THESIS

IN VIVO AND *IN VITRO* CHARACTERIZATION OF THE CANINE NEPHRON AND URINARY PROTEOME

Submitted by

Laura E. Brandt

Department of Microbiology, Immunology and Pathology

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Master's Committee:

Advisor: Andrea Bohn

E. J. Ehrhart
Christine Olver
Jessica Prenni

ABSTRACT

IN VIVO AND *IN VITRO* CHARACTERIZATION OF THE CANINE NEPHRON AND URINARY PROTEOME

Immunohistochemistry allows the localization of proteins to specific regions of the nephron. This study reports the identification and localization of proteins *in situ* within normal canine, feline and mouse kidney by immunohistochemistry, maps their distribution and compares results to previously reported findings in other species. The proteins investigated are aquaporin-1, aquaporin-2, calbindin-d28k, glutathione *S*-transferase-α and Tamm-Horsfall protein. Our findings in the dog are similar to that in other species, and localize aquaporin-1 to the proximal convoluted tubule epithelium, vasa recta endothelium and descending thin limbs, aquaporin-2 to collecting duct epithelium and calbindin-d28k is found within distal convoluted tubule epithelium. Glutathione *S*-transferase-α has variable expression and is found in renal transitional epithelium only in some individuals, the proximal straight tubules only in some individuals or in both locations in others. Tamm-Horsfall protein localizes to thick ascending limb epithelium. These findings are similar in the cat, with the exception that aquaporin-1 is located in glomerular podocytes, in addition to proximal convoluted tubule epithelium, and glutathione *S*-transferase-α is found solely within the proximal convoluted tubule within all kidney samples examined. The mouse kidney is almost identical to the dog, but expresses glutathione *S*-transferase-α in the glomeruli only.

Additionally, we successfully adapted techniques employed in human studies to characterize the normal canine urinary proteome. Both soluble proteins and the exosomal fraction of urine were examined. Greater than 500 proteins were identified in this initial study. Many of these proteins are also found in human urine, but large numbers of proteins also appear to be unique to the canine. Software entitled Biological Networks Gene Ontology (BiNGO) was utilized to characterize canine urinary proteins into respective Gene Ontology categories of Cellular Component, Molecular Function and Biological Process. Exosomal proteins are largely derived from an intracellular location, while soluble proteins are comprised of extracellular and membrane proteins. The majority of proteins in both canine urinary fractions are involved in protein binding. Exosomal proteins are also involved in metabolic process and localization, while soluble proteins are linked to specific localization processes. The distribution of canine urinary proteins within GO functional categories is very similar to that of human urine. Known markers of renal disease, such as aquaporin-1, gamma-glutamyl transferase and retinal binding protein were identified via proteomic techniques in the urine of healthy dogs, as were novel biomarkers like fetuin-A and ubiquitin A-52.

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CHAPTER 1

INTRODUCTION

The in-depth study of renal membrane proteins and urinary proteins in canines has long been over-looked in the veterinary field. These areas of study are important in understanding renal physiology and pathophysiology.

In addition to being popular companion animals, dogs are also used for animal toxicological studies and models of disease. As many developmental drugs are metabolized via the liver and kidney, the definitive localization of renal pathological changes is imperative for accurate histological interpretation of renal changes secondary to drug administration. Additionally, the histological diagnosis of canine renal diseases is often made based on the location of lesions within the nephron. Unfortunately, it can sometimes be challenging to distinguish specific nephron tubules from one another histologically, especially in smaller specimens.

In order to aid in the definitive identification of renal nephron segments, we sought to develop reliable immunohistochemical markers within the normal canine kidney. We chose renal proteins with well-documented nephron locations that would encompass the proximal convoluted tubule (PCT), the thin limbs of the loop of Henle, the thick ascending limb (TAL), the distal convoluted tubule (DCT) and the collecting duct (CD).

As the utility of these markers may be valuable in other veterinary and lab animal species, we expanded our study to include feline and mouse kidneys. The renal proteins

we chose to examine are aquaporin 1 (AQP1), aquaporin 2 (AQP2), glutathione *S*-transferase alpha (GST-α), calbindin D-28k and Tamm-Horsfall protein (THP).

AQP1 (originally called CHIP28) was the first aquaporin discovered in the early 1990's.[66] It was the water channel long sought after by researchers that helped explain water balance within many tissues and organs.[10,12,66-68] AQP1 is found in various tissues, including intestine, brain and erythrocyte membranes.[12] The protein's location in the kidney has been well documented in humans, rats and mice, as well as a few other mammalian and avian species.[9,10,13,50,58,62,66,107] More specifically, AQP1 is found along the apical and basolateral membrane of luminal cells within the PCT, with highest expression in Segment 3 (S3) of the PCT, as well as within type II distal thin limb (DTL) epithelium and to a lesser extent in type III and type I DTL epithelium and the descending portion of the vasa recta.[9,12,62,66,67,92] It is not found in regions of the nephron that are impermeable to water.[9,12,62,66,67,92]

The location and biophysical properties of AQP1 overwhelmingly support that it is essential for proximal nephron water handling and urinary concentration. People deficient in AQP1 and AQP1 knock-out (KO) mice are unable to concentrate their urine, even in the face of water deprivation and vasopressin administration.[106] AQP1 allows isoosmolar glomerular filtrate to pass freely into the PCT.[12,67] Once the filtrate has passed to the descending thin limb of the loop of Henle, it becomes hyperosmolar as water is reabsorbed into the interstitium via AQP1.[12,67] Meanwhile, Na^+, Cl^- and urea are secreted into the filtrate.[12,67] Additionally, it is thought that AQP1 within the descending vasa

recta helps maintain the osmotic gradient established by the countercurrent exchanger, which is responsible for the concentration of urine.[12,67]

Aquaporin 2 was selected as our renal marker for the CD, as its location has been well-documented in various species and its function heavily studied.[9,12,54,62,66,67,91,92] As in AQP1 studies, primary research has utilized human, rat and mouse kidney tissue to study AQP2. Aquaporin 2 is located in the principal cells of the collecting duct and to a lesser extent the connecting tubules in these species.[9,62,67,91,92] Specifically, AQP2 is found chiefly along the apical border of the principal cells, but can also be seen within the cytosol.[9,62,67,91,92] In the CD, vasopressin acts on AQP2, inducing a shuttle mechanism to move the water channel from intracellular vesicles to the apical plasma membrane.[9,62,67,91,92] Water is then reabsorbed through AQP2 and urine is successfully concentrated.[9,62,67,91,92]

More recently, AQP1 and AQP2 expression in the kidney and excretion into the urine have been examined in disease. A 2000 study created a rat model of cisplatin-induced polyuria in order to evaluate the expression of renal aquaporins.[50] The late-phase polyuria associated with cisplatin toxicity correlated with the loss of AQP1 from the PCT, especially within S3, the thin descending limb (TDL) and descending vasa recta (demonstrated via IHC).[50] These findings correlated with H&E stained histological sections that demonstrated tubular necrosis, loss of microvilli and vacuolization within the S3 segment and with decreased expression of AQP1 in the inner medulla by semiquantitative blotting (no changes were observed histologically within the medulla).[50]

AQP2 was also negatively affected by the administration of cisplatin and demonstrated significantly decreased expression in the inner medulla via immunoblotting.[50] The decreased expression of these proteins leads to the inability to concentrate urine.

Other studies have discovered that loss of AQP2 is responsible for some forms of nephrogenic diabetes insipidus.[77,84] Endotoxemia-induced acute kidney injury in AQP1 KO mice appeared to predispose mice to more severe renal injury.[111] In rat models for ischemic renal injury, both AQP1 and AQP2 expression is reduced in the kidney and is thought to be the cause for ischemia damaged kidneys' inability to concentrate urine.[88] Aberrations in AQP1 and AQP2 expression in the kidney and urinary excretion can also occur with extra-renal diseases, such as congestive heart failure and liver cirrhosis.[39,114]

Glutathione *S*-transferase-alpha is a member of the glutathione *S*-transferase (GST) family. Many classes of these enzymes exist in various tissues and are highly prevalent in the liver and kidneys.[33,46] In general, GSTs aid in detoxification processes of cytotoxic drugs, chemicals, carcinogens and other substances within tissues.[33,46] Glutathione *S*-transferase-α has been localized to the epithelium of the PCT in rats and humans, where the cellular distribution is described as both cytoplasmic and nuclear in these species.[17,33] Very mild IHC staining has been seen in medullary tubules, thought to be thin limbs of the loop of Henle.[17,33]

GST-α is not found in the urine of normal, healthy individuals, but is found in significant amounts in those that have incurred tubular damage.[22,33] Urinary GST-α levels

have been found to be increased in individuals with the following disorders: cyclosporine induced PCT damage, acute tubular necrosis, renal transplant infarct, ischemia, cisplatin toxicity, gentamicin toxicity and after heavy metal exposures.[17,22,33,37,115] The enzyme's expression may be directly related to the body's ability to handle oxidative stress.

Calbindin D-28k (formerly referred to as calcium binding protein) is found in many tissues, including kidney, intestine, pancreas and bone, in such species as reptiles, amphibians, fish, chicks, rats, rabbits, monkeys and humans.[35,73,94] This protein has been localized to the DCT in many of these species (including chicks, rabbits, rats and humans), with sparse staining observed in the CD.[35,73,94] A recent toxicological study did localize calbindin D-28k to renal tubules within the canine kidney, but did not further classify the location of the protein.[3] Cellular staining is described as cytosolic and nuclear.[35,73,94]

The function of calbindin D-28k appears to be dependent on vitamin D metabolites in some tissues, as in the kidney, but not in all.[35,73] The functions of calbindin D-28k could be widely different, therefore, within different tissues. It is known, however, that calbindin is a strong calcium binding protein and is thought to be key to calcium regulation, although the exact mechanism is not yet fully understood.[35,73] Potentially, calbindin D-28k has many functional roles within the kidney itself, such as selective calcium resorption, calcium transportation and calcium buffering.[35]

Tamm-Horsfall protein, a glycoprotein, is the most abundant protein found in urine and is believed to have many functions.[51,86] The protein has been localized to the luminal cells of the TAL of the outer medulla in humans and dogs.[37,40,85,86] Immunostaining is reportedly most intense at the apical margins of cells in the TAL.[85] Within the DCT, small numbers of positively staining cells appear intermittently, which demonstrate a homogenous cytoplasmic expression.[85] Similar findings have been found in humans.[37,40,85,86]

Tamm-Horsfall protein is thought to regulate renal cytokines and be a component of the body's defense against urinary tract infection, specifically type 1 fimbriated *Escherichia coli*.[7,72] More recently, it has been studied in conjunction with the formation of urine crystals and stone formation in THP knockout mice (which are more prone to calcium oxalate formation) and people with a family history of calcium oxalate stone formation and concurrent excessive THP excretion.[40,64] Decreased THP excretion has also been noted in children with newly diagnosed diabetes mellitus, as well as in adults who later developed end-stage renal and cardiovascular disease secondary to diabetes.[37]

The above described features of AQP1, AQP2, GST-α, calbindin D-28k and THP make these proteins excellent candidates for markers of various regions of the nephron. Additionally, the variation of expression of these proteins (within the kidney and urine) in diseased states may prove useful to diagnostic medicine, as well as toxicological studies.

While the definition and criteria for diagnosing acute renal disease is sometimes controversial, in veterinary medicine the tried and true diagnosis is generally based on the acute elevation of serum urea nitrogen (sUN) and serum creatinine (sCrt) with concurrent inappropriately concentrated urine. This combination of diagnostics indicates renal azotemia. However, in order to see evidence of renal azotemia via elevated sUN and sCrt, almost two-thirds of the functional capacity of the kidneys must be lost.[89] As it has been shown repeatedly in human medicine that early diagnosis and treatment for ARD is imperative to reducing morbidity and mortality, waiting for two-thirds of the kidney to be affected by a pathological process in order to diagnosis the disease is unacceptable.[83] As our understanding of renal pathology and treatment of disease has progressed, the hunt for additional biomarkers of early renal disease is on. Renal biomarkers should be easy to accurately and reproducibly quantify, have high sensitivity in indicating renal injury or response to treatment, be cost-effective, add to the knowledge obtained by conventional diagnostic methods, and be applicable to a wide population of individuals.[96]

Serum cystatin-C (sCysC) has recently been utilized in human medicine as a sensitive, early biomarker for altered renal function and is superior to sCrt in early detection of renal disease.[21,53,96] Cystatin-C is produced at a steady rate by all nucleated cells and is released into the blood stream at a steady rate.[53] Under normal conditions, cystatin-C is cleared by the kidneys and excreted in the urine.[53] However, if renal function is impaired, sCysC levels increase.[21] Current studies in the dog have not yet proven any significant advantage to measuring sCysC over traditionally measured sCrt in diagnosing ARD.[112]

One of the criticisms of serum biomarkers of renal disease, is that they indicate renal impairment, but do not directly indicate renal injury.[2] Urinary biomarkers have several potential advantages over serum biomarkers. Except in oligouric renal failure, urine is freely and easily obtainable. As urine is continually voided from the bladder, the protein content of urine also provides a snap-shot view of what physiological and pathophysiological processes are occurring at a specific point in time.[43] Urinary proteins originate from glomerular filtrate, renal tubular secretion of soluble proteins, sloughing of whole cells into the urinary space, glycosylphosphatidyl inositol anchored detachment (as in THP) and secretion of exosomes.[38] Therefore, voided urine can contain biomarkers from any region of the urinary tract, including kidneys, ureters, bladder, urethra and potentially prostate gland, which can directly shed light upon the site of renal injury and organ pathophysiology. Additionally, as systemic proteins enter the glomerular filtrate, urine can contain biomarkers for extrarenal diseases. [38]

Human medicine still relies on serum markers like sCrt and sCysC to make a definitive diagnosis of renal disease in the majority of cases. However, increased concentrations of albumin, due to glomerular and proximal tubular damage, are typically seen in a variety of renal diseases prior to increases in sCrt or other renal functional markers.[96] Additionally, the presence of various immunoglobulins indicate various renal pathology. As immunoglobulin-γ (IgG) and immunoglobulin-μ (IgM) are large molecular weight proteins, they are not usually seen in urine unless glomerular damage is present.[43] Urine IgM is also a predictor of progressive and end-stage renal disease.[5,6] Increased

urine IgA correlates to the severity of renal damage incurred by IgA nephropathy in humans.[93] Also, N-acetyl-β-D-glucosaminidase (NAG), a lysosomal protein, is sensitive for tubular interstitial damage in humans.[8,31] The iron-transporting protein, neutrophil gelatinase-associated lipocalin (NGAL), has also become a reliable biomarker for acute renal injury in human patients. Urine levels are known to increase after toxic or ischemic insults, indicating defective proximal tubular resorption of the protein.[63]

Many novel urinary biomarkers are on the horizon and their utility and reliability in diagnosing and monitoring for disease is increasing. For example, nephrin and podocin, which specifically indicate damage to glomerular podocytes, are increased in the urine of patients suffering from diabetic nephropathy and lupus nephritis.[109,110] Another podocyte-specific marker, podocalyxin, is increased in the urine of patients with IgA nephropathy, lupus nephritis and post-streptococcal glomerulonephritis.[44] Kidney injury molecule-1 (KIM-1) is only expressed on the luminal surface of proximal tubular epithelium when the kidney has sustained injury. The presence of this protein in the urine indicates tubulointerstitial damage.[31,104] Liver-type fatty acid-binding protein (L-FAB) is shed by damaged proximal tubular epithelial cells in response to hypoxic injury.[42,75] Increasing amounts of urinary L-FAB correlates with decreasing renal function and is a sensitive marker of both acute and chronic renal disease.[42,75]

