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Insights into the immuno-molecular biology of *Angiostrongylus vasorum* through transcriptomics - prospects for new interventions

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ABSTRACT

Angiostrongylus vasorum is a metastrongyloid nematode of dogs and other canids of major clinical importance in many countries. In order to gain first insights into the molecular biology of this worm, we conducted the first large-scale exploration of its transcriptome, and predicted essential molecules linked to metabolic and biological processes as well as host immune responses. We also predicted and prioritized drug targets and drug candidates. Following Illumina sequencing (RNA-seq), 52.3 million sequence reads representing adult *A. vasorum* were assembled and annotated. The assembly yielded 20,033 contigs, which encoded proteins with 11,505 homologues in *C. elegans*, and additional 1,933 homologues in various other parasitic helminths for which curated datasets were publicly available. Functional annotation was achieved for 11,752 (58.6%) proteins predicted for *A. vasorum*, including peptidases (4.5%) and peptidase inhibitors (1.6%), protein kinases (1.7%), G protein-coupled receptors (GPCRs) (1.5%) and phosphatases (1.2%). Contigs encoding excretory/secretory and immuno-modulatory proteins represented some of the most highly transcribed molecules, and encoded enzymes that digest haemoglobin were conserved between *A. vasorum* and other blood-feeding nematodes. Using an essentiality-based approach, drug targets, including neurotransmitter receptors, an important chemosensory ion channel and cysteine proteinase-3 were predicted in *A. vasorum*, as were associated small molecular inhibitors. Future transcriptomic analyses of all developmental stages of *A. vasorum* should facilitate deep explorations of the molecular biology of this important parasitic nematode and support the sequencing of its genome. These advances will provide a foundation for exploring immuno-molecular aspects of angiostrongylosis and have the potential to underpin the discovery of new methods of intervention.

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1. Introduction

Angiostrongylus vasorum (Baillet, 1866) is a metastrongyloid nematode of dogs and other canids, including various species of fox, wolf, coyote and jackal (definitive hosts) (Koch and Willesen, 2009). This parasite causes angiostrongylosis, usually characterized by progressively worsening signs of respiratory and/or cardiac disease, and occasionally by coagulopathies and neurological signs, with a lethal outcome in sever cases (Koch and Willesen, 2009; Schnyder et al., 2010). *A. vasorum* has emerged as an important pathogen of canids in various countries in Europe and North America (Bwangamoi, 1972; Bolt et al., 1992; Lima et al., 1994; Conboy, 2004; Morgan et al., 2008; Taubert et al., 2009; Di Cesare et al., 2011; Jefferies et al., 2011; Guardone et al., 2013). This nematode has an indirect life cycle: the dioecious adults live in the pulmonary arteries and heart of the definitive, canid host; here, the oviparous females produce eggs that become entrapped in the capillaries of the lungs, after which first-stage larvae (L1s) hatch and penetrate the alveoli. L1s then migrate *via* the mucociliary escalator to the oropharynx, after which they are swallowed and then excreted in the faeces. L1s infect a molluscan intermediate host (snail or slug), either when ingested together with faeces or by penetration of the epidermis of the mollusc, and then develop, under favourable environmental conditions, to third-stage larvae (L3) within ~16 days (Guilhon and Bressou, 1960; Guilhon, 1963). Frogs may act as paratenic hosts, following the ingestion of infected snails or slugs (Bolt et al., 1993). L3s within an infected intermediate or paratenic host are then ingested by canids, penetrate the gut wall and then migrate to the abdominal lymph nodes, where they moult to fourth-stage (L4), and then, early fifth-stage larvae (L5s); these larvae enter the portal circulation, migrate through the liver parenchyma to eventually reach the right ventricle and pulmonary arteries, where they develop to adults (Koch and Willesen, 2009). The pre-patent period of *A. vasorum* is reported to be 38-57 days (reviewed by Bolt et al., 1994).

With an apparent spread and increasing clinical relevance of canine angiostrongylosis, particularly in Western Europe and Canada (Koch and Willesen, 2009), there has been a focus on improving treatment and control. Emphasis has been placed on the targeted treatment of clinical cases with anthelmintics, such fenbendazole, milbemycine oxime and moxidectin (Conboy, 2004; Willesen et al., 2007; Schnyder et al., 2009). However, in spite of knowledge of some basic aspects of the parasite's life cycle and disease in dogs (e.g., Guilhon and Cens, 1969; Chapman et al., 2004), almost nothing is known about the molecular biology of *A. vasorum* itself, with the exception of some recent immuno-proteomic data (Jefferies et al., 2011). Nonetheless, there are some transcriptomic studies of related strongylid nematodes, including *A. cantonensis* (see Chang et al., 2011) and *Dictyocaulus viviparus* (see Ranganathan et al., 2007; Strube et al., 2007a,b; Cantacessi et al., 2011a). Exploring the molecular biology of *A. vasorum* should improve our understanding of mechanisms that underlie the parasite's biology, physiology (including feeding and neurophysiology) as well as its ability to modulate host immune responses, which could open up avenues for designing new interventions. Such investigations could also provide a platform for the prediction of essential genes and/or gene products in *A. vasorum* as drug targets or vaccine candidates, and the subsequent validation *in vitro* and *in vivo* of rationally designed nematocides and/or immunogens, paving the way for improved approaches for the treatment, prevention and/or control of canine angiostrongylosis. In the present study, we take the first step toward a better understanding of *A. vasorum* at the molecular level by undertaking a detailed transcriptomic exploration of the adult stage of this parasite using an advanced sequencing and bioinformatic platform.

2. Next-generation technologies

2.1. RNA-seq (Illumina)

The method of paired-end RNA-seq (Bentley et al., 2008) was used to sequence the transcriptome of *A. vasorum* adults. The adult specimens of *A. vasorum* were collected from infected dogs at the Vetsuisse Faculty of the University of Zurich, Switzerland. The Cantonal Veterinary Office of Zurich granted ethical approval (permit no. 13/2008), in accordance with Swiss animal ethics laws. Worms were obtained by reverse lung perfusion 56-59 days after experimental inoculation (Schnyder et al., 2010). The worms were washed extensively in physiological saline (five times in 200 ml) and then transferred to RNAse/DNAse-free cryotubes, snap-frozen in liquid nitrogen and stored at -80 ºC until RNA isolation. The identity of selected specimens of *A. vasorum* was verified by PCR-based amplification and sequencing of the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA using an established method (Gasser et al., 2006). Then, *DN*ase I-treated total RNA was isolated from adult worms (both sexes) using the TriPure reagent (Roche) (Young et al., 2011). RNA amounts were estimated spectrophotometrically (NanoDrop Technologies), and RNA integrity was verified using a 2100 BioAnalyzer (Agilent). Polyadenylated (polyA+) RNA was purified from 10 µg of total RNA using Sera-mag oligo(dT) beads, fragmented to a length of 100-500 bases, reverse transcribed using random hexamers, end-repaired and adaptor-ligated, according to the manufacturer's protocol (Illumina). Ligated products of ~ 300 bp (mean: 336) were excised from agarose and PCR-amplified (15 cycles) (Young et al., 2011). Products were purifed over a MinElute column (Qiagen) and paired-end sequenced (100 bp; non-normalized cDNA) on a Genome Analyzer II (Illumina), according to manufacturer's instructions.

2.2. Assembly and quality assurance of RNA-seq data

The transcriptome of *A. vasorum* was assembled from raw RNA-seq data and annotated using a bioinformatic pipeline. Firstly, low quality reads were eliminated using the program Trimmomatic (Lohse et al., 2012) (sliding window: 4 bp, minimum average quality: 20; leading and trailing bp: 3; minimum read length: 40 bp), and redundant reads were eliminated using the khmer package (http://ged.msu.edu/pubs.html). The assembly was optimised by using a range of *k*-mer values from 19 to 55 nucleotides (nt) (step-size: 2), and minimum fold-coverage thresholds from 5 to 21 (step-size: 2) in the Oases program (Schulz et al., 2012). The *k*-mer and fold-coverage parameters govern the accuracy with which the assembled transcriptome represents the raw paired-end sequence data (Mangiola et al., 2012). For each transcriptome assembly, the average contig length was determined using the program seqstat (Durbin et al., 1998), and the mean redundancy of contigs in the dataset was calculated as the average number of significantly similar contigs (BLASTn, E-value cut-off: $\leq 10^{-30}$; Altschul, 1997) for any given contig. Finally, the proportion of raw paired-end and unpaired reads mapping to contigs for each assembly was quantitated using the program BWA (Li and Durbin, 2009). The number of contigs and proportion of paired-end reads mapping as pairs to each transcriptome assembly were displayed in an X-Y scatter plot. The transcriptome comprising the largest number of contigs to which a large proportion (> 80%) of raw paired-end reads mapped was selected as the final assembly for the subsequent annotation and analyses. A

normalised measure of transcriptional abundance for each contig was calculated as fragments per kilobase per million reads (FPKM) (Trapnell et al., 2012).

