1 DATASET BRIEF

2 **Revisiting the *Ancylostoma caninum* secretome provides new information on** 3 **hookworm-host interactions**

4 Taylor Morante^a, Catherine Shepherd^b, Constantin Constantinoiu^c, Alex Loukas^{b#} and
5 Javier Sotillo^{b#}

6
7 ^a College of Public Health, Medical & Veterinary Sciences, James Cook University,
8 Cairns, Queensland, Australia.

9 ^b Centre for Biodiscovery and Molecular Development of Therapeutics, Australian
10 Institute for Tropical Health and Medicine, James Cook University, Cairns,
11 Queensland, Australia.

12 ^b College of Public Health, Medical & Veterinary Sciences, James Cook University,
13 Townsville, Queensland, Australia.

14
15 [#] Corresponding Authors:

16 Javier Sotillo. Centre for Biodiscovery and Molecular Development of
17 Therapeutics, James Cook University, Cairns, 4878, Queensland, Australia. Email:
18 javier.sotillo@jcu.edu.au

19 Alex Loukas. Centre for Biodiscovery and Molecular Development of
20 Therapeutics, James Cook University, Cairns, 4878, Queensland, Australia. Email:
21 alex.loukas@jcu.edu.au

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25 ABREVIATIONS

26 AcES: *Ancylostoma caninum* excretory/secretory products

27 DTT: Dithiothreitol

28 ESPs: excretory/secretory products

29 GST: Glutathione-s-transferase

30 IAM: iodoacetamide

31 TIMPs: Tissue inhibitor of metalloproteases

32 SCPs: SCP/Tpx-1/Ag5/PR-1/Sc7 domain containing proteins

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34 **KEYWORDS:** *Ancylostoma caninum*, hookworm, proteomics, secretome,
35 excretory/secretory products, vaccines, immunomodulation.

36

37 ABSTRACT

38 Hookworm infection is a major tropical parasitic disease affecting almost 500 million
 39 people worldwide. These soil-transmitted helminths can survive for many years in the
 40 intestine of the host, where they feed on blood, causing iron deficiency anaemia and
 41 other complications. To avoid the host's immune response the parasite releases
 42 excretory/secretory products (ESPs), a complex mixture of glycans, lipids and
 43 proteins that represent the major host-parasite interface. Using a combination of
 44 separation techniques such as SDS-PAGE and OFFGEL electrophoresis, in
 45 combination with state-of-the-art mass spectrometry we have reanalysed the dog
 46 hookworm, *Ancylostoma caninum*, ESPs (AcES). We identified 315 proteins present
 47 in the AcES, compared with just 105 identified in previous studies. The most highly
 48 represented family of proteins is the SCP/TAPs (90 of the 315 proteins), and the most
 49 abundant constituents of AcES are homologues of the tissue inhibitors of
 50 metalloproteases (TIMP) family. We identified putative vaccine candidates and
 51 proteins that could have immunomodulatory effects for treating inflammatory
 52 diseases. This study provides novel information about the proteins involved in host-
 53 hookworm interactions, and constitutes a comprehensive dataset for the development
 54 of vaccines and the discovery of new immunoregulatory biologics.

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57 Soil-transmitted helminthiases (including trichuriasis, ascariasis and hookworm
58 infections) are debilitating parasitic diseases that affect more than two billion people
59 worldwide [1], with increased incidence occurring in impoverished and
60 underdeveloped societies. Hookworms alone affect almost 500 million people in
61 tropical regions of South America, Africa and Asia [2], and chronic infections result
62 in iron-deficiency anaemia and even physical and intellectual retardation in young
63 children [3]. Adult hookworms live in the intestine of vertebrate hosts where they
64 feed on blood, and constantly release products into their surrounding environment
65 through excretion and secretion mechanisms (excretory/secretory products, ESPs).
66 The ESPs contain proteins that facilitate a parasitic existence, notably penetration of
67 and migration within a host, feeding on host tissues, and evasion of the host immune
68 response [4]. In addition, recently, hookworm ESPs have been shown to contain
69 immunoregulatory properties that can protect mice against inflammatory diseases
70 such as inflammatory bowel diseases and asthma [5-9].

71 Due to the difficulty in obtaining samples from the human hookworm *Necator*
72 *americanus*, the dog hookworm *Ancylostoma caninum* has been extensively used as a
73 model to study hookworm infections. The first proteomic characterisation of the ESPs
74 produced by *A. caninum* (AcESP) was performed by Mulvenna et al. in 2009 [10];
75 however, herein we revisit this data since the *A. caninum* genome was not available at
76 the time and the sensitivity of mass spectrometers has improved dramatically since
77 this last study was conducted.