N-acetyl-β-glucosaminidase and gamma-glutamyl transferase (GGT) are the two most commonly used urinary biomarkers in veterinary medicine. Both have been used in canine toxicologic studies, but have not been relied upon heavily in the clinical

setting.[15,43] N-acetyl-β-glucosaminidase is found within renal tubular epithelial lysosomes and GGT is located in the brush border of proximal tubular epithelial cells.[43] Both enzymes increase in urine prior to an increase in sCrt with ARD.[15,43] However, NAG and GGT are particularly susceptible to biological variation and can be ureliable.[15,43] Additionally, neither of these enzymes provide information about glomerular disease. Other biomarkers under study in canine renal disease include C-reactive protein (CRP), immunoglobulin gamma (IgG) and retinol binding protein (RBP). Recent studies associated increases in these urinary proteins with chronic renal disease and acute, transient nephropathy in dogs with pyometra.[57,87]

Many of these individual proteins are promising biomarkers for diagnosing ARD in both humans and dogs. Due to high individual variation, though, urinary biomarkers can be susceptible to erroneous results. Therefore, the creation of a panel of urinary biomarkers would be ideal to increase the sensitivity and specificity of diagnosing specific renal diseases, monitoring response to treatment and delivering an accurate prognosis.[2,16]

To this aim, proteomic analysis of urine has become a rapidly expanding field in the last ten years. Simply put, proteomics is the large-scale study of proteins, generally employing various techniques to concentrate and isolate proteins and using mass spectrometry to obtain an all-inclusive account of a substance's protein content. In 2002, Thongboonkerd, et. al., employed protein concentration and isolation techniques (both acetone precipitation and ultracentrifugation), 2D gel-electrophoresis and mass

spectrometry to analyze the human urinary proteome, or the entire complement of proteins within human urine.[101] This analysis yielded the identification of 47 unique proteins. Thongboonkerd also discovered that different techniques yielded different proteins.[101] For example, acetone precipitation yielded more acidic, hydrophilic proteins, while ultracentrifugation isolated basic, hydrophobic membrane proteins.[101]

In 2004, Mark Knepper's group identified 295 proteins via improved techniques.[74] His group also confirmed the presence of urinary exosomes in the ultracentrifuged fraction of human urine.[74] Exosomes are the internal vesicles of cellular multivesicular bodies (MVBs). They are formed in a unique manner. Many exosomal proteins begin as cellular membrane proteins that are endocytosed into a cell.[45,74] The endocytic vesicle then fuses with a MVB's membrane and is then endocytosed into the MVB.[45,74] Under the appropriate conditions, MVBs will then bind with the cellular membrane, releasing internal vesicles, now called exosomes, into the urinary space. [45,74] As a result of this process, exosomal membranes have a cellular cytoplasm inward orientation, as do MVBs.[74,98] Exosomes are very small, often only 40-100 nm.[38] They commonly contain transporter or membrane proteins responsible for membrane trafficking and cell-to-cell signaling.[26,74,98] The unique construction and function of exosomes makes them a prime candidate biomarker of disease. Additionally, exosomes can provide unique information about real-time renal pathophysiology.

As proteomic techniques have advanced, so has the database of proteins comprising the human urinary proteome. More recent studies have identified

approximately 1500-2000 unique urinary proteins.[1,49] These advancements are a result of a combination of factors, including the removal of overly abundant urinary proteins from samples (such as albumin and THP), the use of protease inhibitors and improvements in mass spectrometers and search engine software.[117]

Proteomic profiling for disease has added much to the cannon of renal physiology and pathophysiology. For example, proteomic techniques have been employed extensively by Mark Knepper's group to study AQP2 and renal tubular water balance.[36,103] Urine proteomics has been used to study the pathophysiology of diseases like hypertension and diabetic nephropathy.[99,100] Additionally, urinary exosomes are now known to contain various disease associated proteins, such as AQP2 (associated with nephrogenic diabetes insipidis), podocin (associated with podocyte damage), angiotensin-converting enzyme (associated with hypertension), and $Na^{+}K^{+}2Cl^{-}$ cotransporter (associated with Antenatal Bartter syndrome, type I), which have been identified utilizing proteomic techniques.[38]

Most recently, urine proteomics has lead to the discovery of several novel, potential urinary biomarkers for renal disease: fetuin-A, cleaved forms of β2-microglobulin and ubiquitin fusion protein (UbA52). Fetuin-A is a negative acute-phase protein produced by the liver and found in the exosomal fraction of urine. Urine concentrations of fetuin-A are documented to increase prior to an increase in sCrt, making it a potential marker for ARD.[71,116] β2-microglobulin requires an acidic pH, like that of the urine of individuals with acute tubulointerstitial rejection of renal allografts, to

cleave into the specific fragments found in Schaub's recent proteomic study.[82] Ubiquitin fusion protein is released into the urine of patients with diabetic nephropathy, but was not found in the urine of normal people or patients with proteinuria due other causes, making UbA52 a viable marker to diagnose diabetic nephropathy.[24]

Due to the dog's important role as a companion animal and laboratory subject, we attempted to characterize the canine urinary proteome. In our evaluation, we sought to adapt previously reported techniques used on human urine to canine urine. Our goal was to identify and characterize the proteins comprising the canine urinary proteome and make a concise comparison of the canine urinary proteome to that of human urine. Additionally, we attempted to identify canine urinary proteins that may prove to be useful biomarkers for AKD based on review of the literature and the performance of such proteins in the human medical field.

CHAPTER 2

IN VIVO LOCALIZATION OF CANINE, FELINE AND MOUSE RENAL PROTEINS

Immunohistochemistry (IHC) utilizing aquaporin 1 (AQP1), aquaporin 2 (AQP2), glutathione S-transferase-α (GST-α), calbindin D-28k and Tamm-Horsfall protein (THP) antibodies has been used to localize these proteins to segments of the nephron in various species, including human and rat kidneys.[12,33,51,62,66-68,73,92,94] Identification of specific segments of the nephron can be challenging histologically. AQP-1, AQP-2, THP and calbindin D-28k have been used as nephron segment markers and utilized in double-labeling IHC to more exactly localize other proteins within the nephron of humans and rats.[55] By localizing proteins of interest within the nephron we can further elucidate the function of these proteins in renal physiology and pathophysiology and identify nephron segments in disease states.

Our study establishes the *in situ* location of proteins that have a distribution well documented in other species and identify these proteins as nephron segment markers in the dog, cat and mouse kidney. The proteins evaluated include: AQP1 for localization of the proximal convoluted tubule (PCT), AQP2 for the collecting ducts (CD), GST-α for the PCT, calbindin D-28k for the distal convoluted tubule (DCT) and THP for the thick ascending limb (TAL) of the loop of Henle.[12,33,51,62,66-68,73,92,94]

Materials & methods

Five disease-free kidneys from each of the three species (dog, cat and various strains of sentinel mice) were obtained from recently euthanized animals and were formalin fixed. Tissues were routinely processed and paraffin embedded. Four micrometer sections were placed on positive-charged slides. Sections were deparaffinized with xylene and rehydrated by a series of graded baths in alcohol and PBS buffer.

Heat induced epitope retrieval (HIER) was performed on all slides, except those for Tamm-Horsfall protein IHC.[76] Sections for HIER were heated to 125˚C, under pressure, for approximately 1 minute in target retrieval solution (pH 6.0 of low pH, Dako, Carpinteria, CA for AQP1; pH 9.0 of high pH, Dako, Carpinteria for AQP2, calbindin d28-k and GST-α). Non-specific antibody binding was blocked for 5 minutes in Background Sniper (Biocare Medical, Concord, CA). Endogenous peroxidase activity was quenched in 3% hydrogen peroxide. The samples were washed with Tris-buffered saline (TBS).

All samples were incubated with their primary antibody overnight at 4˚C. The following primary antibodies were used: rabbit anti-human AQP-1 polyclonal antibody (1:500, Chemicon, Temecula, CA), rabbit anti-rat AQP-2 affinity purified polyclonal antibody (1:500, Millipore, Billerica, MA), anti-human GST-α monoclonal antibody (1:50, Novocastra, Bannockburn, IL), anti-bovine calbindin D-28K monoclonal antibody (1:1000, Sigma-Aldrich, St. Louis, MO) and sheep anti-human Tamm-Horsfall glycoprotein polyclonal antibody (1:2500, Chemicon, Temecula, CA). Sections for THP

immunostaining were then incubated with an HRP-labeled anti-sheep secondary antibody for 1 hr at 4˚C.[76] All other slides were incubated with Envision+ System (Dako, Carpinteria, CA). Immunoreactive complexes were identified with DAB and the sections were counterstained with hematoxylin.

Renal tubules and other microstructures were identified based on light microscopic features and anatomic location. In the renal cortex, PCTs have a greater external diameter than DCTs and the luminal epithelium has a brush border giving the tubular lumen a "star-shaped" appearance.[79] These features are absent in the DCT.[79] These morphological features are carried into the medullary rays, where the PCT becomes the straight portion of the proximal tubule.[79] Also within the medullary rays is the beginning portion of the DCT, known as the TAL, sharing the morphologic features of cortical DCT.[79] Running in parallel with these structures are the collecting ducts. These tubules are similar in diameter to proximal tubules, but have smaller, cuboidal epithelium with prominent cell borders, exhibit more nuclei per cross section and do not have a brush border.[79] Thin limbs are within the medullary rays and identified by their moderately sized lumen and thin, low cuboidal to squamous epithelium. Small blood vessels throughout the kidney are thin-walled, endothelial-lined structures containing erythrocytes. The more prominent vessels in the medullary rays, running in tandem with renal tubules are the vasa recta.

Western blot analysis to confirm appropriate species cross-reactivity was also performed. Freshly collected kidney tissue from all three species was flash frozen and

stored at -80˚C. Later, RIPA lysis buffer (Santa Cruz Biotech, Santa Cruz, CA) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were added to small pieces of thawed, homogenized renal tissue. Centrifuged supernatants were aliquoted and stored at -20˚C until thawed for immediate analysis.

Protein concentration was quantified using a modified Lowry technique. The amount of protein loaded onto pre-made SDS gels (Bio-Rad Laboratories, Hercules, CA) varied by the protein to be isolated and species of animal. Gels were run for 1 hour at 180V, after which gels were transferred to PVDF membranes (Millipore, Billerica, MA) overnight at 35V in a 4˚C cold room. Membranes were then blocked in 5% milk buffer for one hour prior to incubating with the primary antibody for three hours. Membranes were incubated with an appropriate biotinylated secondary antibody in 5% milk buffer for 1 hour. For THP blots, a directly conjugated rabbit anti-sheep HRP secondary antibody (Abcam, Cambridge, MA) was used.[76] Endogenous peroxidase activity was blocked using hydrogen peroxide, after which the remaining samples were incubated for thirty minutes in streptavadin horseradish peroxidase. Protein bands were identified using a chemiluminescent kit (Thermo Scientific, Rockford, IL).

Results

Aquaporin-1

Staining for AQP-1 was positive in the canine kidney within the luminal epithelial cells of the PCT and proximal straight tubules (PST), the vasa recta endothelium, the descending thin limbs of the loop of Henle and erythrocyte membranes (Figure 2.1 and

Figure 2.2). Within the outer cortical PCT, staining was intense along the cells' apical margin with a less intense, finely granular cytosolic component (Figure 2.1). Within the inner cortical medullary rays, AQP-1 stained the luminal cells of the PST. These cells had strong staining along their basolateral margin and even more intense staining along the apical margin. There was only faint cytosolic staining in these cells. Nuclei did not stain. The endothelium of the vasa recta was intensely stained. Within the medulla, intermittent thin limbs showed strong, diffuse staining of their luminal cells (Figure 2.2). The thin limbs that stained for AQP1 are likely to be the descending thin limbs (DTL), based on localization of AQP1 to the DTL in other species.[9,13,62,67,68]

Findings were similar in the renal epithelium of the cat with some minor differences. Staining for AQP-1 was strong in the PCT luminal cells, but was equally intense along the basolateral and apical membranes in the outer cortex (Figure 2.3). There was no staining noted in the cytoplasm or nuclei of these cells. Glomerular podocytes also stained positive for AQP-1 in cats only (Figure 2.3). As seen in the dog, cat PST epithelium within the medullary rays stained more intensely than in the outer cortex along the basolateral and apical membranes of the luminal cells. The endothelium of the vasa recta stained strongly for AQP-1. Within the medulla, intermittent thin limbs were intensely stained, similar to previously reported positive staining of the descending thin limbs in other species (Figure 2.4).[9,13,62,67,68]

AQP-1 staining in the murine kidney was identical to that described above for the dog (Figure 2.5 and Figure 2.6). This is in keeping with previous reports in rat and mouse kidneys.[67,68,106,111]

Aquaporin-2

Immunostaining for AQP-2 appeared similar within the cortex and medulla of the dog. AQP2 intensely stained the apical border of intermittent luminal cells of the collecting ducts (consistent with the principal cells) (Figure 2.7). There was also less intense, granular staining of the cytoplasm of these cells. The nuclei did not stain for AQP2. No other regions of the nephron had AQP2 staining.

In the cat, intermittent luminal cells, the principal cells, of the collecting duct stained for AQP2 (Figure 2.8). In contrast to the dog, the staining pattern within the cortex was both basilar and apical, becoming primarily apical as the collecting ducts progressed into the medulla. The cat also had less intense, granular staining of the cytoplasm. As in the dog, no other regions of the nephron had AQP2.

Aquaporin-2 localization in the mouse kidney was similar to that described above for the dog. These findings are similar to reports in other mammalian species, including rats and mice (Figure 2.9).[67,77,91,92]

Glutathione S-transferase alpha

GST-α had variable expression within the dog kidney. Interestingly, the transitional epithelium lining the renal papilla of some dogs was positive for GST-α staining along the cell borders and diffusely throughout the nucleus (Figure 2.10). Less intense cytosolic staining is also visible. In some kidney sections with positive GST-α staining of renal transitional epithelium, no staining was found in the tubules (Figure 2.11). Kidney sections from other dogs demonstrated similar positive GST-α staining in the epithelium of PST within the medullary rays with only rare staining of PCT epithelium (Figure 2.12). The amount and intensity of staining in the PST is also variable among individuals. These findings are in contradiction with a previously published study examining the location of GST isoenzymes in the dog kidney, which found only isoenzymes mu and pi, not alpha, within renal tubules.[11]

The epithelial cells of the PCT in the feline kidney had diffuse, cytoplasmic GST-α, as well as nuclear localization (Figure 2.13). However, there was variable intensity of immunostaining among segments of the PCTs. No staining of renal transitional epithelium was observed (Figure 2.14).

Contradictory to previous reports in rats and mice, GST-α was not identified in the PCTs of our mouse kidneys.[17,37] Instead, the glomeruli stained intensely positive for GST-α (Figure 2.15) and no other part of the renal tubule stained positive for the enzyme.

Calbindin D-28k

Calbindin D-28k was present in the DCT of the dog, cat and mouse (Figure 2.16, Figure 2.17 and Figure 2.18, respectively). In the dog and mouse, the tubules in the outer cortex stained the strongest. The inner cortex and outer medulla had lesser numbers of stained tubules. The intensity of the staining of these tubules was somewhat variable in all regions of the kidney. Additionally, there was variability in intensity of individual epithelial cells stained within the same tubular segment. Cellular localization was both cytosolic and nuclear. This staining pattern is consistent with previously reported staining of the distal convoluted tubules, connecting tubules and, to a lesser extent, the proximal collecting ducts in other species.[35,94]

Staining for calbindin D-28k was almost identical in the cat kidney. However, within the outer cortex, there was even more variability in the intensity of individual cell staining within the same tubule. Some cells did not stain at all, while others varied from faint to intense in their expression of calbindin D-28k.