2.3. Bioinformatic processing and functional annotation

Contigs representing the transcriptome of *A. vasorum* were translated in all six open reading frames (ORFs) using the program getORF (EMBOSS v.6.4.0) (Rice et al., 2000), and domain annotations were retrieved using InterProScan (Quevillon et al., 2005) employing default search parameters. Predicted peptides with the most InterPro domain annotations (excluding the generic secondary structure annotations: 'Seg' and 'Coil') were selected. For peptides without predicted domains and those translating in two or more ORFs with equal numbers of annotated domains, the longest sequences were selected for further analysis. Gene ontology (GO) terms that accompanied InterPro annotations were used to group the inferred peptides according to biological process, cellular component and/or molecular function (Botstein et al., 2000). Following the truncation of any N-terminal regions to the first methionine, peptides with signal and transmembrane domains were predicted using Phobius (Käll et al., 2007).

BLASTx (E-value cut-off: $\leq 10^{-5}$, unless otherwise specified) was used to compare inferred *A. vasorum* peptides to those available in public databases: *Canis familiaris* (canid definitive host) (Lindblad-Toh et al., 2005) (http://www.genome.gov/11008069), *Caenorhabditis elegans* (WormBase WS220), *Drosophila melanogaster* (FlyBase) (McQuilton et al., 2011), SwissProt (Boeckmann, 2003), Kinase SARfari (www.ebi.ac.uk/chembl/sarfari/kinasesarfari), MEROPs (Rawlings, 2006), ChEMBL (Gaulton et al., 2012), the transporter classification database (TCDB) (Saier et al., 2009), GPCR-Sarfari (www.ebi.ac.uk/chembl/sarfari/gpcrsarfari), and The Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.kegg.com). Homologues were mapped to conserved biological pathways using the KEGG Orthology-Based Annotation System (KOBAS) (Wu et al., 2006), and those in the GPCR-Sarfari database were filtered based on the presence of a predicted transmembrane domain. Protein sequences predicted from the transcriptomic data for *A. vasorum* were compared with those inferred from the transcriptomes of selected parasitic nematodes (HelmDB.org; Mangiola et al., 2012) (Table 2), those within an in-house, curated database containing proteins linked to haemoglobin digestion, and with sequences of excretory/secretory (ES) proteins identified in proteomic studies of *Ancylostoma caninum* (see Mulvenna et al., 2009), *Haemonchus contortus* (see Yatsuda et al., 2003), *Teladorsagia circumcincta* (see Craig et al., 2006; Smith et al., 2009) and *Brugia malayi* (see Hewitson et al., 2008) using BLASTx and tBLASTx; E-value cut-off: $\leq 10^{-5}$. Matches that were not the closest homologue of an inferred *A. vasorum* protein (determined as the highest bit-score match) were discarded, as were any predicted ES peptides that contained a transmembrane domain. Sequence alignments were performed using eBiox2 (www.ebioinformatics.org/ebiotools/).

In order to predict drug targets in *A. vasorum*, inferred peptides with (a) homologues in *C. elegans* or *D. melanogaster* being associated with a lethal double-stranded RNAi (RNAi) phenotype, and (b) no significant matches to *C. familiaris* peptides, were selected. Prioritized, predicted drug targets were considered to be those with a significant (E-value cut-off: $\leq 10^{-5}$) homologue(s) in the ChEMBL database, and amenable to inhibition (k_i, EC_{50} or IC_{50} value $\leq$ 100,000 nM) with small molecules which were predicted as 'medicinal chemistry friendly' and satisfied the Rule-of-Three (MW < 300 Da; < 3 H donors; < 3 H acceptors; (Congreve et al., 2003). Subsequently, cluster analysis was performed on protein sequences encoding RIO protein kinases (= RIOKs) RIOK-1, -2 or -3 of *A. vasorum*, *Haemonchus contortus*, *C. elegans*, *D. melanogaster*, *Danio rerio*,

Xenopus laevis, *Mus musculus*, *Homo sapiens* and *C. familiaris* using the program Cd-hit (Li and Godzik, 2006). For each RIOK protein family, amino acid sequences were then aligned using the program MAFFT (Katoh et al., 2002), with L-INS-I settings, and adjusted manually. Phylogenetic analysis of the sequence data was conducted by Bayesian inference using the Markov chain Monte Carlo (MCMC) algorithm in the program MrBayes, v.3.2.1 (Ronquist and Huelsenbeck, 2003). The rate matrix for amino acid data was inferred based on sampling of the Markov chain and subsequently selecting the model with the optimum posterior probability (pp) estimate. Generations of the MCMC analysis were performed, sampling trees every 50 generations until the average standard deviation of split frequencies was < 0.01 and the potential scale reduction factor (PSRF) approached one. Summary statistics and consensus trees were generated using the 50% majority rule criterion on bootstrap replicate trees generated with the final 75% of sampled trees.

3. Integrated bioinformatic exploration

3.1. Transcriptome of A. vasorum

A total of 58,561,060 RNA-seq reads were produced for the adult stage of *A. vasorum*; 52,276,380 (89.27%) were retained following quality assurance, including 23,967,571 paired-end read pairs. The transcriptome, to which 82.3% of paired-end reads mapped with a mean depth of coverage (FPKM) of 56.2 ± 362.7, was selected to represent the optimum assembly. This assembly comprised 20,033 contigs (mean length of 1422 ± 1361 nt; range: 100-14,663 nt) and had a mean redundancy of 3.49. In total, 13,967 inferred proteins (69.7% of total) were annotated by homology searches (see Table 1); 11,505 (57.4%) of the predicted proteins had homologues in *C. elegans* and 1,932 had homologues in selected parasitic nematodes (*via* HelmDb), but not in *C. elegans* (see Table 2).

In total, 11,752 (58.6%) proteins inferred for *A. vasorum* were annotated functionally. Using a domain modelling approach, 28,192 InterPro domains (excluding 'Seg' and 'Coil' annotations) were assigned to 10,707 (53.4%) *A. vasorum* proteins; 4,943 unique domain annotations were assigned, of which the most widely represented was the 'protein kinase-like domain' (IPR011009; n = 366) and its subsidiaries: 'protein kinase - catalytic domain' (IPR000719; 323), 'serine/threonine-/dual-specificity protein kinase - catalytic domain' (IPR002290; 297) and 'tyrosine-protein kinase - catalytic domain' (IPR020635; 251). The next most-widely annotated domains were 'armadillo-type fold' (n = 250), 'WD40/YVTN repeat-like-containing domain' (222), 'protein kinase, ATP binding site' (217; also a subsidiary of IPR011009) and zinc finger RING-type' (216) (Supplementary Table 1). GO terms pertaining to 'molecular function' (n = 209), 'biological process' (545) and 'cellular component' (362), were assigned to 8,361 (41.7%) predicted proteins (Fig. 1, Supplementary Table 2). Using KOBAS, 3048 unique KEGG orthology terms were assigned to 6,210 (31.0%) *A. vasorum* homologues in the KEGG database, which mapped to 39 large functional enzyme groups, the most represented of which were 'chromosome' (8.4% of all homologues assigned a K-term), 'splicesome' (7.0%), 'ubiquitin system' (6.4%) and 'protein kinases' (4.6%) (Supplementary Table 3).

Transmembrane and signal peptide domains were identified in 1,911 and 885 of a total of 17,664 full-length proteins, respectively, predicted from the transcriptome of *A. vasorum*. Both domains were present in

119 proteins (Supplementary Table 4); and 34 of 310 proteins representing G protein-coupled receptors (GPCRs) contained at least one transmembrane domain. In addition to domain-based annotation and biological pathway mapping, homology searches showed that the most commonly predicted proteins were peptidases (4.5%), peptidase inhibitors (1.6%), protein kinases (1.7%), GPCRs (1.5%) and phosphatases (1.15%) (Table 1).

An investigation of transcript abundance showed that the top 2,000 most highly transcribed contigs (~10% of all contigs) accounted for 66.4% of total transcription in the adult stage of *A. vasorum* (Fig. 2). In addition, the 40 most highly transcribed molecules accounted for one fifth of all transcribed molecules. Within this group were transcripts encoding the vitellogenins VIT-1, VIT-2, VIT-4 and VIT-6 (combined FPKM = 166,467), excretory/secretory (ES) proteins and proteins involved in intracellular homeostasis, such as ubiquitins, DNA repair molecules and heat-shock proteins (Table 3). Interestingly, the second most-highly transcribed molecule (271 nt; FPKM = 15,202) contained a region that had a perfect match (over 22 nt) to a sequence tract upstream of the gene coding for a tissue-specific metalloprotease inhibitor (GenBank accession no. AF397162.1) of *An. caninum* (see Zhan et al., 2002), but diverged from the coding region. Eight other molecules of this group had no homology to any sequences in any of the databases interrogated.

