered significant. Statistical analyses were performed using Graphpad

164 Prism (GraphPad Software, La Jolla, California, v9). Small RNA-sequencing reads were trimmed

165 using cutadapt, retaining reads between 18 and 30 nucleotides. Reads sharing a unique

166 molecular identifier (UMI) were identified with umi_tools and de-duplified using the "unique"

167 method. Reads were mapped first to the spike-in sequence (cel-miR-39) then unmapped reads

168 were mapped sequentially to mature microRNA sequences in the dog, then human, then mouse

169 genomes (miRBase release 22.1). Mapping was performed using Bowtie, allowing up to 1

170   nucleotide mismatch within a 32 nucelotide seed region and ignoring reads mapping to more than

171   one region.

172   Differential expression analysis was performed in R (version 4.1.3) using edgeR (v3.36.0). Low-

173   abundance reads were filtered out and then counts were normalized using the TMM (trimmed

174   mean of M-values) method. Differential expression was determined using a 2-group generalized

175   log-linear model. Genes were deemed to be differentially expressed if their expression was at

176   least 2-fold different between the control and pancreatitis groups and false-discovery rate was

177   less than 5% after adjustment for multiple testing using the Benjamini & Hochberg method. [34] The

178   heatmap was generated using the pheatmap package (v1.0.12); all other plots were generated

179   using the tidyverse (v1.3.1) and ggplot2 (v3.3.5). The code for our miRNAseq analysis pipeline

180   is publicly accessible at https://zenodo.org/badge/latestdoi/468419301.   Further analysis of the

181   significantly differentially expressed microRNAs was conducted by generating networks through

182   the use of QIAGEN Ingenuity Pathway Analysis. [35]

183   RESULTS

184

185   Dog characteristics

186   There were samples from 17 dogs with pancreatitis (Grade 2 and above) and 19 samples from

187   healthy dogs. Seventeen of the dogs were male (47%) and nineteen were female (53%). Ten

188   (9/17) dogs with pancreatitis had no known concomitant disease at time of presentation.

189   Characteristics of the dogs including sex, age, and breed are summarized in Table 1.

190

191   Small RNA-sequencing Analysis

192   In the raw sequencing dataset, there were a median of 13.7 million (M) reads per library (Inter-

193   quartile range: 12.9M – 14.7M). After trimming and de-duplication of UMIs, median library size

194   was 7.3M reads (6.6M – 8.0M) and the reads mapped to 756 unique mature microRNA

195   sequences. After filtering out microRNAs expressed at very low abundance, median library size

196   was 444,000 and reads mapped to 196 mature microRNAs. Of these, 113 had higher expression

197   in the pancreatitis group and 83 had lower expression in the pancreatitis group.   Seven

198   microRNAs were significantly differentially upregulated in dogs with pancreatitis; one microRNA

199   was downregulated (Table 2; log $_2$ (fold-change) +2, FDR <5%, P<0.05). Of these four mapped

200   to the canine (cfa) genome, three to murine (mmu) and one to human (hsa) genomes. We present

201   these data as a table (Table 2), Volcano plot (Figure 1), heatmap (Figure 2) and dotplots to show

202   the expression patterns of each differentially-regulated microRNA (Figure 3).   Correlation plots

203   between the microRNAs showed expression was significantly correlated with two sets of

204   microRNAs: miR-222, miR-221 and miR-23a; and miR-23a, miR-484 and miR-1290

205   (Supplementary Figure 1). Principal component analysis was conducted and revealed that the

206   control and pancreatitis groups formed (overlapping) clusters illustrating global changes in dogs

207   diagnosed with pancreatitis (Figure 4). The third principal component, which accounted for 11.2%

208   of the total variance, was most significantly associated with disease status (P = 0.00014 by

209   ANOVA).

210

211   Using Qiagen IPA databases, analysis was performed on the network of potential interactions

212   among the set of eight differentially expressed genes, focusing on molecular pathways involved

213   in inflammation of the pancreas. A Qiagen IPA network/My Pathways is a graphical representation

214   of the molecular relationships between molecules. All edges (relationship between two nodes)

215   are supported by at least one reference from the literature, from a textbook, or from canonical

216   information stored in the Qiagen Knowledge Base. Human, mouse, and rat orthologs of a gene

217   are stored as separate objects in the Qiagen Knowledge Base, but are represented as a single

218   node in the network. Four of eight microRNAs (miR-23a, miR-101a-3p, miR-205, miR-221) were

219   identified as potentially involved in different (one or more) pancreatitis-related pathways (Figure

220   5).