Tamm-Horsfall Protein

The distribution of THP was identical in all three species. The epithelia of the TAL in the loop of Henle were intensely stained, while there was less intense staining along cells lining the DCT (Figures 2.19-2.21). Tamm-Horsfall protein was expressed along the apical border of the epithelial cells in both the TAL and DCT. There was also diffuse cytoplasmic staining in these cells. Nuclei did not stain. No other regions of the nephron stained positive for THP.

Western blot analysis

Western blot analysis of homogenized kidney tissue from all three species was performed for AQP1, AQP2, GST-α, calbindin D-28k and THP in order to further validate species antibody cross-reactivity.

Immunoblotting of dog, cat and mouse kidney tissue confirmed the presence of two protein species of approximately 25 and 37 kDa when probed with rabbit anti-human AQP1 polyclonal antibody (Chemicon, Temecula, CA), which is consistent with molecular masses of the unglycosylated (28 kDa) and glycosylated (35-60 kDa) forms of AQP1 previously reported. (Data not shown.)

Immunoblotting using anti-human GST-α monoclonal antibody (Novacastra, Bannockburn, IL) confirmed the presence of two bands of approximately 17 kDa and 25 kDa in the kidney homogenate of all three species. Previous studies have shown a molecular weight of 26-31 kDa for GST-α in rabbits, rats and humans.

Bands of approximately 25 kDa were found using immunoblotting with mouse anti-bovine calbindin D-28k monoclonal antibody (Sigma-Aldrich, St. Louis, MO) in kidney homogenate in all three species. As the name implies, calbindin d-28k is known to have a molecular weight of 28 kDa.

Aquaporin-2 and Tamm-Horsfall protein could not be isolated via immunoblotting.

Discussion

AQP1 & AQP2

AQP1 was the first aquaporin discovered in the early 1990's. It is the water channel that contributes to water balance within many tissues and organs, especially in the kidney, where AQP1 is vital to water absorption by the PCT.[10,12,66-68]

Since the discovery of AQP1, nine other aquaporin isoforms have been discovered in the kidney, including AQP2.[10,12,66-68] Aquaporin-2 is the chief target for vasopressin in the collecting duct. Vasopressin induces a shuttling mechanism that moves AQP2 from intracellular vesicles to apical plasma membranes.[77,84,92] Once AQP2 has relocated to the apical plasma membrane, water can be reabsorbed through the water channel, concentrating urine.[77,84,92] Conversely, in the absence of vasopressin, AQP2 is endocytosed back into the cytoplasm and water is excreted in the urine.[77,84,92]

Aquaporin-1 and AQP2 have been studied extensively in human medicine, primarily relying upon rat and mice models. Understanding the influence of various disease states on these renal proteins has greatly increased our understanding of renal water balance. For example, late-phase polyuria associated with cisplatin toxicity results from the loss of AQP1 from the PCT, as well as the loss of AQP2 from the collecting ducts, leading to the inability to concentrate urine.[50] Another study shows that the

reduction of AQP1 and AQP2 in ischemic acute renal failure models probably leads to the lack of urine concentrating ability after the insult.[111] Changes in AQP1 and AQP2 expression in the kidney and their excretion in the urine can also be affected by extrarenal diseases, such as congestive heart failure and liver failure.[39,114]

Aquaporin-1 has been localized to the kidney in humans, rats and mice, as well as the musk shrew and house sparrow.[9,13,58,67,68] More specifically, AQP1 is found along the apical and basolateral membrane of luminal cells within the PCT. The highest expression is in S3, the straight portion of the proximal tubule that descends into the outer medulla. AQP1 is also found within type II descending thin limb (DTL) epithelium and to a lesser extent in type III and type I DTL epithelium and the descending portion of the vasa recta.[9,66,68] Type II DTL epithelium is found in long-looped nephrons within the cortical labyrinth. This specialized epithelium has tall epithelium, short, blunt microvilli and small numbers of organelles. Type I and type III DTL epithelium is simpler in structure and found in short-looped nephrons and within the inner medulla, respectively.

Less research on aquaporin expression has been done in dogs and cats. AQP1 expression was evaluated via immunoblotting in tissue from Shiba Inu dogs. AQP1 was found in the lung, kidney and spleen of these dogs.[69] To the authors' knowledge, however, the exact location and characterization of AQP1 within the dog kidney has not been previously reported. Nor has any information regarding renal AQP1 expression in felines been reported. We localized AQP1 within the dog and cat to sites reported for other species, the PCT, DTL and vasa recta (Figures 2.1-2.4). Interestingly, the cat also

expressed AQP1 in the glomerular podocytes, indicating a potential difference in renal water management for the cat (Figure 2.3). Additionally, we reconfirmed the presence of mouse AQP1 in these locations (Figures 2.5 and 2.6).

Aquaporin-2 has also been identified in many mammalian and avian species, with most research investigating human, rat and mouse kidney tissue.[9,54,58,77,91,92,107] Renal expression of AQP2 in dogs and cats has not been previously reported. Our findings are identical to that of previously studied species. We found that AQP2 is located in the principal cells of the collecting duct and to a lesser extent the connecting tubules in both dogs and cats (Figures 2.7 and 2.8). These findings were reconfirmed in mice (Figure 2.9). Specifically, AQP2 is found chiefly along the apical border of the principal cells, but can also be seen within the cytosol.

The localization and characterization of renal AQP1 and AQP2 expression confirms the role of these proteins in water regulation in dogs, cats and mice. Similar distribution of AQP1 and AQP2 in these species would be expected given the physiology of water management is likely conserved between most mammalian species. This confirmation is the initial step in evaluating the roles of AQP1 and AQP2 in both renal and extrarenal diseases of cats and dogs. Additionally, these proteins can reliably serve as *in situ* markers for their respective regions of the nephron, which can be difficult to distinguish from other tubules histologically.

GST-α

Glutathione *S*-transfersases are a family of enzymes found in various tissues, especially the liver and kidney. They are integral in detoxification of many substances, including cyclosporine, other cytotoxic drugs, chemicals and carcinogens.[22,33,37] There are a few different classes of the enzyme (GST-α, GST-μ, GST-π) that have unique locations within human and rat kidneys.[22,33,37]

GST-α is not found in the urine of normal, healthy individuals, but is found in the urine of individuals that have incurred renal tubular damage prior to an increase in serum creatinine.[22] Specifically, increased GST-α levels have been seen in urine from individuals with the following disorders affecting the PCT epithelium: cyclosporine-induced PCT damage, acute tubular necrosis, renal transplant infarct, ischemia, cisplatin toxicity, gentamicin toxicity and after heavy metal exposures.[22,33,37] There is promise in its use as an early marker of acute renal disease or to monitor individuals undergoing certain medical treatments, although additional research is needed.[22,33,37,46,115]

GST-α is present in the cells of the proximal convoluted tubule of rabbits, rats and humans.[33] In humans, the cellular localization of GST-α is described as cytoplasmic and nuclear within individual cells.[33] Minimal GST-α has also been noted in medullary tubules believed to be the thin limbs of the loops of Henle in humans.[33]

Distribution was quite different in the dog kidney. The transitional epithelium along the renal papilla showed vibrant, strong cytoplasmic and nuclear GST-α expression in some individuals (Figure 2.10). Overall, sections of dog kidney did not demonstrate significant GST-α staining in the PCT (Figure 2.11). Only rare PCT epithelial cells stained positive in some individuals. However, some kidney samples showed staining of the PST epithelium within in the medullary rays (Figure 2.12). Two kidneys (from different individuals) had staining of both the renal transitional epithelium and PST epithelium.

To date, few studies concerning GST expression in dog renal tissue have been performed. Bohets, et. al., identified the presence of isoenzymes GST-μ and GST-π in homogenized dog kidney tissue and the cytosol of an established dog renal cell line using affinity chromatography and immunoblotting.[11] In contrast to our results, this study found no GST-α in the canine kidney.

The variable, but positive staining of the transitional epithelium and PST epithelium in our study suggests that there may be individual or breed variation in GST isoenzyme expression within renal tissues. This theory for variation in individual GST-α expression may be further substantiated by the findings of researchers investigating the role of P450 and GST-α in trichloroethylene renal toxicity in rats. Cummings, et. al., found that their strain of male F344 rats varied from other previously studied rats in that they possessed GST-α in both their PCT and DCT and suggested this variation in GST-α distribution may be due to the strain of rat being used.[17]

In contrast to the dog, the feline kidney showed expression of GST-α similar to that previously reported in humans and rats. Staining was shown in all PCT cells, but was of variable intensity between individual cells (Figure 2.13). Renal papilla transitional epithelium did not demonstrate the presence of GST-α in the cat (Figure 2.14). Also, individual variation in feline renal expression of GST-α was not seen in this study.

The mouse kidney also had strikingly different results than expected. Rats have been utilized extensively for GST research and while glutathione s-transferase class expression appears slightly variable among rat strains, GST-α remains confined to renal tubules.[17,22,28,37] In the mouse kidney, glomeruli stained positive for all individuals sampled and no tubular staining was appreciated (Figure 2.15). Again, these findings in the murine kidney, as well as the dog kidney, highlight the importance of understanding species variations in tissue protein expression, especially before relying on them as markers for disease.

Calbindin D-28k

Calbindin D-28k is a calcium binding protein, with a very strong affinity for calcium.[35,73,80] In the kidney, there are several theories on the role of calbindin D-28k, and it is therefore possible that the protein has multiple functions within this organ alone.[35,73,80,94] Although approximately 60% of calcium is reabsorbed in the proximal tubule, it is in the distal convoluted tubule that selective calcium reabsorption occurs.[35] 1,25-dihydroxyvitamin D_3 is made in the proximal tubule, but its actions take place in the

distal tubule where calbindin is expressed.[35] This has long formed a strong association between selective calcium reabsorption and calbindin. As renal calbindin expression appears dependent on and influenced by many factors, including 1,25-dihydroxyvitamin D_3, PTH and calcitonin, it seems certain that the protein is involved in calcium regulation, but the exact involvement is yet unknown.[35] Some theories suggest calbindin acts as a calcium shuttle, as a buffer (keeping intracellular calcium below a toxic level) or plays a role in the basolateral membrane calcium pump of renal epithelial cells.[35]

Calbindin D-28k is found in many tissues in many species, including various reptiles, amphibians, fish, chicks, rats, rabbits, monkeys and humans.[73,80,94] The protein has been commonly found in kidney, intestine, pancreas and bone in many of these species.[73,80,94] It has also been heavily studied in retinal and brain tissue from various species, including the cat and dog.[20,30] Its expression and function appears to be dependent on vitamin D metabolites in some tissues, as in the kidney, but not in all.[20,30,35,73,80,94] Therefore, the functions of calbindin D-28k could be widely different within these tissues.

In previous studies, calbindin D-28k has been localized to the distal convoluted tubule of several mammalian species, including rabbits, rats and humans.[35,94] A 1998 toxicology study examining the nephrotoxic effects of cyclosporine A on dogs did localize calbindin D-28k to renal tubular epithelium via IHC, but did not go as far as to state which renal tubules stained positive for calbindin D-28k.[3] The staining is seen diffusely in the cytosol and nucleus. Calbindin D-28k has also been found in the

connecting tubules and in the collecting ducts with cytosolic and nuclear expression.[73,80,94] There is progressively less expression of calbindin in these regions.[73,80,94] However, there does appear to be some species variation in the abruptness of this change.[73,80,94]

In our species of interest, the dog, cat and mouse, renal expression of calbindin was similar to that seen in other species. Within the distal convoluted tubules, intermittent expression of calbindin D-28k was seen in all three species (Figures 2.16-2.18). Staining was cytosolic and nuclear in the renal epithelial cells of the DCT. Some cells in the DCT showed no positive staining. It is difficult histologically to definitively differentiate the DCT from the connecting tubules and it is certainly possible that some of the tubules with positive staining are, indeed, connecting tubules. The collecting ducts do not contain positively stained cells.

Tamm-Horsfall Protein

Tamm-Horsfall protein, a glycoprotein also called uromodulin, is the most abundant protein found in urine and is thought to have many functions.[51,86] Possible functions include regulation of renal cytokines and defense against urinary tract infection, specifically targeting type 1 fimbriated *Escherichia coli*.[7,72] The protein also appears to help protect against the formation of calcium oxalate crystals.[64] It has also been implicated as a contributor to tubulointerstitial renal disease and cast nephropathy in multiple myeloma patients.[86]

A THP-like protein has been studied in dogs specifically because of the species' urinary excretion of vitamin A.[76,85] Humans reabsorb retinol, but dogs (and it is thought carnivores in general) excrete retinol and retinyl esters in their urine.[76,85] These products are hydrophilic and need a carrier in urine, which has recently been shown to be a Tamm-Horsfall like protein.[85] In studying this canine phenomenon, THP has previously been localized to the luminal cells of the thick ascending limb (TAL) of the loop of Henle in the outer medulla and the cells comprising the cortical distal convoluted tubule.[76,85] In previous reports, immunohistochemical staining was most intense at the apical margins of cells in the TAL.[76,85] Within the DCT, positive cells appeared intermittently and had a homogenous cytoplasmic expression.[76,85] Similar findings have been found in humans.[37]

We obtained identical results in our dog, cat and mouse kidneys to that described above in other species (Figures 2.19-2.21).

Western blot analysis

Species antibody cross-reactivity was confirmed for our antibodies via Western blot analysis. Homogenized tissue was used from dog, cat and mouse kidney and probed with each antibody used for IHC.

When probed with rabbit anti-human AQP1 polyclonal antibody (Chemicon, Temecula, CA), two species of approximately 25 and 37 kDa were found in dog, cat and mouse tissue. These proteins' molecular weights are consistent with the molecular weight of the unglycosylated (28 kDa) and glycosylated (35-60 kDa) forms of AQP1. These

findings, coupled with IHC localization that is strongly compatible with reports in other species, give strong evidence for species-specific cross-reactivity of the anti-AQP1 antibody used in this study.

Immunoblotting using anti-human GST-α monoclonal antibody (Novacastra, Bannockburn, IL) confirmed the presence of two protein species of approximately 17 kDa and 25 kDa in the kidney homogenate of all three species. Previous studies have shown a molecular weight of 26-31 kDa for GST-α in rabbits, rats and humans. The 25 kDa protein identified could easily be GST-a, especially given the intense staining seen in the feline kidney that is consistent with previous reports of GST-α localization. As we were using whole, homogenized renal tissue and not cell cultures, we hypothesize that some proteins may have been further cleaved, resulting in a lower molecular weight protein fragments of 17 kDa.

Immunoblotting using anti-bovine calbindin D-28k monoclonal antibody (Sigma-Aldrich, St. Louis, MO) on homogenized kidney tissue from all three species yielded consistent results for dog, cat and mouse tissue. A single band of 25 kDa was found for all three animal species. This protein is compatible with the known 28 kDa molecular weight of calbindin d-28k. The strikingly consistent IHC localization of calbindin d-28k to the DCT, as reported in various other species, gives further evidence of good species cross-reactivity of our anti-calbindin D-28k antibody.

Unfortunately, our attempts at immunoblotting were not as successful for AQP2 and THP. Despite many attempts, definitive bands could not be identified with our anti-rat AQP2 antibody probing of homogenized tissue. The failure of immunoblotting may be due to the fact that antibodies are conformationally dependent and the epitopes could vary in arrangement from more linear in tissue homogenate as compared to more complex tertiary folding in tissue sections. However, the consistent and robust localization of AQP2 to the collecting ducts of all three species is in strong accordance with that reported in other species and strong evidence of species antibody cross-reactivity in tissue section.

Supporting cross-reactivity of our antibody for THP is a previous study in which the same antibody was used for identifying THP in the urine of dogs via Western blotting.[85] Additionally, our IHC staining consistently localized THP to the TAL of all three of our species of interest.