3.2. Molecules predicted to be involved in parasitism

Various groups of molecules identified to be highly represented in adult *A. vasorum* might relate to parasitism, host interactions and/or parasite feeding. For instance, 16 of the 32 ES homologues encoded were amongst the top 10% most highly transcribed molecules in adult *A. vasorum* (Table 4). Represented amongst these highly-transcribed molecules were homologues of extracellular superoxide dismutase (*H. contortus*), fatty-acid and retinol-binding protein (FAR) (*C. elegans*), aspartyl protease inhibitors (*P. tenuis*) (Table 4), high mobility-group protein and gamma-glutamyl transpeptidase precursor of *Brugia malayi*, *Ancylostoma*-secreted proteins 1 and 6 (*An. caninum*) and the nematode polyprotein antigen *Dv*A-1 (*D. viviparus*) (Tables 4 and 5). In addition, eight proteins, encoded by 55 transcripts in *A. vasorum*, were homologues of enzymes implicated in haemoglobin digestion (Table 5); these included aspartyl/cysteine peptidases, glutathione-S-transferases and *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP) homologues (Table 5). One predicted protein showed a particularly high sequence identity to APR-1 of *An. duodenale* (74.6% over 445 amino acids, including residues identical to the catalytic site and the 'protective epitope' ('$A_{291}Y$'), with a single amino acid substitution (A<–>V) in the latter (Pearson et al., 2010) (Fig. 3). In addition, two highly-transcribed cysteine peptidases (combined FPKM = 585), contained a haemoglobinase motif (YWLIANSW--DWGE) present almost exclusively in cathepsins B of blood-feeding nematodes (Baig et al., 2002). Interestingly, in spite of this region being conserved, overall, these inferred cysteine peptidases were most similar in sequence to cathepsin B-like homologues of *C. elegans* and *C. briggsae*. Similarly, a single peptide predicted for *A. vasorum* showed considerable sequence identity (54.6% over 340 amino acids) to a metalloprotease of *N. americanus* (SwissProt accession no. B1Q144), proposed to be involved in haemoglobin digestion (Ranjit et al., 2008). However, again, its closest homologue (55.6% identity over 782 amino acids) was neprysilin-2 of *C. elegans* (SwissProt accession no. O16796). Also notable among the haemoglobin digestion enzymes predicted was a single, highly transcribed molecule (FPKM = 330) with 93.5% sequence identity (over 280 amino acids) to β-galactoside-binding lectin (β-galectin) of *H. contortus*. Considering chemosensory mechanisms in *A. vasorum*, homologues of *C. elegans* proteins involved in metabotropic ion channel activation in chemosensory ASE neurons were identified, such as receptor

guanylate cyclases-1, -6, -7 and -22, the G-protein GPA-13 (accession no. CCA65551.1), the cyclic-nucleotide-gated olfactory channel TAX-4 (CAB63418.2) and the membrane-protein chaperone ODR-4 (CCD70713.1) (Bargmann, 2006; Ortiz et al., 2009).

3.3. Drug target predictions

Thirteen inferred peptides of *A. vasorum* had homologues in *C. elegans* linked to lethal knock-down phenotypes, with homologues in the ChEMBL database and known inhibitors whose IC_{50}, EC_{50}, or k_i values are known to be ≤ 0.1 mM based on tests in mammalian cell-based assays or biochemical assays. Amongst the predicted drug targets were gamma–amino butyric acid (GABA) and acetylcholine-gated neuro-receptors, a cation channel, which is widely distributed in *C. elegans* tissues, a dihydroorotate dehydrogenase, and cysteine proteinase-3 (CP-3) (Table 6). The small molecules identified from the ChEMBL database both satisfied the Rule-of-Three (Congreve et al., 2003) and were also predicted to be 'medicinal chemistry friendly.' In the present data set, a set of RIOKs was also predicted; these atypical kinases have been proposed as drug target candidates in *H. contortus* (a related strongylid nematode) using an *in silico* drug-docking approach (Campbell et al., 2011). Inferred *A. vasorum* RIOK-1 (two isoforms), RIOK-2 and RIOK-3 (one inferred protein each) showed > 70% identity to homologous full-length inferred proteins of *H. contortus*, respectively. For individual nematode RIOKs, optimal clustering was achieved at a cluster redundancy of 3 and a similarity threshold of 0.6 (see Fig. 4). The sequence alignment of RIOK-1 of *A. vasorum* with that of *H. contortus* revealed the presence of both conserved catalytic site residues, and residues Tyr-159, Ser-275 and Ser-292 (Campbell et al., 2011), which are conserved between these nematodes but divergent from mammals (including the canid host). For individual RIOKs, this divergence between nematode and non-nematode proteins was supported by a phylogenetic analysis of amino acid sequence data, in which each RIOK family of *A. vasorum, H. contortus* and *C. elegans* grouped together, to the exclusion of those of *D. melanogaster, Da. rerio, X. laevis, M. musculus, H. sapiens* and *C. familiaris* homologues (Fig. 4).

4. Conclusions and implications - immuno-molecular biological insights provide exciting prospects for disease intervention and biotechnological outcomes

4.1. Advances made through investigating the transcriptome of A. vasorum

The present study elucidates the complement of molecules transcribed in the adult stage of *A. vasorum*. The high quality of raw data produced using Illumina sequencing of paired-end reads, combined with a thorough bioinformatic processing, incorporating quality assurance, enabled the assembly of a transcriptome with an average contig length of 1422 nt, to which more than 80% of raw reads could be mapped. Methodologically, the use of a quantitative analysis of different assemblies allowed the selection of a final, representative transcriptomic data set with a large number of contigs (n = 20,033) to which a large proportion (82.3%) of paired-end reads mapped, with minimal redundancy. This systematic approach of optimizing and

assessing individual assemblies prior to bioinformatic analyses improves considerably on previous methods which rely on the selection of single assembly parameters *a priori* (Cantacessi et al., 2011b). Indeed, the annotation of > 70% contigs representing the transcriptome of *A. vasorum* using high stringency homology searches of nematode and non-nematode databases (E-value cut-off: $\leq 10^{-5}$) indicates a high quality of the optimized assembly.

The present study predicted 11,505 proteins (57.5% of all contigs) in *A. vasorum* with homologues in *C. elegans*. This finding agrees with previous reports, suggesting that 35-52% of proteins predicted in strongylid nematodes have homologues in *C. elegans* (see Cantacessi et al., 2010a,c; 2011a), and with comparative genetic or transcriptomic analyses of nematodes, which have shown that at least 60% of genes are shared between strongylid nematodes and *C. elegans* (see Blaxter et al., 1998; Ruvkun, 1998; Parkinson et al., 2004). This degree of homology suggests that the free-living *C. elegans* provides a useful surrogate system for investigating the functions of key genes and gene products of strongylids, as has been indicated by various functional genomic studies (Redmond et al., 2001; Britton and Murray, 2006; Hu et al., 2010; Stepek et al., 2010).

4.2. Abundance of kinases and key roles

Conserved protein domains were identified among ~ 50% of inferred peptides of *A. vasorum*, with kinase, armadillo-type and WD40 domains amongst the most ascribed InterPro motifs. The annotation of 366 'protein kinase-like domains' in inferred *A. vasorum* peptides is supported by similar results from homology searches (332 kinases) *via* Kinase SARfari, KEGG orthology and ontology-based gene clustering. Specifically, 236 peptides related to 'protein kinases' (ko:01001; Supplementary Table 2) by KOBAS and 659 to 'phospho-transferase activity' by GO clustering. Taken together, these results suggest that 1-3% of the transcriptome of adult *A. vasorum* encode molecules with kinase activity, which accords with previous findings that kinases account for ~ 2% of the eukaryotic proteome (Manning, 2005). At least 438 kinases have been described for *C. elegans*, ~50% of which are in classes that are greatly expanded in this nematode compared with other animal phyla (Manning, 2005). For instance, kinases implicated in chemosensation, such as receptor guanylyl cyclases (Ortiz et al., 2009), are expanded in *C. elegans* (see Morton, 2004). Indeed, chemosensory pathways in *A. vasorum* are well supported by several pieces of evidence. Firstly, close homologues of proteins involved in each stage of metabotropic signal transduction in the chemosensory ASE neurons of *C. elegans* (reviewed by Bargmann, 2006), including receptor gyanyl cyclases, were identified in *A. vasorum.* Second, a reasonable proportion (1.1%) of proteins inferred for *A. vasorum* contained WD40 repeat-like domains, which are involved in G-protein binding activity (Li and Roberts, 2001), and 34 GPCR homologues with predicted transmembrane domains were identified. Third, 52 peptides were predicted by GO clustering to have 'transmembrane signalling receptor activity' **(**Supplementary Table 1). Therefore, based on this information, it is highly likely that adults of *A. vasorum* utilise metabotropic signalling, including chemosensation, previously suggested to be important for host detection, feeding and tissue migration in parasitic nematodes (Jex et al., 2011; Dillman et al., 2012).

4.3. ES proteins, host-parasite interactions and immunomodulation

While chemosensation is essential in directing parasitic behaviour, ES proteins of parasitic nematodes are likely to play key roles in parasite survival within the host, relating to digestion of host tissues or blood for feeding, as well as tissue migration, and the modulation and/or evasion of host immune responses. ES proteins are exposed to the host immune system and thus could represent intervention or diagnostic targets. In the present study, predicted ES proteins, which comprised ~1% of the predicted proteome, were represented by almost 21% of all transcribed molecules in *A. vasorum*. Indeed, these data, coupled to the strong transcriptional bias in the transcriptome of *A. vasorum* (Fig. 1), suggest that the adult stage of this parasite might rely heavily for survival on a relatively small number of proteins, including ES proteins. Recent evidence (Schnyder et al., 2011) has shown also that circulating ES antigens in blood are an effective target for the specific diagnosis of patent *A. vasorum* infection in dogs.