221 DISCUSSION

222

223 This study used small RNA-sequencing to globally assess circulating microRNAs in dogs with

224 naturally occurring chronic pancreatitis. We identified eight significantly differentially expressed

225 microRNAs. Pancreatic injury induced using a caerulein infusion in two dog studies identified

226 microRNAs which could be utilized for pancreatitis diagnosis. [36,37] Higher levels of miR-216a,

227 miR-216b, and miR-217, miR-375 and miR-148a are detected in dogs when pancreatitis is

228 induced. [37] Of twenty dogs with naturally occurring acute pancreatitis, a significant difference in

229 the serum expression of cfa-miR-375 was found between dogs with acute pancreatitis (median:

230 3.59) and healthy dogs (0.81; $P < .001$), but miR-216a was not significantly elevated in this

231 population. [38] None of these microRNAs were in the top twenty differentially expressed

232 microRNAs in our study cohort. The difference in microRNA expression profiles between these

233 studies and the current work could be explained by the acute pancreatitis phenotype and the use

234 of the caerulein infusion, which is more a model of acute pancreatitis. [10] Caerulein infusion can

235 result in highly variable fibrosis and infusion rates vary between studies, which could also

236 contribute to the altered microRNA profiles. [6] The differences between these studies supports

237 the investigation of naturally occurring pancreatitis in dogs rather than injury models and

238 differentiating between acute and chronic pancreatitis.

239

240 The unbiased sequencing approach used in this study identified significantly differentially

241 expressed microRNAs that are consistent with a potential mechanistic role in the pathogenesis of

242 pancreatitis, as in humans. [27,39] The conserved miR-221/222 cluster is an important regulator in

243 multiple cellular processes, which is encoded tandemly in chromosome Xp11.3, and are highly

244 homologous miRNAs sharing the same seed sequence and as a result were not differentiated by

245 the Qiagen IPA analysis. [40] A dog in this study had the highest levels of both miR-211 and miR-

246 222, although high outliers of other microRNAs were from different dogs. Hsa-miR-221 is a distinct

247 biomarker of human early chronic pancreatitis predicting early chronic pancreatitis with AUC of

248 100.0%. [29] MiR-221/222 are regulators of proliferation of pancreatic β-cells and inhibit insulin

249 production of pancreatic β-cells in mice so their higher levels during pancreatic inflammation in

250 these dogs would make biological sense. [41] Qiagen IPA linked miR-221/222 with inhibiting

251 intracellular adhesion molecule- 1 (ICAM-1). Pancreatic acinar cells upregulate the expression of

252 ICAM-1 as part of tumorigeneses to attract macrophages, so there could be a similar role for

253 miR-221/222 to translate ICAM-1 during chronic pancreatic inflammation. [42]

254

255 MiR-23a was higher in dogs with pancreatitis. MiR-23a and miR-23b concentrations are higher in

256 human patients with severe acute pancreatitis (alongside hsa-miR-1260b, -762, -22-3p). [43] MiR-

257 23 promotes proliferation of pancreatic cancer cells and block apoptotic pathways. [44] Qiagen IPA

258 implicated an association with miR-23a and the Smad family (Smad3 and Smad4). Activation of

259 this pathway by miR-23a and a role in pancreatic inflammation makes biological sense as Smad

260 family proteins have a central role in pancreatic fibrosis by activating the proliferation of

261 pancreatic stellate cells. [45–47]

262 MiR-484 has utility as a biomarker for a number of diseases including pancreatic adenocarcinoma

263 and putatively targets the *NOTCH3* gene in humans with chronic pancreatitis. [48,49] The Notch

264 signaling pathway is active during pancreatic development and the reactivates during injury. [50]

265 *NOTCH3* expression is higher in the ducts of patients with chronic pancreatitis and is directly

266 associated with fibrosis in other organs. [48,51,52]

267 MiR-101-3p was the only gene down-regulated in this study. Qiagen IPA indicated a role of miR-

268 101 upregulating inducible co-stimulator (ICOS) and prostaglandin-2 (PTSG2). ICOS and PTGS2

269 are over-expressed in the pancreas of humans with autoimmune pancreatitis and chronic

270 pancreatitis, so this is also considered a relevant target to further examine in dogs with

271 pancreatitis. [53–56]

272 The limitations of this study include small sample groups, dog variability, lack of histopathology in

273 all dogs and complicating concomitant disease in some of the dogs, which potentially influenced

274 the pancreatitis scores assigned.[6,36,37,57] The McCord scale is validated for acute pancreatic

275 disease, not the more chronic phenotype of the dogs in this study, so this could have also

276 negatively influenced pancreatitis grade scoring. Canine pancreas-specific lipase

277 immunoreactivity was not measured in the control dog group, given the very poor sensitivity (21%)

278 in dogs with mild pancreatitis. [14,24] Most of these limitations reflect the difficulties of researching

279 pancreatitis in naturally occurring clinical disease.

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