One disadvantage to evaluating histologic sections only is that nephron segment identification is based solely on morphology. For example, the descending versus ascending thin limbs of the loop of Henle cannot be definitively identified histologically. As some of the thin limbs did not express AQP1, it can be assumed that these sections represent the ascending thin limbs, while the thin limbs that did express AQP1 are likely the descending thin limbs, based on findings in other species.[9,66,68] Additionally, the connecting tubules cannot be differentiated reliably from the collecting duct morphologically. It is certain that the collecting ducts express AQP2, but we must

suppose, based on information from other species, that the connecting tubules also express some AQP2. [9,54,58,77,91,92,107] Electron microscopy with immunolabeling could help to more definitively localize and characterize the distribution of renal AQP1 and AQP2 localization in our samples.

To the author's knowledge, with the exceptions of THP, AQP1 and calbindin D-28k in the dog, and AQP1 and AQP2 in the mouse, the proteins examined here have not been previously localized to the kidneys of our species. Additionally, except for THP in the dog and the aquaporins in the mouse, these proteins have not been localized *in situ* within the dog, cat and murine kidney.

We have localized five renal proteins in three different species using immunohistochemistry and examination by light microscopy. Aquaporin-1 was found in the luminal epithelial cells of the PCT and the thin limbs of the loop of Henle within the dog, cat and mouse kidney. Aquaporin-2 was expressed exclusively in the collecting ducts of all species. Glutathione s-transferase-α had rare staining in the cells of the canine PCT, but was highly expressed in PST epithelium and renal transitional epithelium, with variable expression among individuals. The cat demonstrated strong expression of GST-α within the PCT, while murine glomeruli stained positively. Calbindin D-28k was localized to the DCT of all species. Finally, THP was highly expressed in the TAL of the loop of Henle, with lesser expression in the DCT, in all species.

The renal proteins identified and discussed above are quite varied in their function within the kidney. Describing their location in these additional species may aid further study of their behavior in these animal species. Additionally, some of these proteins can now serve as reliable, renal nephron segment markers to help definitively identify segments of the nephron histologically. Further study of these renal proteins' IHC localization in different diseased states is warranted. Results of such studies may prove invaluable in several ways. Foremost, in drug research and development, researchers may be able to utilize these IHC techniques to definitively identify what part of the renal tubule is affected in toxicological studies. If the expression of these proteins in the urine of healthy and diseased animals can be studied, it may also be possible to correlate the location of renal damage to specific proteins found in urine, which could negate the need for renal biopsies in some cases. Additionally, antibodies to these proteins can be used in double-labeling IHC to localize other proteins of interest to specific regions of the nephron, without utilizing more costly and time consuming methods.[55] With the localization of AQP1, AQP2, GST-α, calbindin d-28k and THP in the dog, cat and murine kidney, many interesting avenues of study are now opened.

Figures

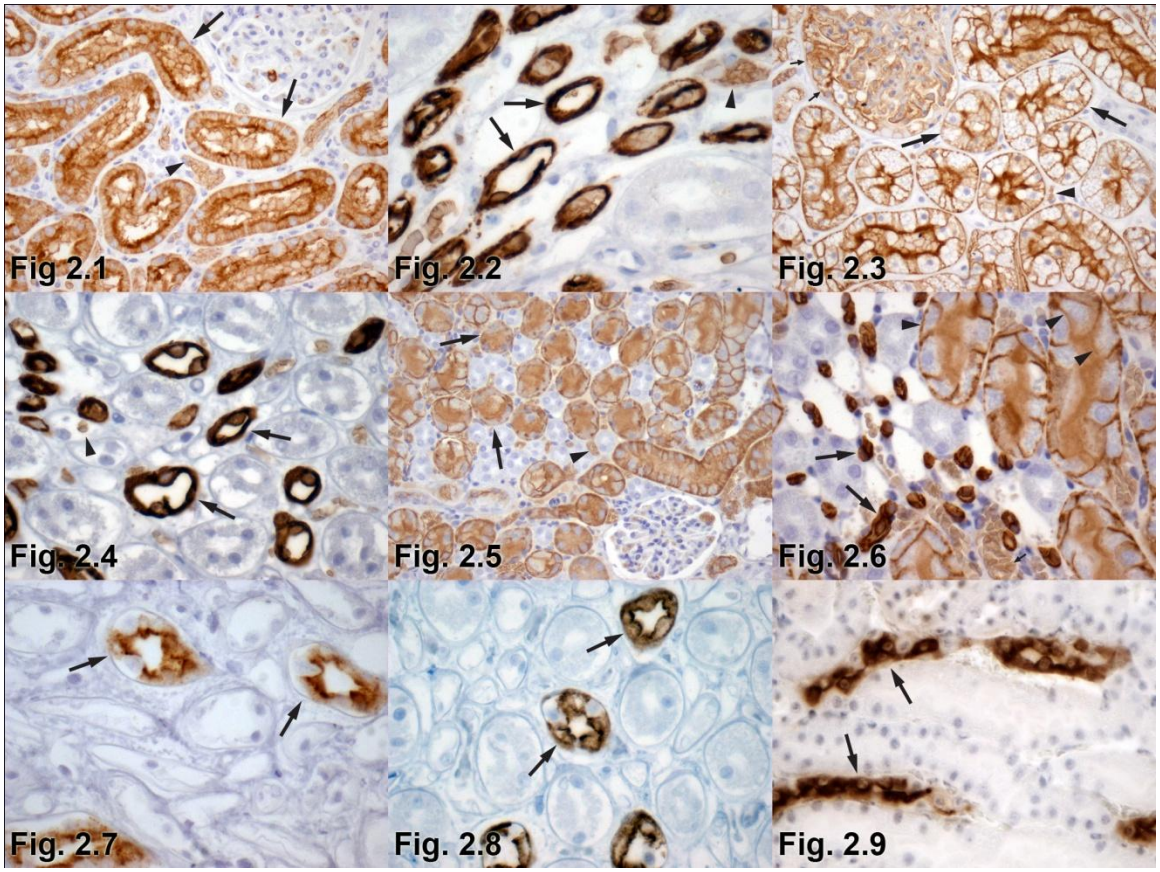

Figure 2.1. Renal cortex; 200x; dog. AQP1 staining of PCT epithelium (arrows), demonstrating intense staining along the cells' apical margin with a less intense, finely granular cytosolic component. Erythrocyte membranes also stain positive for AQP1 (arrowhead). Immunohistochemistry with rabbit anti-human AQP-1 polyclonal antibody (1:500, Chemicon, Temecula, CA), DAB, and H&E counter stain.

Figure 2.2. Renal medulla; 400x; dog. Diffuse AQP1 staining of the DTL luminal epithelium (arrows). Erythrocyte membranes also stain positively for AQP1 (arrowhead). Immunohistochemistry with rabbit anti-human AQP-1 polyclonal antibody (1:500, Chemicon, Temecula, CA), DAB, and H&E counter stain.

Figure 2.3. Renal cortex; 200x; cat. AQP1 staining of the PCT epithelium (big arrows) is shown, with equally strong staining along the apical and basolateral borders of the cells. Glomerular podocytes (small arrows) and erythrocyte membranes (arrowhead) also stain positive for AQP-1. Immunohistochemistry with rabbit anti-human AQP-1 polyclonal antibody (1:500, Chemicon, Temecula, CA), DAB, and H&E counter stain.

Figure 2.4. Renal medulla; 400x; cat. Diffuse AQP1 staining of the DTL luminal epithelium (arrows) and of erythrocyte membranes (arrowhead). Immunohistochemistry

with rabbit anti-human AQP-1 polyclonal antibody (1:500, Chemicon, Temecula, CA), DAB, and H&E counter stain.

Figure 2.5. Renal cortex; 200x; mouse. AQP1 staining of PCT epithelium (arrows). Erythrocyte membranes also show positive staining for AQP1 (arrowhead). Immunohistochemistry with rabbit anti-human AQP-1 polyclonal antibody (1:500, Chemicon, Temecula, CA), DAB, and H&E counter stain.

Figure 2.6. Renal medulla; 400x; mouse. Diffuse AQP1 staining of the DTL luminal epithelium (large arrows). Also shown is AQP1 staining of PCT epithelium with localization to the cells' apical brush border and basolateral cell membrane (arrow heads). Erythrocyte membranes (small arrows) stain for AQP1. Immunohistochemistry with rabbit anti-human AQP-1 polyclonal antibody (1:500, Chemicon, Temecula, CA), DAB, and H&E counter stain.

Figure 2.7. Renal medulla; 400x; dog. Intense AQP2 staining is seen along the apical border of principal cells of the collecting ducts, as well as less intense, granular, cytosolic staining (arrows). Immunohistochemistry with rabbit anti-rat AQP-2 polyclonal antibody (1:500, Millipore, Billerica, MA), DAB, and H&E counter stain.

Figure 2.8. Renal medulla; 400x; cat. AQP2 staining is visible along both the apical and basolateral border of the principal cells of the collecting ducts (arrows). Less intense, granular, cytosolic staining is also present. Immunohistochemistry with rabbit anti-rat AQP-2 polyclonal antibody (1:500, Millipore, Billerica, MA), DAB, and H&E counter stain.

Figure 2.9. Renal medulla; 400x; mouse. As seen in the dog, intense AQP2 staining is seen along the apical border of the principal cells of the collecting ducts (arrows). Cytosolic staining is less intense and granular. Immunohistochemistry with rabbit anti-rat AQP-2 polyclonal antibody (1:500, Millipore, Billerica, MA), DAB, and H&E counter stain.

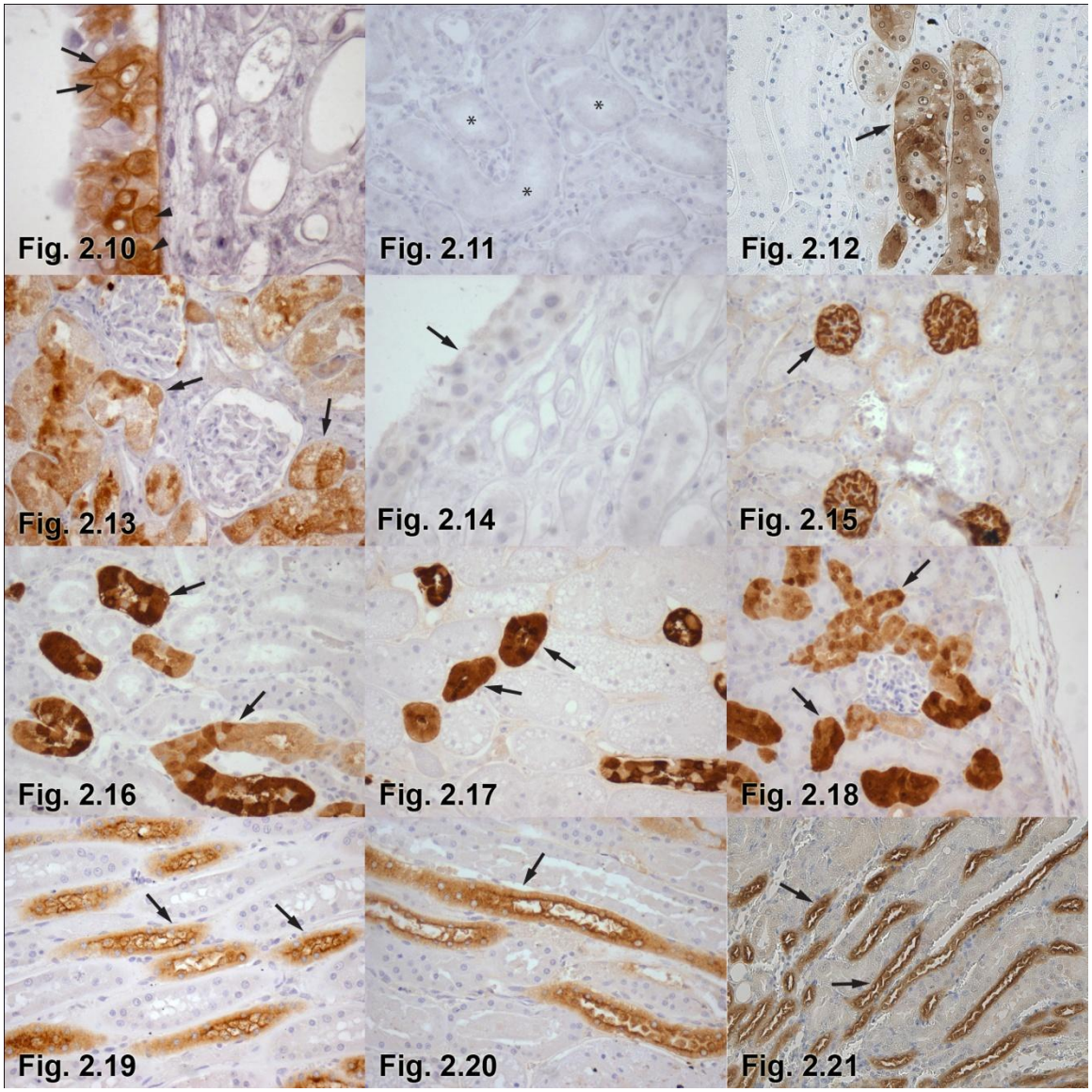

Figure 2.10. Renal papilla; 400x; dog. The transitional epithelium lining the renal papilla demonstrates positive staining for GST-α. Staining is most prominent along cell (large arrow) and nuclear borders (arrowheads). Less intense cytosolic and diffuse nuclear staining is also visible. Immunohistochemistry with mouse anti-human GST-α monoclonal antibody (1:50, Novocastra, Bannockburn, IL), DAB and H&E counter stain.

Figure 2.11. Renal cortex; 200x; dog. Staining was absent for GST-α in the majority of canine PCT epithelium (asterisks denote non-staining PCTs). Immunohistochemistry with mouse anti-human GST-α monoclonal antibody (1:50, Novocastra, Bannockburn, IL), DAB and H&E counter stain.

Figure 2.12. Inner renal cortex; 200x; dog. Within medullary rays, the PST epithelium exhibits positive staining for GST-α (arrow). The staining pattern of individual cells is

similar to that of the renal transitional epithelium and is both nuclear and cytoplasmic. Immunohistochemistry with mouse anti-human GST-α monoclonal antibody (1:50, Novocastra, Bannockburn, IL), DAB and H&E counter stain.

Figure 2.13. Renal cortex; 200x; cat. The PCT epithelium shows positive staining diffusely throughout the cytoplasm, as well as throughout the nucleus for GST-α (arrows). Immunohistochemistry with mouse anti-human GST-α monoclonal antibody (1:50, Novocastra, Bannockburn, IL), DAB and H&E counter stain.

Figure 2.14. Renal papilla; 400x; cat. No staining of renal transitional epithelium for GST-α was observed (arrow). Immunohistochemistry with mouse anti-human GST-α monoclonal antibody (1:50, Novocastra, Bannockburn, IL), DAB and H&E counter stain.

Figure 2.15. Renal cortex; 200x; mouse. Only the glomerular membrane complex stained positive for GST-α in the mouse (arrow). Immunohistochemistry with mouse anti-human GST-α monoclonal antibody (1:50, Novocastra, Bannockburn, IL), DAB and H&E counter stain.

Figure 2.16. Renal cortex; 200x; dog. Positive staining of calbindin D-28k is variable in intensity between individual epithelial cells of the DCT (arrows). Staining is both nuclear and cytosolic. Immunohistochemistry with mouse anti-bovine calbindin D-28k monoclonal antibody (1:1000, Sigma-Aldrich, St. Louis, MO), DAB and H&E counter stain.

Figure 2.17. Renal cortex; 200x; cat. Calbindin D-28k staining is evident in the epithelial cells of the DCT (arrows). Staining is both nuclear and cytosolic with variable intensity between individual cells. Immunohistochemistry with mouse anti-bovine calbindin D-28k monoclonal antibody (1:1000, Sigma-Aldrich, St. Louis, MO), DAB and H&E counter stain.