In accordance with a number of previous studies of nematodes (Spieth and Blumenthal, 1985; Craig et al., 2006; Mulvenna et al., 2009), vitellogenins were highly represented amongst ES proteins, accounting for 14.8% of the total transcription in *A. vasorum*. Vitellogenins are secreted glycol-lipoproteins that nourish developing embryos (Chen et al., 1997), and are localised in the gut of the adult, egg-laying hermaphrodite of *C. elegans* (see Spieth and Blumenthal, 1985). For *A. vasorum*, it is likely that the abundant transcription of vitellogenin genes relates to female adults, consistent with findings for other dioecious strongylid nematodes (Nisbet and Gasser, 2004; Cottee et al., 2006; Campbell et al., 2008). Although vitellogenins of such nematodes are not usually reccognised to interact with the host animal as do classical ES proteins, these proteins have been detected in ES products of some strongylids (Vercauteren et al., 2003; Mulvenna et al., 2009), and could be actively secreted by the worms or may emerge from damaged worm tissues (Craig et al., 2006). Other genes found to be highly transcribed in *A. vasorum* encoded homologues of ubiquitin, a heat shock protein homologue of *Nippostrongylus brasiliensis* and the *xpc-1* DNA repair gene homologue of *C. elegans* (cf. Table 3), possibly reflecting the heavy protein-folding and chaperon requirements of vitellogenin-producing tissues. The high level of transcription relating to a fatty acid and retinol binding protein (FAR) homologue of *C. briggsae* was also observed. This finding is corroborated by the previous identification of FAR in a proteomic analysis of antigenic proteins of adult *A. vasorum* (see Jefferies et al., 2011). FARs are secreted by parasitic nematodes and function to import lipid metabolites from the host that are lacking from parasites (Jordanova et al., 2009).

A particularly important group of predicted ES proteins identified in the present study are immunomodulatory proteins, which are released by parasites to avert host immune responses and thereby enable a prolonged survival in the host animal (Hewitson et al., 2009). Residing within the vasculature, adults of *A. vasorum* are continuously exposed to humoral and cellular immune responses. Therefore, the ability of this stage to diminish or divert host immune responses is highly likely to be important for parasitism. Consistent with this hypothesis, numerous peptides of *A. vasorum* were identified as homologues of known immunomodulatory proteins. Firstly, two aspins (aspartyl protease inhibitors) were identified, each of which exhibited levels of transcription that were 50-fold greater than the average of all molecules transcribed in the nematode (FPKM: > 2800; Table 4). Aspins of *A. vasorum* and other parasitic nematodes have already been shown to be highly immunogenic (Duffy et al., 2002; De Maere et al., 2005; Jefferies et al., 2011), and can inhibit antigen processing by B cells (Delaney et al., 2005) to dampen host immune responses. Moreover, immunoreactivity against aspin has been suggested to be a predictor of resistance of sheep against *Trichostrongylus colubriformis* (see Shaw et al., 2003). Collectively, this information suggests that these protease inhibitors are immunogens that might represent a possible nexus in the parasite-host relationship.

Similarly, the direct modulation of host leukocyte activity by *A. vasorum* might be achieved through homologues of *B. malayi* high mobility group box protein (BmHMGB1) and of *D. viviparus* antigen 1 (*Dv*A-

1). BmHMGB1 is capable of binding DNA and might modulate the transcriptional program of host macrophages to induce the secretion of pro-inflammatory cytokines (Thirugnanam et al., 2012). On the other hand, the homologue of *Dv*A-1 in *A. vasorum* might interact with CD40 receptors on canine B-cells to elicit the secretion of cytokines involved specifically in a Th2-type immune response, characteristic of parasitic nematode infections – as suggested for a protein (called DiAg) from *Dirofilaria immitis* (Imai et al., 2001). Further modulation of host immune cells might relate to homologues of *An. caninum* ASP-1 and ASP-6, which are key SCP/TAPS proteins. Although the exact roles of these proteins are not yet known, they are abundant in the ES products of many parasitic nematodes (reviewed by Cantacessi et al., 2009), and some can modulate the activity of host leukocytes, as shown for *Na*-ASP-2 (from *N. americanus*, a human hookworm), which is able to recruit mouse neutrophils (Bower et al., 2008) and is posited to act through binding chemokine receptors on those cells.

Besides modulating the activity of host leukocytes, *A. vasorum* may utilize extracellular superoxide dismutase (SOD) to neutralise oxidative attack from host phagocytes (Hewitson et al., 2009). Indeed, neutralisation of oxidative radicals by SOD might be crucial for nematodes to survive in their host(s), as the clearance of *N. brasiliensis* from mice appears to depend on the degree of host-mediated oxidative stress to which parasites are subjected (Smith and Bryant, 1989). Taken together, this information suggests that *A. vasorum* shares many immunomodulatory strategies with other parasitic nematodes, including related strongylids. Furthermore, the exceptionally high transcription levels of many of the genes encoding such proteins suggests that they play essential roles in regulating the interplay between *A. vasorum* and its canid host. It is also important to note that a number of highly transcribed molecules were identified within the ~40% of the *A. vasorum* transcriptome for which inferred homologues were either restricted to uncharacterised parasitic nematode proteins (9.6%) or completely lacking (i.e. orphans; 30.3%). The occurrence of subsets of homologous proteins which are restricted to parasitic nematodes is a common finding in genomic and transcriptomic studies (Ghedin et al., 2007; Cantacessi et al., 2010a,c, 2011b; Godel et al., 2012). Importantly, this information suggests the existence of shared as well as entirely novel sets of proteins that are specifically involved in parasitism. Investigating ES proteins within this group could inform both the biology of *A. vasorum* and provide new avenues for treatment and control.

4.4. Haemoglobin digestion

Living within the vasculature system, *A. vasorum* likely utilizes host blood as a major food source, supported by occasional anaemia in *A. vasorum*–infected dogs (Koch and Willesen, 2009). While being an accessible source of protein nutrients, the potentially reactive haeme by-products of haemoglobin digestion must be detoxified to avoid damage to parasite biomolecules (Brophy and Pritchard, 1992; Hotez et al., 2010). Haematophagous hookworms (e.g., *Necator* and *Ancylostoma* spp.) and *H. contortus* express proteolytic enzymes on the gut brush border which are capable of digesting haemoglobin *in vitro* (Knox et al., 1993; Williamson et al., 2004). Specifically, the cascade of enzymatic processing is proposed to involve the initial cleavage of the haemoglobin tetramer by aspartic proteases, followed by proteolysis by cysteine proteases (globinases) and subsequent proteolysis by metalloproteases (Williamson et al., 2003, 2004; Hotez et al., 2010). Members of each of these classes of peptidases were predicted for *A. vasorum*. Aspartic peptidases homologous to *H. contortus* pepsinogen 1 and *A. duodenale* APR-1 were identified, the latter having a conserved catalytic domain and a relatively conserved, protective epitope (Pearson et al., 2010) (cf. Fig. 3). Furthermore, homologues of *N. americanus* CP-3 in *A. vasorum* contained the same

haemoglobinase-motif reported almost exclusively for blood-feeding nematodes (Baig et al., 2002). The primary sequence conservation between aspartyl and cysteine proteases of blood-feeding nematodes and *A. vasorum* strongly suggests haemoglobin digestion activity in this parasite, although, interestingly, the metalloproteases inferred showed marginally higher identity to *C. elegans* homologues than to those of other blood-feeding nematodes. A possible explanation for the latter observation is that metalloproteases (class M13) might have less substrate specificity, because they act on partially digested haemoglobin, downstream in the digestion cascade (Williamson et al., 2004), reflected in less sequence divergence in this subset of enzymes between nematodes that feed on blood and those that do not (e.g., *C. elegans*). After haemoglobin proteolysis, a glutathione-S-transferase predicted in *A. vasorum*, which is an homologue of *An. caninum* GST-1, might act to neutralise liberated haematin (Zhan et al., 2005; Hotez et al., 2010). The identification in *A. vasorum* of these hookworm homologues, together with protein members (= PEP-1 and β-galectin) of the H-gal-GP complex of *H. contortus*, further supports the proposal that adults of *A. vasorum* utilize blood as a major food source.

4.5. Drug targets

Although fenbendazole and some macrocyclic lactones (ivermectin, milbemycin oxime and moxidectin) are used for the treatment of canine angiostrongylosis (Conboy, 2004; Schnyder et al., 2009), the limited number of approved compound classes and the potential for emergent drug resistance in parasitic nematodes (Van Wyk et al., 1999; Kaplan, 2004; Wolstenholme et al., 2004; Sargison et al., 2007; James et al., 2009; Bourguinat et al., 2011) demand that novel drug targets and drugs are identified. Using a transcriptomic-guided approach, we prioritized drug target candidates, whose gene homologues have lethal RNAi knock-down phenotypes in *C. elegans* or *D. melanogaster* but no close homologue in the canine host (Table 8). Although it is anticipated that these candidates represent selective targets, some of them relate to molecules in mammals identified in the ChEMBL database as drug targets. Nevertheless, the presence of neurotransmitter receptors (GABA and acetylcholine-gated receptors) and the TPRA-1 ion channel among drug-target candidates is promising, because most existing anthelmintics are thought to act *via* a relatively selective perturbation of neural function (Wolstenholme, 2010; Kaminsky and Rufener, 2012). In *C. elegans* TRPA-1 is expressed widely in sensory neurons, head and vulva epithelium (Kindt et al., 2007). Inhibition of this ion-channel in *A. vasorum* might therefore disrupt the function of multiple biological systems in the worm, which is desirable for an anthelmintic. Furthermore, many modulatory compounds are available for mammalian TPRA-1, as this ion channel appears to be involved in nociception and has been the subject of relatively intense research (Baraldi et al., 2010). Also identified amongst possible drug targets was dihydroorotate dehydrogenase, an enzyme involved in the pyrimidine synthesis pathway, which leads to both amino acid and nucleic acid production. The potential of this pathway for chemotherapeutic intervention has been explored in protozoan parasites, yielding kinetic, structural and RNAi data (Arakaki et al., 2008; Cordeiro et al., 2012), which might further inform research into dihydroorotate dehydrogenase in parasitic helminths, including *A. vasorum*. As haemoglobin digestion enzymes are known to be important for the survival of blood-feeding nematodes in their hosts (reviewed by Hotez et al., 2010), the identification of an homologue of the cysteine proteinase CP-3 as a potential drug target is encouraging. Cysteine proteinases are believed to be involved in the second step of haemoglobin digestion by blood-feeding nematodes (Williamson et al., 2003), and, therefore, the chemical inhibition of CP-3 might disrupt the feeding process in *A. vasorum*. The human cathepsin B was identified in the ChEMBL database as the protein most closely

related to a protein predicted for *A. vasorum*, and is potently inhibited by bicyclic carbamates (IC_{50} = 1 nM) (Epple et al., 2007).