78 A total of ~300 *A. caninum* adult worms were obtained from the small intestine of 5
79 fresh cadaver dogs that had been naturally infected. Worms were divided into two
80 different batches and incubated in 5x substrate (Dulbecco's Phosphate Buffered
81 Saline (DPBS) (+) CaCl₂ (+) MgCl₂, 5% antimycotic/antibiotic, 1% Glutamax) for 2 h

82 at 37°C and 5% CO₂ to reduce bacterial contamination. Hookworms were then
83 transferred to 2x substrate and incubated for a further 24 h at 37°C and 5% CO₂ at a
84 rate of ~ 50 worms per 25 ml of media.

85 A total of 50 µg of AcESP from batch 1 was separated by SDS-PAGE and 18 bands
86 were excised from the gel, reduced using dithiothreitol (DTT), alkylated with
87 iodoacetamide (IAM) and digested with trypsin overnight as described previously
88 [10]. One hundred (100) micrograms of AcESP from batch 2 was reduced and
89 alkylated using DTT and IAM, respectively followed by trypsin digestion overnight.
90 Peptides were separated using an OFFGEL fractionator as previously described [10].
91 All samples were desalted using C18 ZipTips after SDS-PAGE or Offgel separation.
92 Peptides were analysed using a Shimadzu Prominence Nano HPLC coupled to an AB
93 SCIEX Triple TOF+ 5600 mass spectrometer and processed using the software
94 Analyst TF 1.6.1. The mass spectrometry proteomics data have been deposited to the
95 ProteomeXchange Consortium via the PRIDE [11] partner repository with the dataset
96 identifier PXD006511 and doi:10.6019/PXD006511.

97 Database searches were performed against a database consisting of the *A. caninum*
98 genome and proteins from the common Repository of Adventitious Proteins (cRAP,
99 <http://www.thegpm.org/crap/>) with Mascot using Mascot Daemon (v.2.5.1, Matrix
100 Science) and X! Tandem (v.2015.12.15.2) and Comet (v.2016.01 rev.2) using
101 PeptideShaker (v.1.11.0) [12].

102 A total of 237 and 289 proteins were identified with 2 or more peptides and FDR <1%
103 using PeptideShaker and Mascot (Supplementary Table 1, 2), respectively, from
104 which 211 were common between both search programs, while 26 and 78 were
105 uniquely found by PeptideShaker and Mascot, respectively, resulting in a final
106 quantity of 315 AcESP proteins (Supplementary Table 3). A total of 67 out of the 105

107 proteins identified by Mulvenna et al. [10] were also found in the present study
 108 (Figure 1A).

109 The top three most abundant proteins found by Mascot (Table 1) were
 110 ANCCAN_13497 (a previously described tissue inhibitor of metalloproteases; TIMP
 111 [13]), ANCCAN_25071 (a hypothetical protein), and ANCCAN_19759 (a sperm-
 112 coating protein; SCP, also called SCP/Tpx-1/Ag5/PR-1/Sc7 domain containing
 113 proteins; SCP/TAPS). The top three proteins found by X! Tandem and Comet using
 114 PeptideShaker (Table 2) were ANCCAN_03259 a platelet inhibitor,
 115 ANCCAN_01699 an SCP protein, and ANCCAN_13497 a TIMP (also found by
 116 Mascot in the top three most abundant proteins). TIMP proteins are a multifunctional
 117 family of inhibitors of matrix metalloproteases (MMP) associated with different
 118 functions in eukaryotic systems such as tissue remodelling, extracellular matrix
 119 turnover, cell proliferation and angiogenesis among others [14, 15]. However, in
 120 parasites, it has been previously suggested that TIMP-like proteins might not be
 121 functioning as MMP inhibitors [16]. The TIMP-like protein ANCCAN_13497
 122 (previously identified as *Ac*-TIMP-1 [13]) was already identified by Mulvenna et al. as
 123 the most abundant (and only TIMP-like) protein in the *Ac*ES [10]. Interestingly, other
 124 *A. caninum* TIMP-like protein, the renamed Anti-Inflammatory Protein-2 (AIP-2), has
 125 been shown to be a potent immunomodulatory protein that suppresses airway
 126 inflammation in a mouse model of asthma [7]. AIP-2 has extensive homology with
 127 other TIMPs found in *Ac*ES, including the highly abundant ANCCAN_26655 and
 128 ANCCAN_24968, suggesting that these two proteins could have similar
 129 immunomodulatory properties. Four different TIMPs are present in *Ac*ES, and are
 130 highly abundant based on the emPAI and spectral count.