Figure 2.18. Renal cortex; 200x; mouse. Staining identical to that of the dog and cat was seen in the DCT epithelium of the mouse with variable nuclear and cytoplasmic localization of calbindin D-28k to the DCT (arrows). Immunohistochemistry with mouse anti-bovine calbindin D-28k monoclonal antibody (1:1000, Sigma-Aldrich, St. Louis, MO), DAB and H&E counter stain.

Figure 2.19. Inner renal cortex; 200x; dog. The epithelia of the TAL of the loop of Henle stain intensely, most prominently along the apical border and diffusely throughout the cytoplasm, for THP (arrows). Nuclei did not stain. Immunohistochemistry with sheep anti-human Tamm-Horsfall glycoprotein antibody (1:2500, Chemicon, Temecula, CA), DAB and H&E counter stain.

Figure 2.20. Inner renal cortex; 200x; cat. As in the dog, the cat epithelium of the TAL stains positive for THP along the epithelial apical border and diffusely throughout the cytoplasm (arrow). Immunohistochemistry with sheep anti-human Tamm-Horsfall glycoprotein antibody (1:2500, Chemicon, Temecula, CA), DAB and H&E counter stain.

Figure 2.21. Inner renal cortex; 200x; mouse. Staining for THP is identical in the mouse to the dog and cat with positive staining of the TAL apical epithelium and cytoplasm (arrows). Immunohistochemistry with sheep anti-human Tamm-Horsfall glycoprotein antibody (1:2500, Chemicon, Temecula, CA), DAB and H&E counter stain.

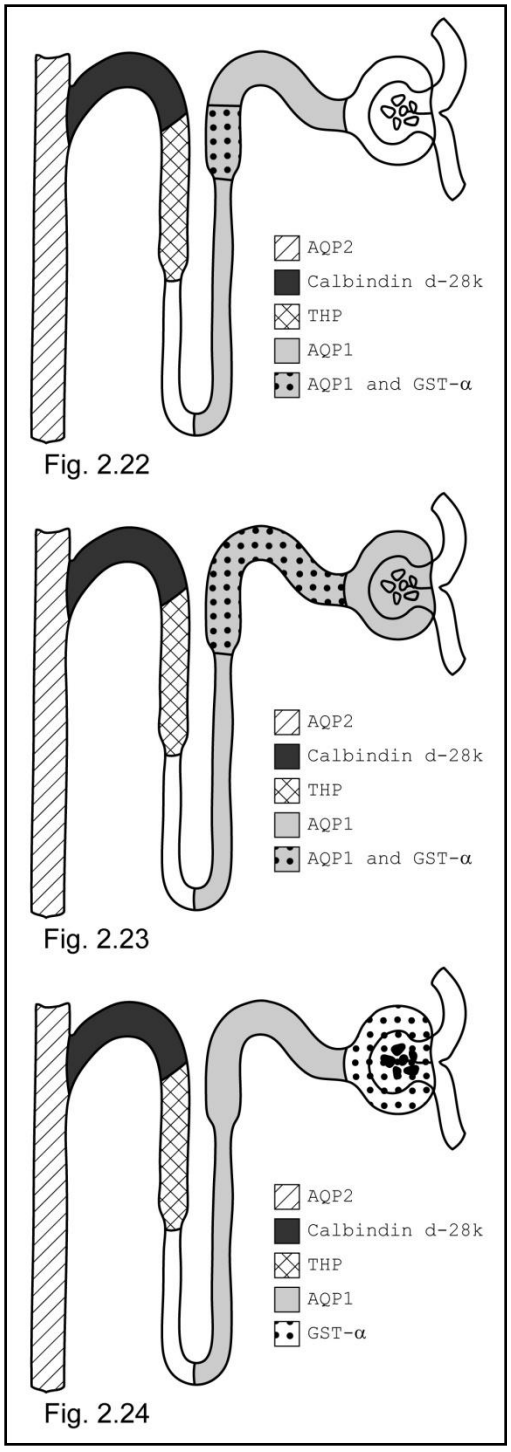

Figure 2.22. Dog. Diagram of immunohistochemistry localization of AQP1, AQP2, GST-α, calbindin d-28k and THP. From left to right, AQP2 is found in the collecting ducts, calbindin d-28k in the DCT, THP in the TAL, AQP1 throughout the DTL and PCT, and GST-α is localized to the PST of the loop of Henle. *Some dogs did not stain positive for

GST-α at this site. Not represented is the variable staining of renal transitional epithelium and rare PCT epithelial cells for GST-α seen in some dogs.

Figure 2.23. Cat. Diagram of immunohistochemistry localization of AQP1, AQP2, GST-α, calbindin d-28k and THP. From left to right, AQP2 is found in the collecting ducts, calbindin d-28k in the DCT, THP in the TAL, AQP1 throughout the DTL, PCT and glomerulus (podocytes), and GST-α in the PCT.

Figure 2.24. Mouse. Diagram of immunohistochemistry localization of AQP1, AQP2, GST-α, calbindin d-28k and THP. From left to right, AQP2 is found in the collecting ducts, calbindin d-28k in the DCT, THP in the TAL, AQP1 in the DTL and PCT and GST-α in the glomerulus.

CHAPTER 3

IN VITRO CHARACTERIZATION OF THE CANINE URINARY PROTEOME

Traditionally, the diagnosis of acute renal disease (ARD) in veterinary medicine is based on the acute elevation of serum urea nitrogen (sUN) and serum creatinine (sCrt) concentrations paired with inappropriately concentrated urine, indicating renal azotemia. However, the major caveat to assessing renal azotemia is that approximately two-thirds of the renal nephron functional capacity must be lost before an increase in sUN and sCrt can be appreciated.[89] In human medicine, early identification of renal injury and assertive intervention has been shown to significantly reduce morbidity and mortality, especially in the acute setting.[83] The challenge in both human and veterinary medicine is to accurately identify instances of ARD in a timely manner that allows for appropriate treatment.

The need for more timely diagnosis and intervention of ARD has prompted researchers in the medical field to seek out additional biomarkers that are better able to diagnosis the earliest stages of renal disease. Although the serum marker cystatin C (sCysC) has been found to be more sensitive in diagnosing mild GFR impairment than sCrt in humans, sCysC has not been definitively proven to be of any additional diagnostic use over sCrt in dogs with ARD. [9,20] As the discovery of reliable serum markers for early detection of ARD has not been forthcoming, recent research has targeted the discovery of novel urinary biomarkers for renal disease. Urinary biomarkers also have the potential to

indicate location of injury, shed light on pathogenesis and be used to monitor response to treatment.

Human medicine has developed many urinary biomarkers that while not yet eclipsing sCrt and sCysC, are proving useful for diagnosing ARD in various specific settings.[2,15,57,87] Some urinary enzymes have also been studied in the veterinary realm in conjunction with renal failure in dogs, most notably N-acetyl-β-glucosaminidase (NAG) and gamma-glutamyl transferase (GGT). Although many urinary proteins do appear to hold promise in the diagnosis of ARD, more biomarkers are needed. Ideally, a panel of biomarkers should be developed to increase sensitivity and specificity of diagnoses under various conditions and to assess response to treatment.

With the streamlining of mass spectrometry techniques, many researchers in the human field have turned to proteomic analysis of urine in order to perform broad spectrum analysis of the protein content of urine. Various studies have been performed and as technology improves, increasing numbers of proteins are being discovered in human urine.[1,16,26,49,74,90,101] Although the data analysis that follows can be cumbersome, some of these forays into the human urinary proteome have gleaned promising biomarkers for specific renal diseases, as well as extrarenal pathological conditions.[16,18,26,38,48,49,70,97]

The goal of our current study is to adapt methods established in human research to canines and to characterize the canine urinary proteome by examining both the exosomal

and soluble urine fractions. Additionally, we sought to make an informative comparison of the canine urinary proteome to the human urinary proteome. Lastly, we discuss the features of several relevant proteins discovered in canine urine that may prove to be useful biomarkers for acute renal disease in dogs.

Materials and methods

Canine urine collection and protein concentration

Inclusion criteria required that dogs be between 1-10 years of age, clinically normal, not taking any medications or supplementations and fed a commercial adult dog food diet. The health of all dogs was further evaluated by routine CBC, including evaluation of blood film, serum chemistry profile, urinalysis and urine protein to creatinine ratio (UPC).

Fifty milliliters of urine was collected via free catch into sterile urine collection cups from all dogs. A protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added at a ratio of 1:10 to each sample immediately after collection to avoid protein degradation.[117] Samples were then immediately frozen to -80˚C and stored until all samples were collected and could be batch evaluated.[117]

Samples were thawed in cool water and then kept on ice during processing. After vortexing to reconstitute particles that may have settled during the freezing process, all samples were mixed together. Batched urine was aliquoted into six ultracentrifuge tubes (25x89 mm, Beckman, Brea), and centrifuged at 17,000 x g for 10 minutes at 4˚C (L70

Ultracentrifuge, SW 28 rotor, Beckman, Brea) to remove whole cells and debris.[74] Next, the sample supernatants were collected and again aliquoted into six, clean ultracentrifuge tubes (25x89 mm, Beckman, Brea). The samples were then ultracentrifuged at 113,000 x g for 2 hours at 4˚C (L70 Ultracentrifuge, SW 28 rotor, Beckman, Brea) to obtain exosomal pellets.[74] The supernatant from this ultracentrifugation step was decanted and stored at -80˚C until proteomic analysis of the soluble fraction of the canine urine could be performed.

The exosomal pellets were resuspended in isolating buffer (10mM triethanolamine, 250mM sucrose at pH of 7.6) and each sample was transferred to a 1.6 ml Eppendorf tube. Dithiothreitol (DTT) was added to a final concentration of 200 mg/ml and heated at 95˚C for 2 minutes for depletion of Tamm-Horsfall protein (THP).[74] Samples were then transferred to six ultracentrifuge tubes (14x89 mm, Beckman, Brea). Isolation buffer was used to fill the remaining volume of the ultracentrifuge tubes and the samples were then ultracentrifuged at 200,000 x g for 1 hour at 4˚C (L70 Ultracentrifuge, SW 41 Ti rotor, Beckman, Brea).[74] The final pellets obtained were reconstituted in 40 µl of isolation buffer and combined into three 1.6 ml Eppendorf tubes. 5X-SDS Laemmli buffer containing Bromophenol blue and 60 mg/ml DTT was added to each Eppendorf tube containing combined exosomal pellets at a1:4 ratio, which was then incubated at 60˚C for 10 minutes before boiling for 5 minutes. All exosomal pellets were then frozen at -20˚C until gel electrophoresis could be performed.

One-dimensional SDS-PAGE and in-gel digest of canine urinary proteins

Both urine supernatant and exosomal fractions were thawed on ice. The supernatant was concentrated using Amicon Ultra-15 centrifugal filter units with a molecular weight cut-off of 5 kDa (Millipore, Billerica, MA) per manufacturer instructions. Protein quantification was performed on both soluble and aliquoted Bromophenol blue-free exosomal urine fractions using the Pierce* Coomassie Plus (Bradford) protein assay kit (Thermo Scientific, Waltham, MA) according to the manufacturer instructions. The concentrated supernatant had a protein content of 1.1 µg/µl and the exosomal pellet contained 1.2 µg/µl of protein.

Urine supernatant protein (24.8 µg) and exosomal protein (19.8 µg) was applied to separate lanes on a 4-20% Mini PROTEAN® TGX™ precast gel (BioRad, Hercules, CA). Using 1X Tris/Glycine/SDS running buffer, the gel was run at 175 v for approximately 1 hour. The gel was stained with Imperial Protein Stain (Thermo Scientific, Rockford, IL) according to manufacturer instructions. Prior to in-gel digestion, each lane was cut into 25 pieces, which were then diced into ~1x1 mm squares and stored in 0.6 ml Eppendorf tubes.

In-gel digestion was then performed using a standard trypsin digest protocol to produce peptides. In short, gel pieces were destained and washed, after which DTT reduction and iodacetamide (IAA) alkylation was performed. Finally, proteins were digested overnight with mass spectrometry grade trypsin (Promega, Madison, WI) at 37˚C. Tryptic peptides were then extracted from the gel pieces using 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA). A vacuum centrifuge was used to evaporate

any remaining solvent from the samples. Samples were reconstituted in a buffer of 0.1% acetic acid and 3% ACN.

LC-MS^2 Analysis

Peptides were purified and concentrated using an on-line enrichment column (Zorbax C18, 5µm, 5 x 0.3mm, Agilent, Santa Clara). Subsequent chromatographic separation was performed on a reverse phase nanospray column (1100 nanoHPLC, Zorbax C18, 5µm, 75 µm ID x 150mm column, Agilent, Santa Clara) using a 60 minute linear gradient from 25%-55% buffer B (90% ACN, 0.1% formic acid) at a flow rate of 300 nanoliters/min. Peptides were eluted directly into the mass spectrometer (LTQ linear ion trap, Thermo Scientific, Waltham) and spectra were collected over a m/z range of 200-2000 Da using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Bioworks 3.0 software (Thermo Scientific, Waltham) with an intensity threshold of 5,000 and 1 scan/group. MS/MS spectra were searched against the NCBI Canine protein sequence database containing 69,862 sequence entries using both the Mascot (version 2.3, Matrix Science, Boston) and Sequest (Sorcerer^{TM}-SEQUEST®, Sage-N Research, Milpitas, CA) database search engines.

A peptide mass tolerance of 2 Da and a fragment ion mass tolerance of 1.5 Da were utilized by Mascot searches, while a peptide mass tolerance of 2 Da and a fragment ion mass tolerance of 1.0 was used via Sequest. The following parameters were employed in all database searches: fully tryptic digestion allowing for 1 missed cleavage, variable

modification of methionine oxidation, and a fixed modification of cysteine carbamidomethylation.

Peptide identifications from both search engine results were combined using probabilistic protein identification algorithms implemented in Scaffold software (Proteome Software, Portland, OR). Peptide and protein probability thresholds of 95% and 90% respectively, were applied to the results (2.2% FDR as calculated by Scaffold based on probability statistics). Proteins containing shared peptides are grouped by Scaffold to satisfy the laws of parsimony. Manual validation of MS/MS spectra was performed for all protein identifications above these thresholds that were based on one peptide. Selection by manual validation was based on the following criteria: 1) The peptide must be identified by both search engines (Sequest and Mascot), 2) There must be a minimum of 75% coverage of theoretical y or b ions (at least 5 in consecutive order), 3) There must be an absence of prominent unassigned peaks greater than 5% of the maximum intensity, and 4) Indicative residue specific fragmentation, such as intense ions N-terminal to proline and immediately C-terminal to aspartate and glutamate, were used as additional parameters of confirmation.

Enrichment analysis of GO categories

Analysis of the canine urinary proteome was modeled after a recent paper by Adachi, et. al.[1,59,60] Enrichment analysis of the total canine urinary proteome dataset was performed using the Cytoscape plug-in tool, Biological Networks Gene Ontology (BiNGO).[60] Separate datasets were generated for exosomal versus soluble proteins.

Proteins were appropriately grouped into GO categories and determined to be over- or under-represented in comparison to the complete International Protein Index (IPI) human proteome based on results from a hypergeometric test. A resultant p-value of ≤ 0.001 was considered to be statistically significant.[60] Corrections for multiple term testing were performed automatically by BiNGO using the recommended Benjamini and Hochberg correction.[60]

This analysis was repeated using datasets comprised of canine soluble and exosomal proteins found to be in common with human exosomal proteins, as well as with datasets of soluble and exosomal proteins that were discovered to be unique to canine urine.

For the figures in this document that are based on BiNGO obtained data, GO terms were subjectively paired down to include as many GO terms encompassing as many categorized proteins as possible, while still allowing the data to be presented in a useful manner (see Supplemental Data for complete BiNGO analyses).