Also RIOKs of *A. vasorum* have potential as drug targets, as reported recently for *H. contortus*, another blood-feeding nematode (Campbell et al., 2011). Comparison of the three identified RIOK homologues from these two nematodes revealed > 70% identity (full-length proteins) between respective homologues, particularly in the catalytic domain. As these atypical kinases appear to diverge from mammalian RIOKs, particularly in regions that may complex with ligands, future structural studies are warranted to reveal the details of such molecular interactions, for example, in complex with the phosphate-donating nucleotides, to provide a solid basis for structure-based drug design. To date, the mechanistic aspects of RIOKs are poorly understood, and our current working hypothesis assumes that the two flexible elements in the RIOK domain, the hinge and the flexible loop, serve as docking points for the substrate and might undergo conformational change in the substrate-bound state (Campbell et al., 2011). Such a process might be further aided by phosphorylation of *Hc*-RIOK-1 Ser165 (corresponding to *Av*-RIOK-1-Ser170), which is located in the flexible loop and seems to be a conserved residue for RIOKs. Clearly, crystal structures of substrate-bound and phosphorylated nematode RIOKs will assist in elucidating the structural biology of these proteins and in assessing their potential as valid and selective drug targets.

Taken together, these candidate proteins in *A. vasorum*, which lack close homologues in the canid host and for which inhibitory compound data are available, should provide a basis for the development of novel chemotherapeutics. However, as most of the small molecule inhibitors identified here (Table 6) have been developed for mammalian use, chemical modifications might be needed to limit possible side effects in the host.

4.6. Vaccine prospects

Immunogenicity and/or protection against nematode infection has been reported for a number of *A. vasorum* homologues identified here, including haemoglobin-digestion enzymes (Williamson et al., 2004; Loukas et al., 2006; Hotez et al., 2010), *Dv*A-1 (Britton et al., 1995) and aspins (Duffy et al., 2002; De Maere et al., 2005; Jefferies et al., 2011). In addition, a protective immune response can be achieved against *Dictyocaulus viviparus*, the bovine lungworm, using an irradiated larval vaccine (Jarrett et al., 1960), suggesting prospects for the development of a vaccine against other 'lungworms', such as *A. vasorum*. However, the disadvantages of live vaccines relate to the instability of irradiated larvae, the need to produce larvae in intermediate hosts, and a likely inability to confer sterile immunity and life-long protection. Therefore hypothesis-directed, evidence-based recombinant vaccine trials are warranted. Several of the proteins inferred here to be involved in immunomodulation or haemoglobin digestion might be vaccine candidates against canine angiostrongylosis. For example, the fatty-acid and retinol-binding (FAR) protein is both immunogenic (Jefferies et al., 2011) and highly expressed in the transcriptome of adult *A. vasorum*. Structural and functional information has been published for FAR-7 of *C. elegans* (see Jordanova et al., 2009), which should provide a solid foundation for the characterization of FAR from *A. vasorum*. Furthermore, there has been a focus on developing a vaccine against parasitic trematodes of mammals based on FAR (e.g., Sm14 from *S. mansoni*) (Tendler and Simpson, 2008).

On the other hand, proteins such as haemoglobin-digestion enzymes, expressed in the gut epithelium of haematophagous nematodes (Hotez et al., 2010; Knox, 2011) are considered to be relatively 'hidden' from

the immune system; however, immunization with recombinant antigens can raise antibodies that inactivate these enzymes as blood is ingested (e.g., Williamson et al., 2003). For example, dogs immunized with recombinant *An. caninum* APR-1 showed significantly reduced adult *An. caninum* burdens, and appeared to display reduced clinical signs of hookworm disease (Loukas et al., 2005). Interestingly, the *A. vasorum* APR-1 exhibits an epitope ($A_{306}Y$) that is very similar to the $A_{291}Y$ found in APR-1 of several hookworms (i.e. *N. americanus*, *An. duodenale* and *An. ceylanicum*) (Fig. 3). Furthermore, vaccination of dogs with recombinant *Na*-APR-1 (from *N. americanus*) confers some protection (29% reduction in worm burden) against challenge with *An. caninum* L3s (Pearson et al., 2009), which is proposed to be due to antibodies directed against the $A_{291}Y$ epitope (Pearson et al., 2010), despite this region in *Ac*-APR-1 (from *An. caninum*) displaying greater sequence divergence from that of *N. americanus* than of *A. vasorum* APR-1 (see Fig. 3). Therefore, immunization of dogs with *Na*-APR-1 might also protect against *A. vasorum* infection. More broadly, these findings indicate the relevance of the canine model for vaccine development against *A. vasorum* and related blood-feeding strongylid nematodes, including hookworms.

In addition to hookworm work, there has been a major emphasis on the development of vaccines against *H. contortus* (see Bethony et al., 2006; Knox, 2011). For instance, major effort has been directed at inducing immunity in sheep against proteins expressed in or excreted/secreted from the gut of *H. contortus*, with the aim of disrupting or inhibiting the parasite's digestion of host blood. To date, one of the most effective immunogens is the *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP), which is expressed mainly in the microvillar surface of the parasite's gut, and contains metalloproteases with haemoglobinase activity, pepsinogens, cystatin and galectin (reviewed by Knox et al., 2003). Lambs vaccinated with the native H-gal-GP from adult *H. contortus* showed a ~70% reduction in worm burdens following challenge infection; both the metalloprotease and pepsinogen components of this complex have been shown to elicit a 30-50% reduction in the number of *H. contortus* eggs shed in faeces (reviewed by Knox, 2011). Here, through transcriptomic data, we were able to define in the inferred proteome of *A. vasorum* two components of the H-gal-GP complex, namely a highly transcribed, close homologue of β-galectin, and homologues of pepsinogen 1. The identification of these proteins in *A. vasorum* supports the proposal of a gut associated H-gal-GP-like complex, which might incorporate catalytic enzymes, such as aspartyl and cysteine proteases identified herein (Table 5), and may therefore be a viable immunogen. These proposals require testing.

4.7. Concluding remarks

In conclusion, the present article describes the first comprehensive exploration of the transcriptome of the adult stage of *A. vasorum*, a parasite of major animal health importance. Using an integrated bioinformatic approach and functional information available for key genes/gene products in model and other eukaryotic organisms, this exploration predicts proteins likely to be of central importance in haemoglobin digestion and immunomodulation as well as for chemotherapeutic or vaccine intervention against canine angiostrongylosis. This knowledge and the transcriptomic resource established here, in conjunction with future studies of developmentally regulated transcription in different developmental stages and sexes, will underpin genomic, proteomic and metabolomic investigations of this parasite, and support the development of new treatment and control strategies.

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Table 1. Summary of available nucleotide sequence data for the adult stage of *Angiostrongylus vasorum* determined in the present study.

Description[a]		
Number of paired-end reads		5.98 million
Number of contigs (average nucleotide length ± SD)		20,033 (1422 ± 1361)
Contig length range in nucleotides		100 to 14,663
Number of inferred proteins with predicted InterPro domains (%)		10,707 (53.4)
Number of enzyme functional groups (BRITE) predicted using KOBAS		39
pathways predicted using KOBAS		286
Number of gene ontology terms assigned in total (%)		8,312 (41.5)
Number of specific (lowest level) gene ontology clusters defined within	Biological process	545
	Molecular function	209
	Cellular component	362
Number of inferred proteins in *A. vasorum* with homologues in: *C. elegans (%)*		11,505 (57.5)
C. elegans that are unique (%)		7,056 (35.2)
Parasitic nematodes (%) (see Table 2)		9,936 (49.6)
Parasitic nematodes but no *C. elegans* homologue		1,932 (9.6)
SwissProt database (%)		9,948 (49.7)
KEGG database (%)		11,728 (58.6)
TCDB database (%)		2,095 (10.5)
MEROPS peptidase domain database (%)		902 (4.5)
MEROPS peptidase inhibitor domain database (%)		314 (1.6)[b]
Kinase SARfari database (%)		332 (1.7)
GPCR database (%)		310 (1.5)[c]
Parasitic ES protein and Hb digestion enzymes (%)		151 (0.75)

[a] Abbreviations: KOBAS: KEGG Orthology Based Annotation System; TCDB: Transporter classification database; MEROPS: Peptidase and peptidase inhibitor database; GPCR: G protein-coupled receptor database; Hb: haemoglobin; ES: excretory/secretory. 'Gene ontology clusters' are generated by grouping *A. vasorum*

inferred proteins annotated with specific (lowest level) GO terms. Genome and transcriptome data for parasitic nematodes was available in the HelmDB database (Table 2).