131 The SCP proteins are highly represented in the infective larval stage of hookworms
 132 [17], where a role in larval penetration and infection has been hypothesized (reviewed
 133 by [18]). They have also been speculated to be involved in immunomodulation [19].
 134 For instance, an *A. caninum* SCP-like protein (known as Neutrophil Inhibitory factor;
 135 NIF) is able to inhibit neutrophil function and oxidative stress [20]. Although we
 136 didn't find this protein in the adult secretions, we have found two SCPs having
 137 extensive homology with the NIF sequence deposited in NCBI (accession number
 138 AAA27789.1): ANCCAN_04194 (88% identity and E-value = 6.9×10^{-89}) and
 139 ANCCAN_22933 (94.3% identity and E-value = 1.5×10^{-65}). This could refer to a
 140 missannotation of the genome, since a blast search against the *A. caninum* genome
 141 using the deposited NIF sequence doesn't return any sequence with 100% homology.
 142 SCP-related proteins (including SCPs and SCP-like proteins) contributed to 90 out of
 143 315 (31%) of the overall protein families and were by far the most highly represented
 144 family of proteins in the AcES (Figure 1B). These results suggest that the SCP family
 145 might play a key role in orchestrating a parasitic existence and modulating the host's
 146 immune response, and further studies should focus on this family of proteins.

147 Among the most represented protein domains in AcES was the metallopeptidase
 148 family M13 (11 proteins), the transthyretin-like family (10 proteins), astacin
 149 metalloproteases (7 proteins) and glutathione-s-transferases (GSTs; 6 proteins). The
 150 role of peptidases in the secretome of helminths have been linked to important roles in
 151 parasitism [21]; however, the roles of transthyretin-like proteins are still unknown
 152 (despite their abundance in nematodes in particular). Astacins are a family of
 153 metallopeptidases highly abundant in the secretome of helminths [22]. Indeed, the
 154 human hookworm *N. americanus* has 82 astacin-encoding genes [23], and astacins are
 155 abundantly represented in ESPs of the rat hookworm *Nippostrongylus brasiliensis*

[24] and other nematodes [25]. The role of astacins is not fully understood, although, in nematodes, they have been shown to participate in host invasion and parasite development [26, 27]. It is also noteworthy to highlight the presence of GSTs in the AcES proteome. *Na*-GST-1, a GST secreted by adult stage *N. americanus* that is thought to play a role in feeding by detoxifying the free heme produced after hemoglobin ingestion, is being currently tested as a vaccine against *N. americanus* [28]. Other molecules that have been tested as vaccines against hookworms include aspartic proteases [29] and cysteine proteases [30], which are protein families also represented in our study (Figure 1B). In the present study we found 5 different cysteine proteases (ANCCAN_06649, ANCCAN_06644, ANCCAN_30567, ANCCAN_06619 and ANCCAN_06647) and four aspartic proteases (ANCCAN_18339, ANCCAN_13546, ANCCAN_29459 and ANCCAN_25067). Thus, it is tempting to speculate that the GSTs, cysteine proteases and aspartic proteases identified in the present study could be potential vaccines against the dog hookworm.

In the present study we have reanalysed the protein constituents of AcES in order to gain a more comprehensive snapshot of the hookworm secretome and how this impacts on host-pathogen interactions. We have identified almost three times as many proteins as previously reported in the ESP of this important parasite. In addition, new proteins of interest with potential as both novel immunoregulatory biologics and vaccine candidates have been identified, and clearly warrant future exploration.

177

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271 diminishes the fecundity and growth of worms. *J Infect Dis* 2004, 189, 1952-1961.

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274 **FIGURE LEGENDS**

275 **Fig. 1.** (A) Comparison between the number of *Ancylostoma caninum*
276 excretory/secretory proteins (AcES) identified by Mulvenna et al. [10] and the present
277 study. (B) Top ten most represented protein families in the AcES after a Pfam
278 analysis. CAP: Cysteine-rich secretory protein family.

279

Table 1. Top 20 proteins found by Mascot in the excretory/secretory products of *Ancylostoma caninum* adult worms based on emPAI. Proteins were identified by SDS-PAGE, Offgel, or both. CAP: Cysteine-rich secretory protein family; DOMON: dopamine beta-monooxygenase N-terminal; NTR: UNC-6/NTR/C345C module; SCP: sperm-coating protein; TIMP: tissue inhibitor of metalloproteases.