Results

Dogs

Urine was collected from 15 clinically normal, adult pet dogs. Four dogs were excluded from the study. Despite being clinically normal and having normal laboratory values, two dogs exhibited excessive numbers of acanthocytes on review of their blood films. Additionally, one dog had a mildly elevated alanine transaminase of 435 IU/L

(reference range 10-110 IU/L) and went on to develop lymphoma in the months following urine collection for this study. The fourth dog had marked struvite crystalluria on urine sediment exam.

The remaining 11 dogs had no abnormalities on CBC, blood film review, serum chemistry profile or urinalysis and had urine protein creatinine ratios (UPC) ≤ 0.1. This group of dogs included 6 female spayed dogs, one intact female dog and four castrated male dogs. Dogs ranged in age from 2-9 years of age with a median age of 6 years. Represented breeds included 5 mix-breed dogs, 3 Labrador retrievers, one border collie, one golden retriever and one pug.

Characterization of the canine urinary proteome

Our analysis of both the canine exosomal and soluble urine fractions yielded the identification of a total of 8881 exosomal peptides and 5324 soluble peptides. In total, 563 proteins were identified after the removal of redundant proteins and anionic trypsinogen from the dataset (see Supplemental Data for full list of identified proteins). Three hundred and ninety one exosomal proteins and 214 soluble proteins were identified. Of these, 349 proteins were found in the exosomal fraction only, 172 proteins were located in the soluble supernatant only and 42 proteins were identified in both urine fractions. Among the exosomal proteins, 74 proteins were identified based on a single peptide. The soluble protein fraction contained 50 proteins identified via a single peptide. Manual validation of spectra for these proteins met the inclusion criteria discussed in the Materials and Methods section.

Although fewer proteins were identified in our canine urine versus human urine (recent papers have found 1500-2000 proteins in human urine), the distribution of canine urinary protein molecular weights is similar to that of humans.[1,49] The majority of canine urinary proteins, both exosomal and soluble fractions, have a molecular weight of approximately 10-80 kDa (Figure 3.1), with small numbers of proteins exceeding 80 kDa.

The canine urinary proteome was further successfully characterized using BiNGO, a plug-in for Cytoscape, which searches proteins against Gene Ontology (GO) terms and categories.[4,60] Gene Ontology was developed by the GO Consortium in 2000 to unify the annotation of proteins from various eukaryotic organisms by relying on a stringently defined vocabulary of terms to be included in the larger functional categories of Cellular Component, Molecular Function and Biological Process.[4] The ontological categorization of proteins is further enhanced through the GO's links to various other gene and protein databases, such as SwissPROT and GenBank, which helps the GO protein classifications remain current.[4]

Six of our identified proteins (Chain A, Grp94 N-terminal bound to geldanamycin [exosomal], Na^+/K^+ ATPase alpha chain [exosome], IgG heavy chain B [exosome & soluble], IgG heavy chain D [soluble], IgG heavy chain A [soluble] and DLA class I histocompatibility antigen, A9/A9 alpha chain [soluble]) could not be categorized using BiNGO, as standard gene name abbreviations are not established for these proteins, which are necessary to input datasets. For the remaining proteins, two datasets were

made, one comprised of 388 exosomal proteins and the other of 171 soluble proteins. These original datasets were individually searched against GO annotation terms within the functional categories. For the exosomal fraction, 335, 331 and 324 proteins were linked to a minimum of one annotation term within the GO categories of Cellular Component, Molecular Function and Biological Process, respectively. Within the soluble urine fraction, 190, 175 and 168 proteins were successfully linked to at least one annotation term within the Cellular Component, Molecular Function and Biological Process functional categories, respectively. Due to the large amount of data obtained, the figures discussed below contain edited data displayed in as relevant a manner as possible.

Over-represented canine urinary exosomal proteins within the Cellular Component category are displayed in Figure 3.2. Exosomal proteins were annotated to GO terms related primarily to internal or membrane cellular structures, such as cytoplasm (266), organelle (231) and plasma membrane (118). Smaller numbers of proteins were annotated to more specific cellular locations including Golgi apparatus (34), endosome (29) and lysosome (15), similar to findings in human urine.[1] In contrast, the soluble fraction of canine urine contained large numbers of over-represented proteins from the extracellular region (110) within the Cellular Component category (Figure 3.3). In this fraction, proteins were annotated to the plasma membrane (65), cell surface (22) and brush border (5). Relatively small numbers of proteins in the soluble fraction were also over-represented and annotated to GO terms pertaining to intracellular components.

Under-represented canine urinary exosomal proteins were categorized to only two GO terms within the Cellular Component category (Figure 3.4). Fifty-nine proteins were found to be integral to membrane and 67 proteins were intrinsic to membrane, which is somewhat surprising as exosomes contain cellular membrane components within their membranes.[74,117] Under-represented proteins of the soluble fraction were annotated to seven GO terms, some of which were redundant (Figure 3.5). Four GO terms encompassed the majority of proteins allocated to under-represented GO terms: cell part (141), nucleus (25), intracellular part (96), and membrane-bounded organelle (68).

Within the Molecular Function category, over-represented canine urinary exosomal proteins were annotated to 120 GO terms (Figure 3.6). The majority of these proteins were linked to protein binding (234), including terms such as cytoskeletal protein binding (36), enzyme binding (26), coenzyme binding (16) and MHC protein binding (4). Nucleotide binding (102), lipid binding (29) and vitamin binding (9) were also over-represented. Catalyase activity (194), a parent term to hydrolase activity (61) and lyase activity (13), were over-represented, as well, in addition to other proteins.

Similarly, protein binding (164) was over-represented in the soluble fraction of canine urine (Figure 3.7). Daughter terms included identical protein binding (16), glycoprotein binding (4) and syndecan binding (2). Calcium ion binding (30), carbohydrate binding (24), lipid binding (16) and vitamin binding (6) were over-represented in the soluble fraction. Enzyme regulator (29) and enzyme inhibitor activity (21) were significantly increased in canine urine soluble fraction, as was peptidase

activity (18). Within peptidase activity, proteins were annotated to peptidase regulator activity (18), endopeptidase activity (12) and exopeptidase activity (6) in the soluble fraction.

Under-represented proteins from the canine urinary exosomal fraction were categorized to ten GO terms (Figure 3.8) within the Molecular Function category. These proteins were annotated to transition metal ion binding (25), nucleic acid binding (21), zinc ion binding (16), receptor activity (12), transcription regulator activity (11), transmembrane receptor activity (6), transcription factor activity (4), sequence specific DNA binding (1), G-protein coupled receptor activity (1) and sequence-specific DNA binding (1). Likewise, fewer proteins were under-represented in the soluble fraction of canine urine (Figure 3.9). These proteins were annotated to 12 GO terms, some of which have very similar definitions. The streamlined list includes terms linked to nuclear activities, such as nucleic acid binding (9), nucleotide binding (4), DNA binding (2) and transcription regulator activity (3).

The Biological Process category contained the largest number of over-represented proteins in both the exosomal and soluble fractions (Figures 3.10 and 3.11, respectively). Within the exosomal fraction, major parent terms included cellular process (254), metabolic process (175), localization (125) and transport (109). Daughter terms of metabolic process included small molecule metabolic process (99), nitrogen compound metabolic process (72), organic acid metabolic process (42), carbohydrate metabolic process (40) and alcohol metabolic process (39), among others. Localization

encompasses macromolecule localization (62), protein localization (57), cellular location (38) and regulation of location (30). Transport is a parent term to protein transport (52), vesicle-mediated transport (37) and intracellular transport (27). In contrast to human urine, only small numbers of proteins were annotated to immunological terms, such as natural killer cell mediated immunity (3), leukocyte degranulation (3) and leukocyte mediated cytotoxicity (3).[1]

The soluble fraction of canine urine was also heavily annotated to GO Biological Process terms, including multicellular organismal process (85), developmental process (76) and response to stimulus (63), among other terms. Within multicellular organismal process, multicellular organismal development (67) and regulation of multicellular organismal process (36) were over-represented. Developmental process encompasses many over-represented terms, including organ development (47), regulation of developmental process (23), vasculature development (17) and tube development (11). Response to stimulus is a parent term to response to stress (45), regulation of response to stimulus (26), response to extracellular stimulus (15), response to hormone stimulus (14) and response to oxidative stress (7), to name a few.

As in the Molecular Function category, the majority of under-represented proteins annotated to Biological Process terms from both the exosomal and soluble fractions are related to nuclear biological processes. In the exosomal fraction, under-represented proteins were annotated to sixteen GO terms (Figure 3.12). A consolidated list includes regulation of cellular metabolic process (56), cellular macromolecule metabolic process

(43), regulation of biosynthetic process (36), regulation of gene expression (28), regulation of transcription (24), nucleic acid metabolic process (9), gene expression (7) and RNA metabolic process (4). Soluble proteins were annotated to only nine GO terms within the Biological Process category (Figure 3.13). These terms included regulation of biosynthetic process (12), regulation of gene expression (8), regulation of transcription (7) and regulation of RNA metabolic process (4).

Comparison of the canine versus human urinary proteome via Gene Ontology annotation

Upon comparison of our canine data to recently published human data, we found that 52% (205/391) of our canine urinary exosomal proteins and 62% (133/214) of the canine soluble proteins were also found within the human urinary proteome.[1] Interestingly, 48% (186/391) of canine exosomal and 38% (81/214) of soluble proteins are unique to the canine urinary proteome.

To further compare the canine urinary proteome to human data, we subdivided our exosomal and soluble protein datasets into datasets containing canine proteins in common with human urinary proteins and proteins unique to the canine urinary proteome. BiNGO analysis was repeated with these new datasets, searching for over- and under-represented terms within the GO functional categories of Cellular Component, Molecular Function and Biological Process.

Within the Cellular Component group, the canine exosomal proteins in common with the human urinary proteome shared a very similar distribution of overrepresented

GO terms as our original canine dataset. The majority of over-represented proteins were linked to intracellular locations (Figure 3.14). The analysis of shared proteins within the canine soluble fraction localized the majority of proteins to GO terms such as plasma membrane (50) and cell surface (20) (Figure 3.15), which is similar to the original canine soluble fraction dataset. Gene Ontology analysis of the human urinary proteome contained many soluble proteins annotated to extracellular terms, with fewer numbers annotated to intracellular terms.[1] Canine urine contains both intracellular and extracellular components. However, intracellular proteins were present in greater number in the exosomal fraction, while extracellular proteins were more common in the soluble fraction.

When canine urinary exosomal proteins were compared to human urinary proteins, there were no common, statistically significant under-represented proteins annotated to GO Cellular Component terms. In contrast, under-represented soluble canine proteins in common with human urinary proteins were annotated to five GO terms (cell [103], cell part [103], nucleus [18], intracellular [69] and intracellular part [69]) (Figure 3.16), which followed a similar distribution as the original, combined canine urine dataset. Many of these GO terms are linked to under-represented proteins in human urine, as well.[1]

When BiNGO analysis was performed on exosomal and soluble proteins unique to the dog, a similar distribution to our original canine datasets was obtained. Although these proteins identified in canine urine were not found in human urine, over-represented

canine exosomal proteins still tended to annotate to intracellular locations (Figure 3.17).[1] The over-represented soluble proteins unique to the dog were similar in distribution of GO terms to our original urinary soluble protein dataset and were largely annotated to GO terms related to extracellular components, as well as cytoskeletal elements and components of filtrated plasma (Figure 3.18) As stated previously, the total human urinary proteome contains more over-represented proteins annotated to extracellular and membrane locations and fewer proteins annotated to intracellular terms, in relative comparison to the canine urinary proteome.[1]

The under-represented exosomal proteins that are unique to the dog were annotated to the same Cellular Component terms as the original canine exosomal dataset (integral to membrane [15] and intrinsic to membrane [15]) (Figure 3.19). No under-represented soluble proteins unique to the canine were annotated to GO Cellular Component terms. This is somewhat in contrast to findings in the human urinary proteome, which contains many under-represented proteins annotated to intracellular components, such as organelle, nucleus and ribosome.[1]

The exosomal and soluble canine urinary datasets that contain proteins in common with the human urinary proteome are heavily annotated to the GO Molecular Function category. Within these groups, the majority of over-represented exosomal proteins are involved with binding (178), protein binding (139) and catalytic activity (123) (Figure 3.20). This distribution of proteins is very similar to our original canine exosomal dataset's Molecular Function annotation. Likewise, the annotated GO terms

from the canine soluble proteins in common with the human urine proteome are also quite similar to the original canine soluble protein annotation (Figure 3.21). Among the canine GO terms in common with the human urinary proteome (both exosomal and soluble), many proteins are linked to the same Molecular Function terms as are human urinary proteins (such as peptidase activity and various peptidase activity daughter terms). However, the relative number of proteins annotated to GO terms differs.[1] For example, canine urine has less proteins annotated to signal transducer activity and enzyme inhibition than human urine.[1]

As in human urine, the majority of under-represented annotated terms in our original exosomal and soluble protein datasets, as well as the datasets comprised of proteins found in common with human urine (Figure 3.22 and Figure 3.23), are related to nuclear activities, such as DNA binding.[1]

The overrepresented exosomal proteins unique to canine urine are heavily annotated to binding terms, such as protein binding (95), nucleotide binding (49) and cytoskeletal protein binding (17), as well as hydrolase activity (25) and various other molecular functional activities (Figure 3.24). Many of these GO Molecular Function terms are shared between the unique canine exosomal proteins and the original canine exosomal dataset, as well as with the human urinary proteome. Although the annotated proteins unique to the canine urine are not found in human urine, their molecular functions can be categorized similarly. This may indicate some variation in molecular function pathways between canines and humans.

As expected, the over-represented exosomal urinary proteins unique to the canine are also annotated in small numbers to a few exclusive GO terms, such as structural molecule activity (18), SNARE binding (3), xylulokinase activity (1) and purine-nucleoside phosphorylase activity (1) (the latter two terms being daughter terms of transferase activity).

The over-represented soluble urinary proteins that are unique to the canine (Figure 3.25) share an almost identical distribution of over-represented GO annotation terms as the original, combined canine urine soluble fraction. Both of these canine soluble protein datasets contain similar annotated terms to the human urinary proteome, but the relative number of proteins linked to the GO Molecular Function terms vary between the canine and human datasets.

The under-represented urinary exosomal proteins unique to the canine were annotated to six Molecular Function GO terms (Figure 3.26). These terms included nucleic acid binding (11), transition metal ion binding (5), molecular transducer activity (4), signal transducer activity (4), zinc ion binding (3) and receptor activity (1). Similar annotated terms were under-represented in the dataset of the original canine urinary exosomal fraction and the canine exosomal proteins in common with human urine datasets. The under-representation of proteins annotated to signal and molecular transducer activities does, once again, highlight the apparently decreased transducer activity in canine urine versus human urine. No statistically significant under-represented

proteins were found in the dataset comprised of soluble proteins unique to the canine in this GO category.

As with our original canine urinary datasets, the GO Biological Process category resulted in the largest lists of annotated terms for statistically significantly over-represented canine urinary proteins from both the exosomal and soluble protein datasets that were in common with human urinary proteins. Not surprisingly, the protein annotation from the canine urinary exosomal fraction (Figure 3.27) shared an extremely similar distribution of Biological Process annotation terms with the original canine exosomal dataset. Proteins were annotated most heavily to cellular process (152), metabolic process (116), localization (79), response to stimulus (68) and transport (71). A similar trend was followed with the canine urine soluble fraction dataset comprised of proteins common to the human urinary proteome (Figure 3.28), in that many proteins were annotated to the same Biological Process categorical terms as the original canine soluble protein dataset. Multicellular organismal process (70), developmental process (60), response to stimulus (57), multicellular organismal development (54) and anatomical structure development (48) contain the largest number of annotated proteins in the canine urine soluble dataset (comprised of proteins in common with human urine). Although some of the canine exosomal and soluble protein annotated terms are also highly annotated in human urine, terms such as cell communication, defense response and immune response contained more annotated proteins in human urine versus canine urine.[1]

Many of the under-represented terms, which are shared between the canine exosomal and soluble protein groups, also have datasets comprised of proteins shared between the canine and human urinary proteome (Supplemental data, Figures 3.29 and 3.30, respectively). The under-represented Biological Process terms generally refer to nuclear processes, such as regulation of gene expression and regulation of transcription. Human urine does contain proteins annotated to nucleobase, nucleoside, nucleotide and nucleic acid metabolism, however the majority of under-represented proteins in human urine are annotated to more general terms, such as cell metabolism and regulation of cellular processes.[1] This may indicate that more proteins related to nuclear biological processes are found in human urine versus canine urine.