[b] 101 (0.5%) of MEROPS protease inhibitor domains were predicted within an inferred peptide that also contained a peptidase domain.

[c] 34 inferred *A. vasorum* GPCRs also contained at least one predicted transmembrane domain.

Table 2. Comparison of proteins predicted from the transcriptome of the adult stage of *Angiostrongylus vasorum* with genome and transcriptome data compiled for parasitic nematodes (in HelmDB).

Parasite group	**Species**	**Data type**	**Reference**	**Total number of homologues in *A. vasorum***	**Number of homologues in *A. vasorum* with no close homologue in *C. elegans*** [a]
Metastrongyloidea	*Dictyocaulus filaria*	Transcriptome	Mangiola et al. (2012)	6,801	1,396
Strongyloidea	*Oesophagostomum dentatum*	Transcriptome	Cantacessi et al. (2010b)	1,741	270
Trichostrongyloidea	*Trichostrongylus colubriformis*	Transcriptome	Cantacessi et al. (2010c)	546	112
Ancyclostomatoidea	*Necator americanus*	Transcriptome	Cantacessi et al. (2010d)	415	145
Ascaridoidea	*Ascaris suum*	Genome	Jex et al. (2011)	427	9
Enoplea	*Trichurius suis*	Transcriptome	Cantacessi et al. (2011b)	6	0
				Total: 9,936	**Total: 1,932**

[a] Proteins inferred from nucleotide sequences within HelmDB (Mangiola et al., 2012) were compared with those of *A. vasorum* using BLASTx (E-value cut-off: $\leq 10^{-5}$).

Table 3. Closest homologues (by bit-score) of proteins inferred from the 40 most highly transcribed molecules in *Angiostrongylus vasorum*, in order of abundance.

Accession number[a]	Species	Description	Bit score[b]	FPKM[c]	Percentage of total transcription	Unique to *A. vasorum*	Transmembrane (TM) or signal (S) domain[d]
k|CBG14608	*Caenorhabditis briggsae*	Vitellogenin VIT-2	896	15,753.7	1.402		n
gb|AF397162.1	*Ancylostoma caninum*	Putative tissue metalloprotease inhibitor Aca14	48.1	15,201.7	1.353		n
k|CBG14608	*Caenorhabditis briggsae*	Vitellogenin VIT-2	808	14,210.3	1.265		n
gb|CCD65563.1	*C. elegans*	Vitellogenin VIT-2	343	14,130.6	1.257		n
k|CBG14608	*Caenorhabditis briggsae*	Vitellogenin VIT-2	354	13,759.5	1.224		S
k|CBG14608	*C. briggsae*	Vitellogenin VIT-2	763	13,119.8	1.167		n
k|CBG14608	*C. briggsae*	Vitellogenin VIT-2	885	13,097.9	1.166		n
k|CBG14608	*C. briggsae*	Vitellogenin VIT-2	882	12,736.4	1.133		n
sp|Q94637	*Oscheius brevesophaga*	Vitellogenin VIT-6	1106	12,421.6	1.105		S
sp|Q94637	*O. brevesophaga*	Vitellogenin VIT-6	1114	11,972.9	1.065		n
k|CBG14608	*C. briggsae*	Vitellogenin VIT-2	354	10,831.2	0.964		n
k|CBG06812	*C. briggsae*	Fatty-acid and retinol-binding	190	8,163.91	0.726		n

		protein FAR-1					
gb|CCD71958.1	*C. elegans*	Vitellogenin VIT-4	194	7,901.89	0.703		N/A
NH	-	-	-	5,071.87	0.451	y	n
NH	-	-	-	4,873.04	0.434	y	S
NH	-	-	-	4,625.33	0.412	y	n
k|CBG14608	*C. briggsae*	Vitellogenin VIT-2	211	4,399.3	0.391		n
sp|Q94637	*O. brevesophaga*	Vitellogenin VIT-6	313	4,398.75	0.391		n
sp|Q94637	*O. brevesophaga*	Vitellogenin VIT-6	274	4,039.7	0.359		n
NH	-	-	-	3,936.49	0.350	y	n
NH	-	-	-	3,850.06	0.343	y	n
k|CBG14608	*C. briggsae*	Vitellogenin VIT-2	211	3,717.26	0.331		n
gb|CCD68779.1	*C. elegans*	Protein UBQ-1	225	3,076.91	0.274		n
sp|Q07160	*Nippostrongylus brasiliensis*	Heat-shock protein homologue	152	3,015.96	0.268		S
NH	-	-	-	2,881.94	0.256	y	n
sp|Q9BN10	*Paralephostrongylus tenuis*	Aspartyl protease inhibitor	219	2,870.91	0.255		S
NH	-	-	-	2,829.19	0.252	y	n
k|CBG13962	*Caenorhabditis briggsae*	Hypothetical protein	116	2,818.69	0.251		n
sp|Q9BN10	*Paralephostrongylus tenuis*	Aspartyl protease inhibitor	219	2,802.42	0.249		S

k|CBG14608	*Caenorhabditis briggsae*	Vitellogenin VIT-2	235	2,753.28	0.245		n
NH	-	-	-	2,553.33	0.227		TM, S
sp|P51547	*Haemonchus contortus*	Extracellular superoxide dismutase	181	2,542.68	0.226		n
gb|CCD73179.1	*Caenorhabditis elegans*	xpc-1 DNA repair gene homologue	487	2,541.17	0.226		TM
gb|CCD73179.1	*Caenorhabditis elegans*	xpc-1 DNA repair gene homologue	487	2,369.75	0.211		n
gb|CCD73179.1	*Caenorhabditis elegans*	xpc-1 DNA repair gene homologue	487	2,311.9	0.206		n
gb|AW700240	*Ancylostoma caninum*	similar to *Homo sapiens* ubiquitin	211	2,217.15	0.197		n
gb|CCD73021.1	*Caenorhabditis elegans*	Sodium-dependent acetylcholine transporter	422	2,205.07	0.196		TM
NH	-	-	-	2,194.08	0.195	y	TM
NH	-	-	-	2,154.23	0.192		n
NH	-	-	-	2,130.27	0.190	y	n
Totals				248482.13	22.11		

[a] Accession numbers for GenBank (gb), SwissProt (sp) or KEGG (k) are provided, as are gene IDs (gi). NH: No homologue identified.

[b] Bit-scores are displayed for peptide-peptide comparisons using BLASTx or tBLASTn as appropriate.

[c] FPKM: fragments per kilobase per million reads - a normalised measure of transcript abundance; y: yes; n: none.

[d] N/A: Transmembrane domains and signal peptides could not be predicted due to a lack of methionine in the inferred amino acid sequence.

Table 4. Proteins predicted from the transcriptome of the adult stage of *Angiostrongylus vasorum* known to occur in excretory/secretory products from other parasitic nematodes.

No. of contigs	Accession number of *A. vasorum* homologue[a]	Homologue description[b]	ES product from species with match to *A. vasorum* homologue	Data	FPKM		Bit score		Reference
					Lower range	Upper range	Median	Lower range	Upper range	
2	gb|AF397162.1	Putative tissue metalloprotease inhibitor Aca14	*Ancylostoma caninum*	mRNA	80.52	15,201.7	7,641.11	46.1	48.1	Mulvenna et al. (2009)
2	gb|AF533365.1	Fatty acid- and retinol-binding protein 2 FAR2	*An caninum*	mRNA		181.23	8,163.91 4,172.57	200	275	Mulvenna et al. (2009)
2	sp|Q9BN10*	Aspartyl protease inhibitor	*Parelaphostrongylus tenuis*	Protein	2,802.42	2,870.91	2,870.91	219	219	Duffy et al. (2002)
5	sp|P51547*	Extracellular superoxide dismutase	*Haemonchus contortus*	Protein	7.21	2,542.68	92.6	115	206	Liddell et al. (1998)
2	gi|170579411	Actin	*Brugia malayi*	Protein	571.77	1,688.19	1,129.98	503	756	Hewitson et al. (2008)
9	sp|Q16937	Ancylostoma secreted protein ASP-1	*An. caninum*	Protein	4.44	1,371.98	1,071.47	52	174	Hawdon et al. (1996)
3	gi|71990714	JC8.8 similar to *C. elegans* transthyretin-like	*An. caninum*	Protein	8.77	954.08	54.25	52.8	169	Mulvenna et al. (2009)