Accession Number	Description	No. Validated Unique Peptides		emPAI		Pfam
		SDS	OGE	SDS	OGE	
ANCCAN_13497	TIMP-like	13	9	60.86	16.4	NTR domain (PF01759)
ANCCAN_25071	Hypothetical protein	10	5	26.08	3.15	CAP domain (PF00188)
ANCCAN_19759	SCP	12	6	21.95	2.54	CAP domain (PF00188)
ANCCAN_20483	DOMON domain-like protein	10	2	18.18	0.99	DOMON domain (PF03351)
ANCCAN_03257	Platelet inhibitor	2	-	16.71	-	-
ANCCAN_03259	SCP	3	3	8.03	8.12	CAP domain (PF00188)
ANCCAN_26655	TIMP-like	7	4	10.05	2.89	NTR domain (PF01759)
ANCCAN_23843	Glu/Leu/Phe/Val dehydrogenase	10	-	11.94	-	Glu/Leu/Phe/Val dehydrogenase domain (PF02812)
ANCCAN_26341	Glutamate dehydrogenase	11	3	10.78	0.86	Glu/Leu/Phe/Val dehydrogenase domain (PF02812)
ANCCAN_16282	Hypothetical protein	5	2	9.15	1.54	-
ANCCAN_08479	SCP	8	3	9.19	1.05	CAP domain (PF00188)
ANCCAN_01923	Hypothetical protein	11	3	9.61	0.56	Protein of unknown function (DUF3270) (PF11674)
ANCCAN_17690	SCP-like protein	17	7	8.21	1.05	CAP domain (PF00188)
ANCCAN_21219	SCP	6	3	6.73	2.14	CAP domain

						(PF00188)
ANCCAN_18161	Galactoside-binding lectin family	18	8	7.12	1.43	Galactoside-binding lectin domain (PF00337)
ANCCAN_06585	SCP	4	-	6.84	-	CAP domain (PF00188)
ANCCAN_04963	Apyrase	15	5	6.4	0.7	Apyrase domain (PF06079)
ANCCAN_00673	Hypothetical protein	-	3	-	5.81	-
ANCCAN_24968	TIMP-like	5	3	4.2	1.04	TIMP domain (PF00965)
ANCCAN_03218	Hypothetical protein	-	6	-	4.87	-

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Table 2. Top 20 proteins found by X! Tandem and Comet in the excretory/secretory products of *Ancylostoma caninum* adult worms based on spectrum counting. Proteins were identified by SDS-PAGE, Offgel, or both. AnfO_nitrog: Iron only nitrogenase protein AnfO; CAP: Cysteine-rich secretory protein family; NTR: UNC-6/NTR/C345C module; SCP: sperm-coating protein; TIMP: tissue inhibitor of metalloproteases.

Accession Number	Description	No. Validated Unique Peptides		Spectrum Count		Pfam
		SDS	OGE	SDS	OGE	
ANCCAN_03257	Platelet Inhibitor	4	7	12605.4	3157.9	-
ANCCAN_01699	SCP	3	-	9940.4	-	CAP domain (PF00188)
ANCCAN_13497	TIMP-like	17	15	1674.2	4779.2	NTR domain (PF01759)
ANCCAN_03254	SCP-like protein	7	5	2278	1622.4	CAP domain (PF00188)
ANCCAN_03218	Hypothetical protein	-	5	-	2280.6	-
ANCCAN_03214	Hypothetical protein	7	-	-	2244.7	-
ANCCAN_23152	Hypothetical protein	3	-	-	2060.2	AnfO_nitrog domain (PF09582)
ANCCAN_11008	Secreted protein 4 precursor	2	3	517.8	2046.8	-
ANCCAN_20841	Unknown	4	3	495.5	1982.6	-
ANCCAN_13591	Hypothetical protein	-	4	-	1869.4	-
ANCCAN_16282	Hypothetical protein	5	-	1737.3	-	-
ANCCAN_25071	Hypothetical protein	8	6	1395.2	1587.6	CAP domain (PF00188)
ANCCAN_26655	TIMP-like	3	2	1567.9	1196.3	NTR domain (PF01759)
ANCCAN_10664	TIMP-like	-	2	-	1455.6	TIMP domain (PF00965)
ANCCAN_19423	Hypothetical protein	-	4	-	1238.5	-
ANCCAN_05485	SCP-like protein	3	2	564.8	1093.2	CAP domain (PF00188)

ANCCAN_18168	Kunitz Bovine pancreatic trypsin inhibitor domain	-	3	-	1082.6	Kunitz/Bovine pancreatic trypsin domain (PF00014)
ANCCAN_25718	Excretory secretory protein 1	2	3	1060.7	1394.7	-
ANCCAN_05778	Copper zinc superoxide dismutase	6	5	525.6	947.6	Copper/zinc superoxide dismutase domain (PF00080)
ANCCAN_19037	Hypothetical protein	-	4	-	881.3	-

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A

B