No statistically significantly under-represented exosomal proteins unique to the canine urinary proteome were annotated to any GO Biological Process terms. Within the soluble proteins unique to the canine, proteins were linked to only two GO Biological Process terms, ectoderm development and epidermis development, each containing the same 7 annotated proteins (Figure 3.31). Human data was not annotated to either of these GO Biological Process terms.

Identification of potential urinary biomarkers

Among our extensive list of identified proteins in the canine urine, sixty of these proteins, also found in human urine, are known to be related to specific extra-renal diseases in people (Table 3.1).[26] Many of these proteins are linked to rare human diseases that do not have a recognized canine counterpart, such as hypermethioninemia, Bardet-

Biedl syndrome and Papillon-Lefevere syndrome. However, some proteins linked to human extra-renal disease do have a canine version of the disease. Many proteins related to hypertension are found in human and canine urine, including angiotensin 1-converting enzyme isoform 1 (ACE), ACE2, dimethylarginine dimethylaminaophydrolase 1, glutamyl aminopeptidase, hydroxyprostaglandin dehydrogenase 15-(NAD) and membrane metallo-endopeptidase. [26] Additionally, urinary proteins associated with human disorders like megaloblastic anemia (amnionless protein precursor [AMN]), cystathioninuria (cystathionase isoforms 2 [CTH]), and Charcot-Marie-Tooth neuropathy (N-myc downstream regulated gene 1 [NDRG1] and dynamin 2 [DNM2]) were found in canine urine.

Megaloblastic anemia can result from acquired vitamin B6 deficiency or be secondary to a genetic mutation; it is reported in dogs and appears to have a similar pathogenesis as humans.[34] Cystathioninuria has been reported in daschunds and a Scottish terrier.[25] Charcot-Marie-Tooth-like neuropathies have been diagnosed clinically in dogs, however further research of the pathogenesis is needed to determine if these neuropathies are truly similar to those seen in humans.[27]

The focus of our particular group, however, is the early diagnosis of acute renal injury. We identified fourteen known and potential biomarkers for renal disease in our canine samples (Table 3.2). Known urinary biomarkers of renal disease include albumin, immunoglobulin-γ (IgG) and retinal binding protein 4 (RBP4). Recently discovered and developmental urinary biomarkers for renal disease include clusterin (CLU), aquaporin 1

(AQP1), hemopexin (HPX), fetuin-A (AHSG) and ubiquitin A-52 (UBA52), among others.

Other proteins known to have altered regulation during renal disease, localized to the kidney or involved in genetic renal diseases were also found in our canine urine samples. These proteins are chloride intracellular channel 1 (CLIC1, aka NCC27), solute carrier family 12 ($Na^+K^+Cl^-$ transporters) member 1 (SLC12A1) and myo-inositol oxygenase (MIOX).[47,56,102,113] Other urinary biomarkers found in the urine of dogs with renal disease, such as C-reactive protein (CRP), gamma glutamyl transferase (GGT) and *N*-acetyl-β-D-glucosaminidase (NAG), were not identified in the urine of our selection of clinically normal dogs via proteomic methods.[15,57,87]

Discussion

Characteristics of the canine urinary proteome and comparison to the human urinary proteome

Using techniques developed for human urine, we successfully identified 563 urinary proteins in pooled urine from clinically healthy dogs. Early proteomic identification of human urinary proteins yielded much smaller numbers of proteins. An early study by Thongboonkerd, et. al, yielded 67 proteins.[101] Another 2004 study identified 295 exosomal proteins.[74] As techniques have improved, proteomic protein discovery rates have accelerated. More recent studies have identified 1500-2000 urinary proteins in the urine of normal humans.[1,49] For an initial foray into urinary proteomics in the canine, our comparatively modest yield has produced some interesting information.

Canines are thought to have more THP in their urine than humans, which likely contributed to the reduced number of proteins identified in our study compared with human urine.[85] The presence of large concentrations of a few proteins can essentially mask other proteins present in very low concentrations in a sample analyzed via mass spectrometry. Although we performed THP depletion via previously described methods, these methods were developed for human urine and may not be as efficient for the THP levels found in canine urine. As carnivores, dogs excrete vitamin A in their urine and THP has been identified as the carrier protein in urine of dogs for retinol.[85] Therefore, we could attempt to develop additional and/or alternative methods to more efficiently remove THP from canine urine.[74] Canine urine also contains relatively high levels of albumin. Commercially available kits have been developed for albumin removal and could be optimized for their use with canine urine.[1] Optimization of these techniques could potentially greatly increase our canine urine protein yield. Finally, as the canine genome has only recently been completed, the canine database is not yet fully validated and annotated. Therefore, proteins from our datasets may not have been identified due to their absence in the database.

The molecular weight distribution of canine urinary proteins followed a similar distribution to human urinary proteins (Figure 3.1).[1] It is widely accepted that proteins filtered via the healthy glomerulus are generally < 68 kDa.[89] The majority of canine urinary proteins and human urinary proteins are in the range of 10-79 kDa.[1] Relatively smaller number of proteins with molecular weights ≥80 kDa are present in normal urine

from both humans and dogs.[1] Sixty-four canine exosomal proteins have a molecular weight ≥ 80 kD, while 58 soluble proteins have a molecular weight ≥ 80 kDa. These higher molecular weight proteins may therefore represent post glomerular filtration proteins released or secreted by renal epithelial proteins and this could provide valuable information about real-time renal health intrinsic to renal tissue.

On further analysis of the canine urinary proteome, we found that urinary proteins were largely allocated to the extracellular region (primarily soluble proteins), the cytoplasm and organelles (primarily exosomal proteins), as well as the plasma membrane (both soluble and exosomal proteins). Our sub-datasets of proteins in common with human urinary proteins and unique to the canine also followed this distribution. This is similar to the Cellular Component distribution of human urine.[1] It is also logical that the soluble portion of canine urine would contain more extracellular elements, as well as proteins shed from the plasma membrane, while the exosomal urinary fraction would contain intracellular proteins, as exosomes are small, low-density membrane vesicles, released from epithelial cell surfaces.[1,74]

Exosomal proteins yielded significantly low numbers of proteins that are integral and intrinsic to the membrane. These types of proteins are buried in or penetrate one side of the plasma membrane's lipid bilayer, or span both sides of the lipid bilayer, respectively. It is somewhat surprising that proteins embedded in the plasma membrane would not be present in higher numbers in the exosomal fraction. However, perhaps integral and intrinsic membrane proteins are preferentially retained by the plasma

membrane during exocytosis of exosomal vesicles. Nuclear and intracellular proteins were expectantly under-represented in the soluble protein fraction. It is unlikely that nuclear and vast numbers of intracellular proteins would escape from normal, healthy cells into the renal tubular lumen.

Proteins involved in various Molecular Functions were found in both the exosomal and soluble urine fractions. The canine urinary exosomal fraction contains many proteins related to various types of protein binding and catalase activity. In tissue as metabolically active as the kidneys, it is not surprising that these (intracellular) molecular activities would be amplified in the exosomal fraction of urine. A similar distribution of annotated GO Molecular Function terms was found in canine urine datasets comprised of proteins in common with human urinary proteins and of proteins unique to canine urine. Protein binding and hydrolase activity (a daughter term of catalytic activity) were both significantly over-represented in human urine.[1]

Soluble urinary proteins involved in protein binding were also present in large numbers in canine urine. Additionally, increased numbers of proteins associated with the binding of other substances, such as carbohydrates, glycoproteins, lipids and vitamins, and involved in enzyme regulation and inhibition were identified within the canine soluble urinary fraction. Proteins related to these molecular functions were also present in high numbers within the dataset of canine urinary soluble proteins in common with human urinary proteins and soluble proteins unique to canine urine. As previously stated, proteins involved in protein binding, as well as proteins involved in carbohydrate and

lipid binding, are found in significant numbers in human urine.[1] Additionally, proteins related to peptidase activity are heavily annotated to human urine – more so than in canine urine.[1]

Proteins that were under-represented in all canine urinary proteome datasets were largely related to nuclear activities, such as nucleic acid binding. This is similar to findings in human urine, and again, not surprising as nuclear material and activities should be restricted to the nucleus in intact, healthy cells.[1] In the canine soluble urinary fraction dataset comprised of proteins unique to the canine, proteins related to signal transducer activity were significantly decreased. This is in contrast to human urine, where signal transducer activity is significantly over-represented with 275 annotated proteins.[1] Cellular signal transduction, according to the GO definition, involves conveying a signal across a cell to trigger a response, such as a change in cellular function or state. The significance of this startling difference between the protein composition of canine versus human urine is not yet understood. A GO major daughter term of signal transduction is receptor binding, to which 23 proteins in the canine urinary soluble fraction are annotated. It may simply be that fewer, more specialized metabolic functions occur in canine renal epithelium and glomerular filtrate. Further study is needed to clarify the issue.

Amongst GO Biological Process terms, canine urinary proteins were heavily annotated to terms related to metabolic process, response to stimulus, transport and anatomical structural development. These terms, and related terms, were also heavily

linked to human urinary proteins. However, human urine contained over-represented proteins annotated to terms related to the immune response, which canine urine did not. This may indicate a higher degree of immunological activity in normal human urine versus normal canine urine. Additionally, as the canine datasets comprised of proteins unique to the canine were also categorized to similar Biological Process terms as human urine, this may indicate that alternative pathways are utilized in the dog for these biological processes.

Exosomes and identification of potential urinary biomarkers for renal disease

The search for urinary biomarkers of disease has included examining the urinary proteome for some time.[74,101,117] Urine is easily and non-invasively obtained and is filled with proteins, making it potential diagnostic liquid gold. More recently, however, most groups have tended to focus on the exosomal urinary fraction. Urinary exosomes inherently have the potential to provide very specific information about the cells they come from due to their unique acquisition of vesicular membranes and liquid contents.

Exosomes are formed as plasma membrane proteins are endocytosed into a cell, becoming an endocytic vesicle. The endocytic vesicle then binds to and is incorporated into an intracellular multivesicular body (MVB), where it becomes an internal vesicle. Finally, the MVB is called to the cell surface, where it binds with the cellular plasma membrane, releasing exosomes out of the cell. A unique characteristic of exosomes is that they have an inverted orientation of their membrane, with the "cytoplasmic-side inward orientation," as do MVBs.[98] These unique exosomal characteristics load

exosomes with specialized information about their originating cells. They contain both membranous and cytoplasmic constituents and are generally excreted from cells for very specific reasons, such as cell-to-cell signaling or to act as membrane transport proteins.

The urinary exosomal population is thought to be comprised largely of exosomes from renal and other urinary tract cells. However, exosomes are found in serum and due to their generally small size can be freely filtered by the glomerulus into the urinary space.[74] Theoretically, if a non-renal disease causes an increase of specific exosomes in serum, their proteins may be found in greater abundance in urine. Therefore, urinary exosomes have great potential to provide important information about not just the urinary tract, but about systemic health, as well.

Multiple previous studies involving human urine have definitively identified and isolated urinary exosomes via ultracentrifugation techniques similar to those we used in our study.[74,101,117] As we isolated many proteins in common with human exosomal proteins and followed similar methods, we can feel confident that our methods were adequate to identify canine urinary exosomal proteins. Inevitably, soluble urinary proteins will also be trapped in the exosomal fraction, so the exosomal urinary fraction cannot be termed a pure population of exosomal proteins.

The proteomic analysis of human urine, and especially the exosomal fraction, has been employed to discover biomarkers for various diseases, such as acute pediatric

appendicitis and urethral and bladder cancer, with some success.[14,48,97] One of the main focuses, however, of urine proteomics has been to elucidate biomarkers of renal disease.

As stated in the introduction, ARD leads to high patient morbidity and mortality in humans and veterinary patients.[61] Unfortunately, our currently relied on markers for renal pathology (sUN and sCrt) fall short in early detection of ARD in our patients.[61,89] Early diagnosis and medical intervention has been shown to be much more advantageous in treatment of ARD, lessening morbidity and mortality.[61] Human medicine has increased its reliance on biomarkers such as sCysC, urine and serum neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18) and kidney injury molecule-1 (KIM-1) to identify early acute renal injury due to many underlying causes, to monitor response to treatment or exposure to nephrotoxic substances and potentially differentiate between various renal diseases.[61,96] These proteins were not identified via proteomics techniques in our healthy canine urine, which could indicate that these proteins may be potentially useful in identifying renal injury in dogs.

The expanded discovery and application of urine biomarkers for AKD has been underutilized in canines, both for animal model studies and to aid in the diagnosis and monitoring of renal disease in veterinary patients. Toxicological studies have relied on enzymes such as GGT and NAG to diagnose nephrotoxic damage and renal failure in dogs.[15,43] These proteins have been employed diagnostically as well, but are not utilized on a large scale. GGT is located to the brush border of the proximal tubule of the nephron and indicates PT damage when increased amounts are found in the urine.[15,43] NAG is

localized to lysosomes within the proximal tubule and the renal papilla. Generally, increased levels of uNAG are thought to indicate tubular or generalized renal disease.[15,43] Neither protein was identified via proteomic methods in our normal dog urine. As it has been well established that these urinary proteins are associated with renal injury in dogs, this may be further evidence that our sampled dog urine was obtained from animals with truly healthy kidneys.[43]

Recent canine studies have further explored the utility of urinary biomarkers for renal disease. A study found that uNAG and uRBP were significantly increased in the urine of dogs with chronic kidney disease versus healthy controls.[87] Additionally, increased urine concentrations of IgG, CRP, NAG and RBP are reflective of glomerular and tubular injury in dogs with pyometra-induced renal dysfunction.[57]

Urine proteomics have specifically been responsible for the discovery of urine fetuin-A and urine ubiquitin A-52 ribosomal fusion protein 1 (UBA52) in urine and their development as indicators of ARD.[96,116] This list will inevitably expand as the field is further investigated. Our proteomic analysis of normal canine urine has yielded an extensive list of urinary proteins. Some proteins discovered in canine urine are already being used as urinary biomarkers for renal disease in humans and animal models, such as albumin, retinol binding protein 4 (RBP4) and IgG (Table 3.2). However, proteins like fetuin-A and UBA52 (Table 3.2), which are present in our canine urinary proteome and have not previously been, to the author's knowledge, localized to canine urine, may prove to be useful indicators of AKD in dogs.

Fetuin-A is produced by the liver and is considered a negative acute-phase protein. It is known that fetuin-A suppresses the release of tumor necrosis factor-alpha by lipopolysaccharide and that it inhibits ectopic calcification. Larger amounts of fetuin-A are found in the exosomal fraction of urine; its urine concentration increases 2 days prior to sCrt in animal model studies of ARD and it appears to imply structural damage rather than early renal response to injury.[71,81,116] The exact role of fetuin-A in AKD, however, is yet unknown.[116]

UBA52 is a ubiquitin fusion protein located in renal tubules within the kidney.[24] Reportedly, expression of UBA52 in mouse kidney tubules increases as blood glucose rises.[24] Regulation of this protein in the kidney is influenced by oxidative and carbonyl stress, both of which often occur in diabetic nephropathy.[24] It is thought that UBA52 plays an important role in diabetic nephropathy, but the exact mechanism is not yet elucidated.[24] UBA52 appears to have promising utility as an early marker for development of diabetic nephrotic syndrome.[24]

In conclusion, we successfully adapted human methodology to study the urinary proteome to canines, including the exosomal urinary fraction. By using GO and BiNGO analysis, we were able to effectively characterize the canine urinary proteome and make a relevant comparison to the human urinary proteome. Additionally, we identified several known canine urinary markers of AKD, as well as a few biomarkers being developed in human medicine. It has become apparent in human medicine that the use of multiple

proteins compiled into profiles for AKD will be useful and yield higher sensitivity and specificity in diagnosing disease.[96] This will likely prove to be the same in our canine patients. Our initial foray into canine urinary proteomics will serve as a foundation to build on. More study, especially of the urinary proteome of animals afflicted with renal disease, is clearly indicated.