		protein								
7	gb|AW588425.1	similar to *Homo sapiens* histone H4	*An. caninum*	mRNA	6.14	879.27	37.41	51.5	200	Mulvenna et al. (2009)
10	gb|EX544710.1	similar to *Patella granatina* Histone H2B	*A. caninum*	mRNA	5.7	641.86	29.7	49.6	226	Mulvenna et al. (2009)
1	gi|Bm1_25620A	High mobility group protein	*B. malayi*	mRNA	504.85	504.85	504.85	106	106	Hewitson et al. (2008)
1	gb|EDP35716.1	Hypothetical protein	*B. malayi*	Protein	325.72	325.72	325.72	117	117	Hewitson et al. (2008)
2	gb|AW700814.1	similar to *C. elegans* cytochrome C	*An. caninum*	mRNA	46.94	297.57	172.255	226	272	Mulvenna et al. (2009)
5	gb|BM077319.1	similar to *C. elegans* hypothetical protein	*An. caninum*	mRNA	10.88	274.4	16.5	60.2	106	Mulvenna et al. (2009)
1	sp|A8P0Q4	Major sperm protein	*B. malayi*	Protein	249.18	249.18	249.18	238	238	Hewitson et al. (2008)
5	sp|Q24702*	DVA-1 polyprotein	*Dictyocaulus viviparus*	Protein	57.37	134.14	63.06	573	1427	Britton et al. (1995)
1	gb|EW743110.1	similar to *C. elegans* hypothetical protein F46E10.10a	*An. caninum*	mRNA	115.07	115.07	115.07	490	490	Mulvenna et al. (2009)
1	gb|BM077851.1	transthyretin-like protein precursor (*C. elegans*)	*An. caninum*	mRNA	97.32	97.32	97.32	214	214	Mulvenna et al. (2009)
10	gb|CZ197638.1	similar to histone family member his-35 (*C. elegans*)	*An. caninum*	mRNA	4.76	94.76	13.84	81.8	212	Mulvenna et al. (2009)
1	gi|170574880	DJ-1 family protein	*B. malayi*	mRNA	48.82	48.82	48.82	213	213	Hewitson et al. (2008)

1	gb|BM077473.1	similar to *Nippostrongylus braziliensis* myoglobin	*An. caninum*	mRNA	42.27	42.27	42.27	95	95	Mulvenna et al. (2009)
1	gb|EX535424.1	Similar to *Takifugu rubripes* a-2,8-sialyltransferase	*An. caninum*	mRNA	36.77	36.77	36.77	55.1	55.1	Mulvenna et al. (2009)
1	gb|EX538267.1	similar to *C. elegans* lysozyme LYS-8	*An. caninum*	mRNA	25.33	25.33	25.33	228	228	Mulvenna et al. (2009)
1	gb|EX535002.1	similar to *C. elegans* lysozyme LYS-8	*An. caninum*	mRNA	16.51	16.51	16.51	284	284	Mulvenna et al. (2009)
2	gb|EX547128.1	similar to *C. elegans* lysozyme LYS-8	*An. caninum*	mRNA	9.19	14.64	11.915	71.2	130	Mulvenna et al. (2009)
1	gb|DW718354.1	similar to *C. elegans* lysozyme LYS-8	*An. caninum*	mRNA	14.39	14.39	14.39	171	171	Mulvenna et al. (2009)
1	sp|O97392*	Gamma-glutamyl transpeptidase (precursor)	*B. malayi*	Protein	5.76	5.76	5.76	43.9	43.9	Lobos et al. (1996)
1	gi|170592110	Galectin *Bm*-GAL-2	*B. malayi*	Protein	4.46	4.46	4.46	63.2	63.2	Hewitson et al. (2008)
1	gb|AAO63578.1	*Ancylostoma* secreted protein 6 precursor	*An. caninum*	Protein	4.44	4.44	4.44	52.8	52.8	Mulvenna et al. (2009)
1	gb|AAN78298.1	similar to *Globodera rostochiensis* actin-2	*Teladorsagia circumcincta*	Protein	2.87	2.87	2.87	129	129	Craig et al. (2006)

[a] Accession numbers are for WormBase, SwissProt and GenBank databases. Asterisks denote ES proteins that have been characterised in the listed species. FPKM: fragments per kilobase per million reads - a normalised measure of transcript abundance; Bit score: a normalized probability measure, where larger numbers indicate lower probability.

[b] Descriptions are provided for proteins in publically available databases that are the closest homologue (best bit-score match) of both a predicted protein(s) of *A. vasorum*, and a sequenced ES protein from at least one parasitic nematode.

Table 5. Proteins predicted from the transcriptome of the adult stage of *Angiostrongylus vasorum* that are homologues of proteins involved in haemoglobin digestion by haemotaphagous nematodes.

No. of contigs	Accession number of *A. vasorum* homologue [a]	Homologue description [b]	Species	Data	Peptidase class	FPKM	Bit score [c]		References		
						Lower range	Upper range	Median	Lower range	Upper range	
2	gb|AAT37718.1	Glutathione-S-transferase	*Ancylostoma caninum*	Protein		6.93	477.27	-	253	288	Zhan et al. (2005)
3	gb|ABL85237.1	Cysteine proteinase 3	*Necator americanus*	Protein	C01.101	7.46	431.66	61.43	44.7	85.9	Williamson et al. (2003); Ranjit et al. (2008)
1	sp|B6DVK1	Aspartyl protease (APR-1)	*Ancylostoma duodenale*	Protein	A01.068	380.08	380.08	-		687	Pearson et al. (2009)
1	sp|O44126	β-galactoside-binding lectin*	*Haemonchus contortus*	Protein		329.76	329.76	329.76	549	549	Newlands et al., (1999); Hewitson et al. (2008)
2	gb|ACX53261.1	Glutathione-S-transferase 1	*N. americanus*	Protein		118.89	142.19	-	178	254	Zhan et al. (2010)
4	gb|U18912.1	Cathepsin-B proteinase*	*An. caninum*	mRNA	C01.101	84.98	127.86	111.53	53.8	287	Harrop et al. (1995); Mulvenna et (2009);
10	gb|AY605283.1	Glutathione-S-transferase	*An. caninum*	mRNA		2.63	123.05	6.635	44.1	54	Zhan et al. (2005)
32	gb|AF079402.1	Pepsinogen (PEP-1)*	*H. contortus*	mRNA	A01.053	0	22.38	2.755	29.9	67.9	Longbottom et al. (1997); Redmond et al. (2001); Yatsuda et al. (2003)

[a] sp: SwissProt ; gb: GenBank; FPKM: fragments per kilobase per million reads - a normalised measure of transcript abundance.

[b] The homologues displayed showed the greatest similarity (i.e. highest bit-score) to *A. vasorum* contigs or inferred peptides.

[c] Bit score: a normalized probability measure, where larger numbers indicate lower probability. Asterisks denote proteins that have also been identified in the ES products of the listed species.

Table 6. Protein targets and associated small molecule compounds from the ChEMBL database. Targets are homologous to proteins inferred from the transcriptome of *Angiostrongylus vasorum* that are likely to derive from essential genes, based on RNA interference studies in *Caenorhabditis elegans*.

No. contigs	WormBase Gene ID [a]	Closest homologue description	Species	FPKM [b]	TMD [b]	ChEMBL target ID	ChEMBL target description [a]	Published activity	Conc. (nM) [b]	Compound name [c]	Reference
1	6784	γ-aminobutyric acid (GABA) receptor	*Caenorhabditis elegans*	5.91	Y	2746	γ-aminobutyric acid (GABA) receptor subunit pi	IC50	9.40E-06	Desmethyldiazepam	Fatmi et al. (1984)
1	785	Cysteine proteinase-3	*Necator americanus*	431.66		4072	Cathepsin B	IC50	1	cis-4-(2,3-dimethylphenoxy)-6-oxa-1-aza-bicyclo[3.2.1]octan-7-one	Epple et al. (2007)
1	8304	Serine protease inhibitor	*C. elegans*	4.74		2487	Amyloid β A4 protein	EC50	810	2-amino-4-cyclohexyl-1-methyl-4-phenyl-1H-imidazol-5(4H)-one	Nowak et al. (2010)
1	10079	similar to glutathione-S-transferase	*C. elegans*	11.55		5879	Hematopoietic prostaglandin D synthase	IC50	920	3-(3-phenylisoxazol-5-yl)-1H-pyrazole	Hohwy et al. (2008)
1	15364	Hypothetical protein	*Caenorhabditis briggsae*	4.8		218	Cannabinoid receptor 1	Ki	1,000	Tacrine	Lange et al. (2010)
1	18880	Acetylcholine-gated chloride channel AAC-3	*C. briggsae*	3.89		343	γ-aminobutyric acid (GABA) receptor subunit α-1	IC50	1,620	9H-β-Carboline	Hagen et al. (1987)
1	7801	Transient receptor potential cation channel TRPA-1	*C. elegans*	4.74	Y	1075310	Transient receptor potential cation channel subfamily A	EC50	2,580	Isovelleral	Baraldi et al. (2010)

1	366	TAZ zinc finger family protein	*Brugia malayi*	3.48		3784	Histone acetyltransferase p300	IC50	3,000	2-(4-Pyridyl)-isothiazol-3(2H)-one	Gorsuch et al. (2009)
1	20932	Dihydroorotate dehydrogenase	*C. elegans*	3.49		1966	Dihydroorotate dehydrogenase	IC50	10,000	5-Methyl-isoxazole-4-carboxylic acid (4-trifluoromethyl-phenyl)-amide	Albert et al. (1998)
1	10877	β-lactamase	*C. briggsae*	6.74	Y	2026	β-lactamase	IC50	10,000	3-(4-Dimethylamino-benzylidene)-1,3-dihydro-indol-2-one	McGovern et al. (2002)
1	13805	β-carbonic anhydrase	*C. briggsae*	5.94		5767	Probable transmembrane carbonic anhydrase	Ki	12,100	4-(2-hydroxyethyl)phenol	Davis et al. (2011)
1	20012	Hypothetical protein	*C. briggsae*	14.85		3616	Protein kinase C ETA type	IC50	15,000	5-Bromo-6-methoxy-9H-β-carboline	Castro et al. (2003)
1	3635	Zinc-finger DNA-binding protein	*C. elegans*	8.58		5398	Hepatocyte nuclear factor 4-α	EC50	100,000	naphtho[2,1-b]furan-2-carboxamide	LeGuevel et al. (2009)

[a] *A. vasorum* predicted proteins with homologues in *C. elegans* (WormBase Gene ID) that are associated with lethal RNAi phenotypes, but which have no close homologues in the canid host were compared with the ChEMBL database. The closest match for each predicted protein identified in all searched databases is given. The closest homologue (highest bit score match) of *A. vasorum* drug target candidates are provided for description.

[b] Abbreviations: FPKM: fragments per kilobase per million bases – a normalised measure of transcript abundance; TMD: predicted transmembrane domain. Conc.: concentration.

[c] All listed compounds satisfy the Rule-of-Three for drug-like molecules (Congreve et al., 2003), do not violate any of the five Lipinski rules (Lipinski et al., 2001), and are deemed 'medicinal chemistry friendly'; trade names for compounds are provided where appropriate.

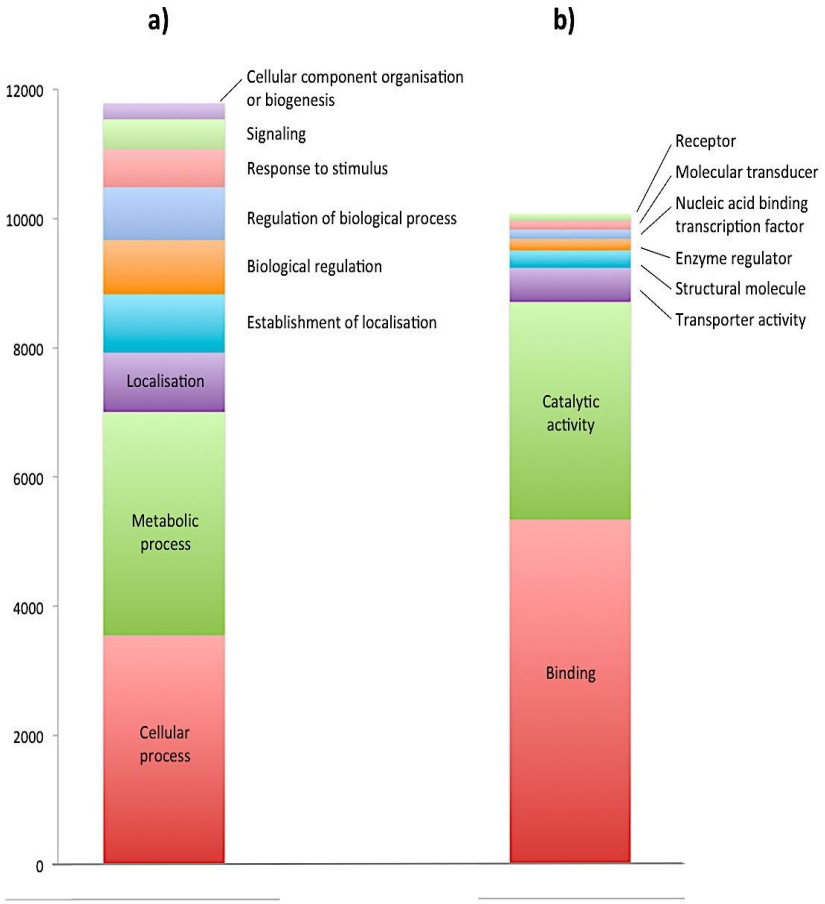

Fig. 1. Major clusters of proteins encoded in *Angiostrongylus vasorum* inferred using gene ontology (GO) clustering of (a) molecular functional groups, and (b) biological processes. As multiple GO terms can be assigned to a single transcript, the clusters displayed are not mutually exclusive; therefore the y-axis is not cumulative. In total, 8,361 inferred peptides were assigned GO terms.

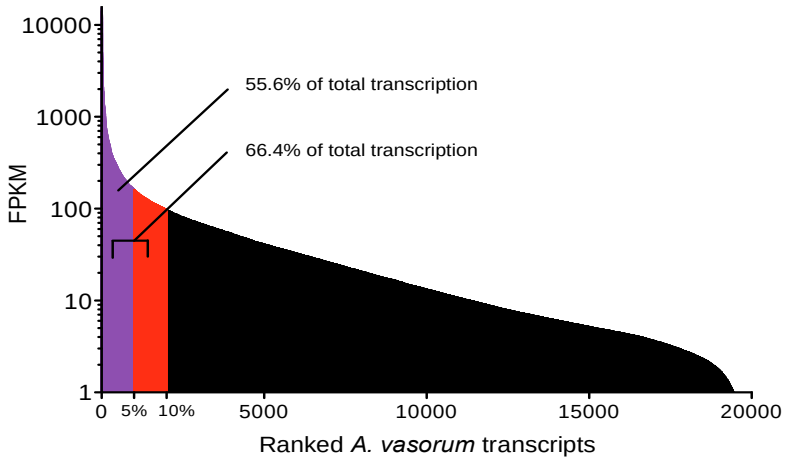

Fig. 2. *Angiostrongylus vasorum* transcripts ranked by fragments per kilobase per million reads (FPKM; i.e. transcriptional abundance); 5% of all contigs account for 55.6% of all transcribed molecules (purple area); 10% of all transcripts account for 66.4% of total all transcribed molecules (red and purple areas). The x-axis

represents ranked individual contigs. The y-axis indicates the number of FPKM - a normalized measure of transcript abundance for each contig on a logarithmic scale. The 10th (10%) and 5th (5%) percentile are indicated on the x-axis.

Fig. 3. Alignment of the APR-1 catalytic domain of the inferred *Angiostrongylus vasorum* APR-1, and APR-1 of other parasitic nematodes, after (Pearson et al., 2010). The catalytic domain determined for the *N. americanus* APR-1 is outlined in black, and the protective epitope *Na*-A$_{291}$Y is outlined in red. There is sequence conservation in the protective epitope between anthropophilic hookworms and *A. vasorum*, and divergence in sequence between *N. americanus* and *An. caninum*. Despite this divergence, immunization of dogs with recombinant *N. americanus* APR-1 achieved protection against challenge infection with *An. caninum* (Pearson et al., 2009).

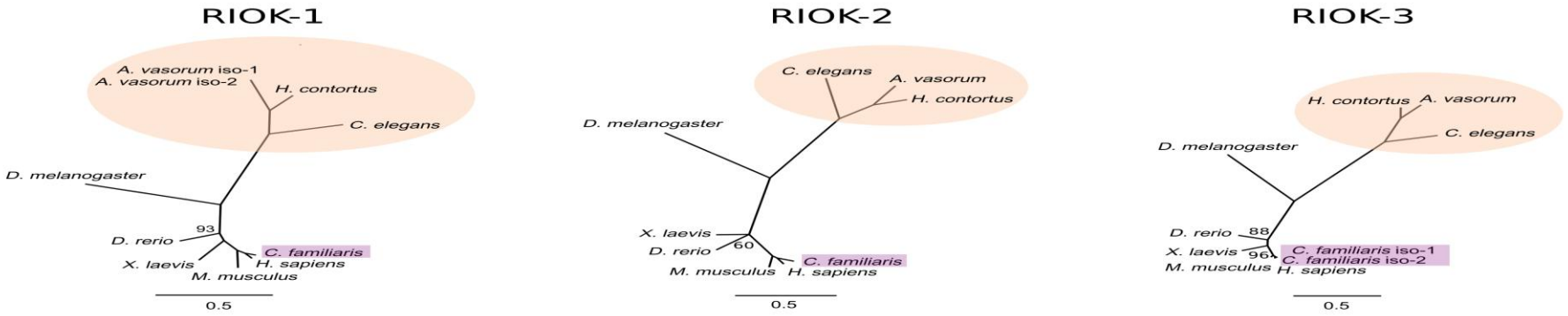

Fig. 4. Phylogenetic analysis of amino acid sequence data for RIO protein kinases (RIOK-1, RIOK-2 and RIOK-3) of *Angiostrongylus vasorum*, *Haemonchus contortus*, *Caenorhabditis elegans* (nematodes), and other organisms *Drosophila melanogaster, Danio rerio, Xenopus laevis, Mus musculus, Homo sapiens* and *Canis familiaris* (i.e. the canid host of *A. vasorum*) by Bayesian inference. Bootstrap values for all branches were 100%, except where otherwise indicated. The unrooted tree shows that RIOKs from nematodes cluster together (shaded pink) to the exclusion of RIOKs from the other organisms included in the analysis, including the canid host (shaded purple). Accession numbers of RIOKs sequences used in then analysis: CCD67367.1, CAC70109.2, CAA80180.1, XP_535878.1, XP_536291.3, XP_003639236.1, XP_003639235.1, NP_648489.1, NP_651365.1, NP_608871.1, NP_998160.1, NP_998719.2, NP_001003614.1, ADW23592.1, ADW23593.1, ADW23594.1, NP_113668.2, EAW96088.1, NP_003822.2, NP_077204.2, NP_080210.1, NP_077144.2, NP_001116165.1, NP_001088220.1, NP_001087045.1.