Figures

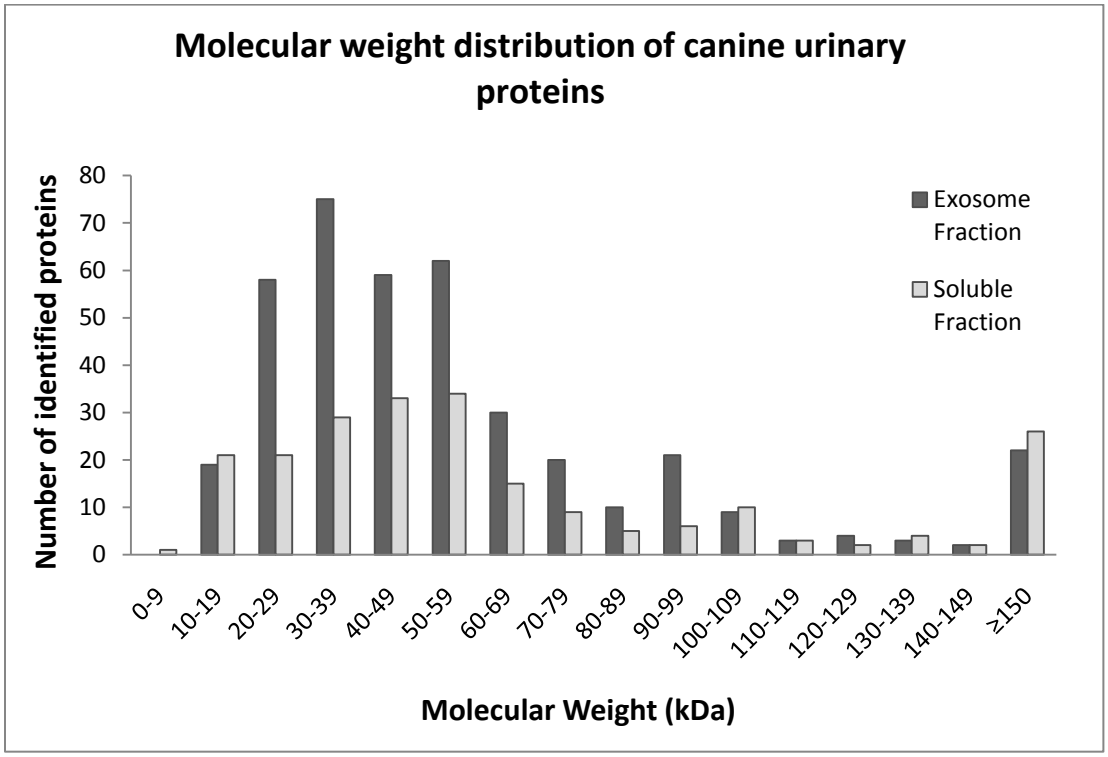

Figure 3.1. The molecular weight distribution of canine urinary exosomal and soluble proteins is shown here

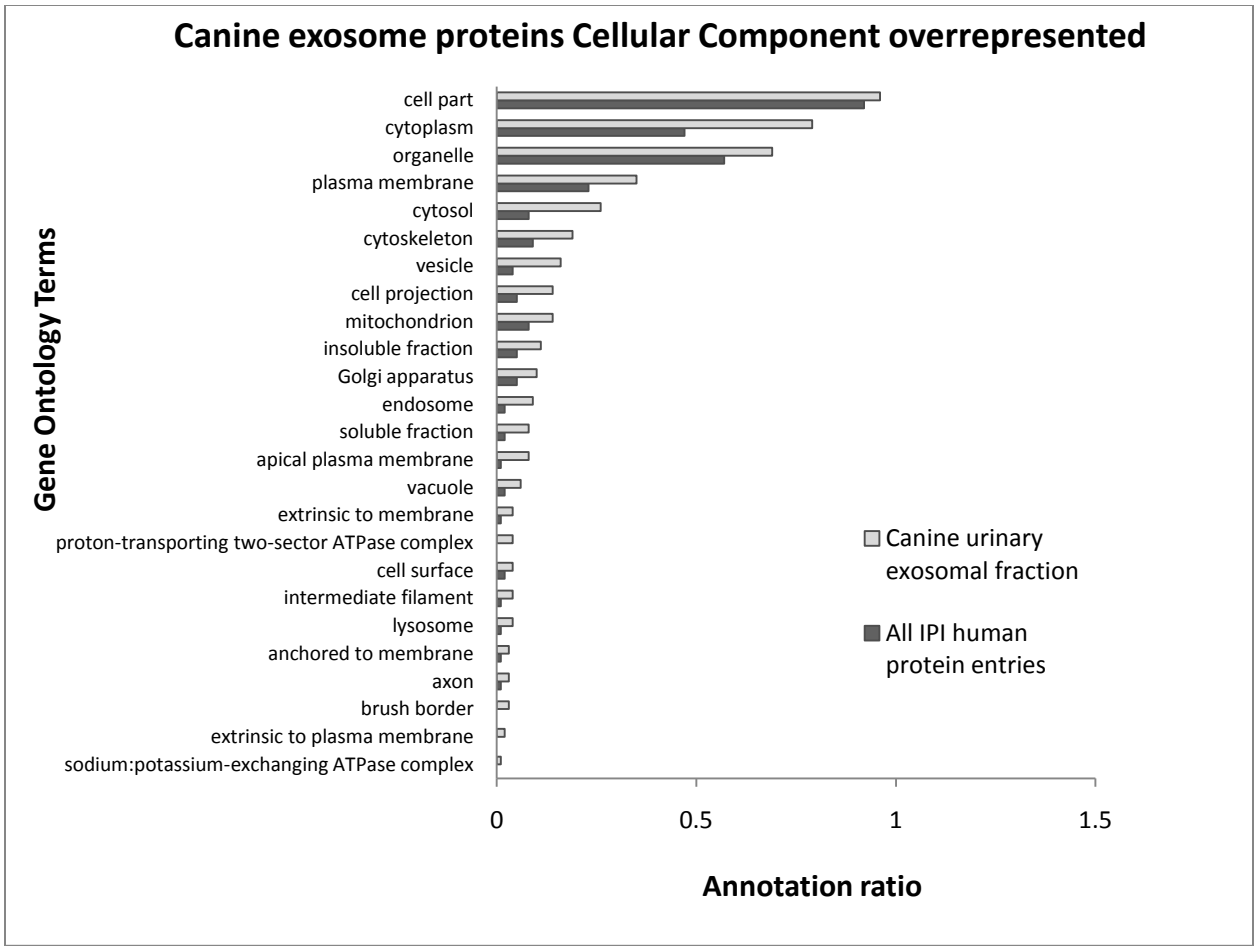

Figure 3.2. Gene Ontology (GO) Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine urine exosomal fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is the number of canine exosomal proteins associated with a specific GO term divided by the total number of canine exosomal proteins annotated to the specific functional annotation group by GO. The annotation ratio is calculated in the same manner for IPI human proteins and is defined as the number of IPI proteins associated with a specific GO term divided by the total number of IPI human proteins annotated by GO within a functional group. The canine urinary exosomal and IPI human protein annotation ratios are displayed side-by-side.

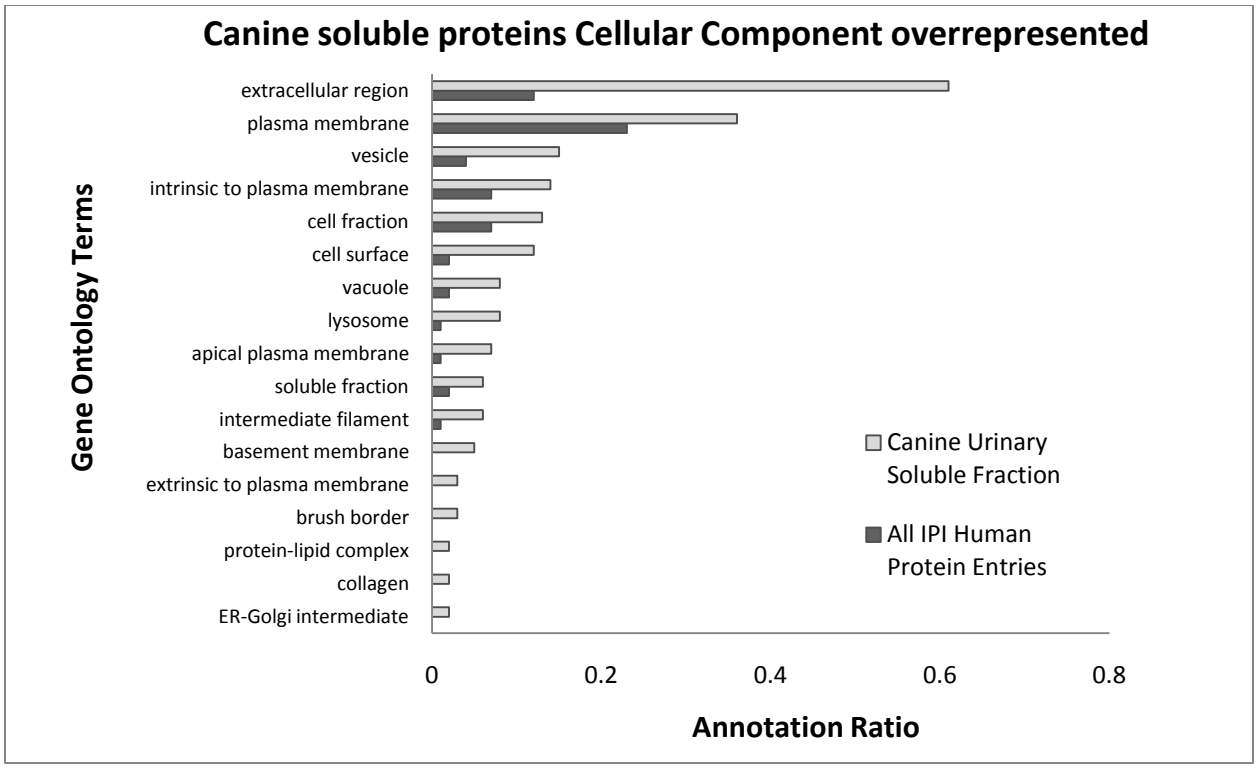

Figure 3.3. GO Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine urinary soluble fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2. The canine urinary soluble fraction and IPI human protein annotation ratios are displayed side-by-side.

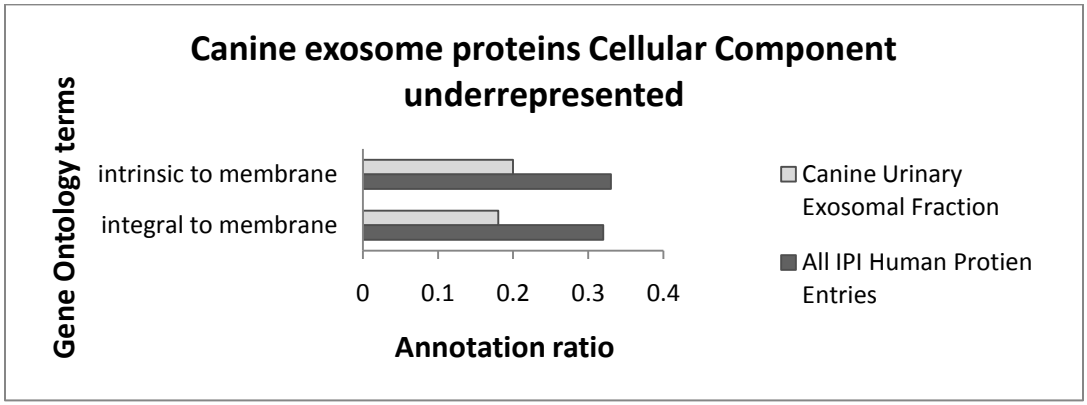

Figure 3.4. All GO Cellular Component terms that are significantly under-represented ($P < 0.001$) in the canine exosomal fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

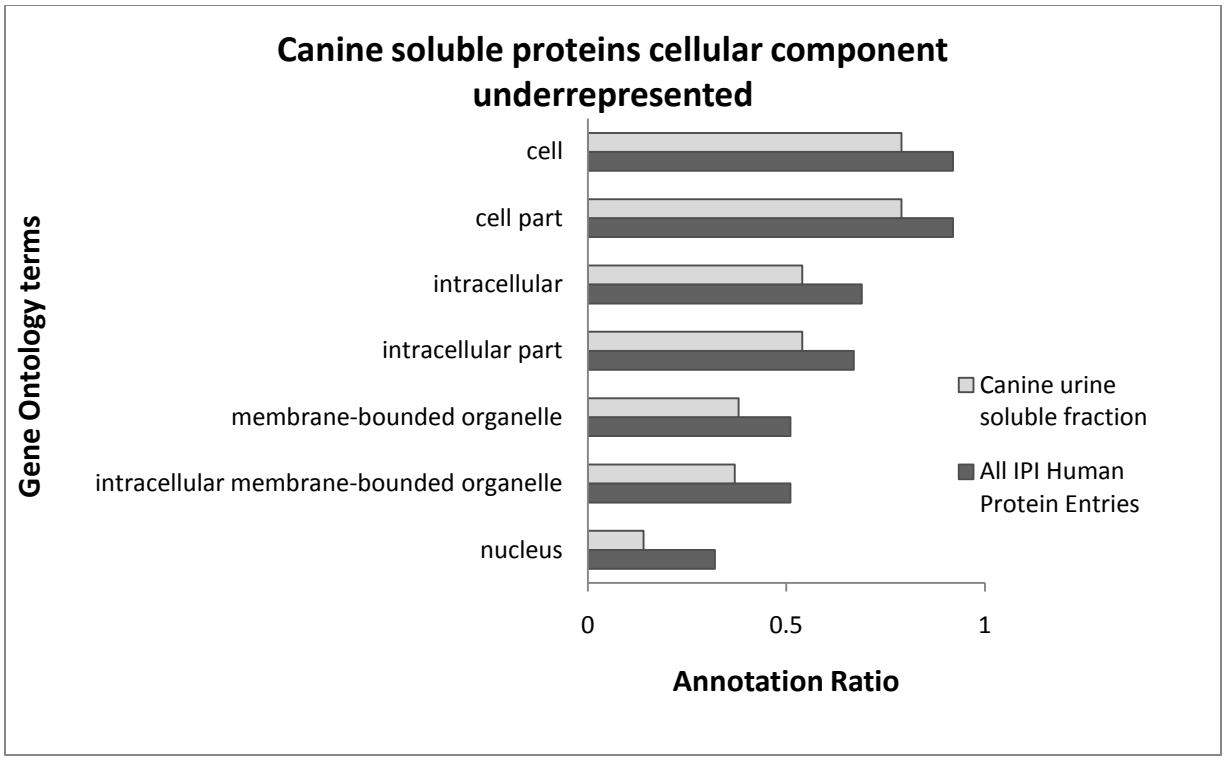

Figure 3.5. All GO Cellular Component terms that are significantly under-represented ($P < 0.001$) in the canine soluble fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

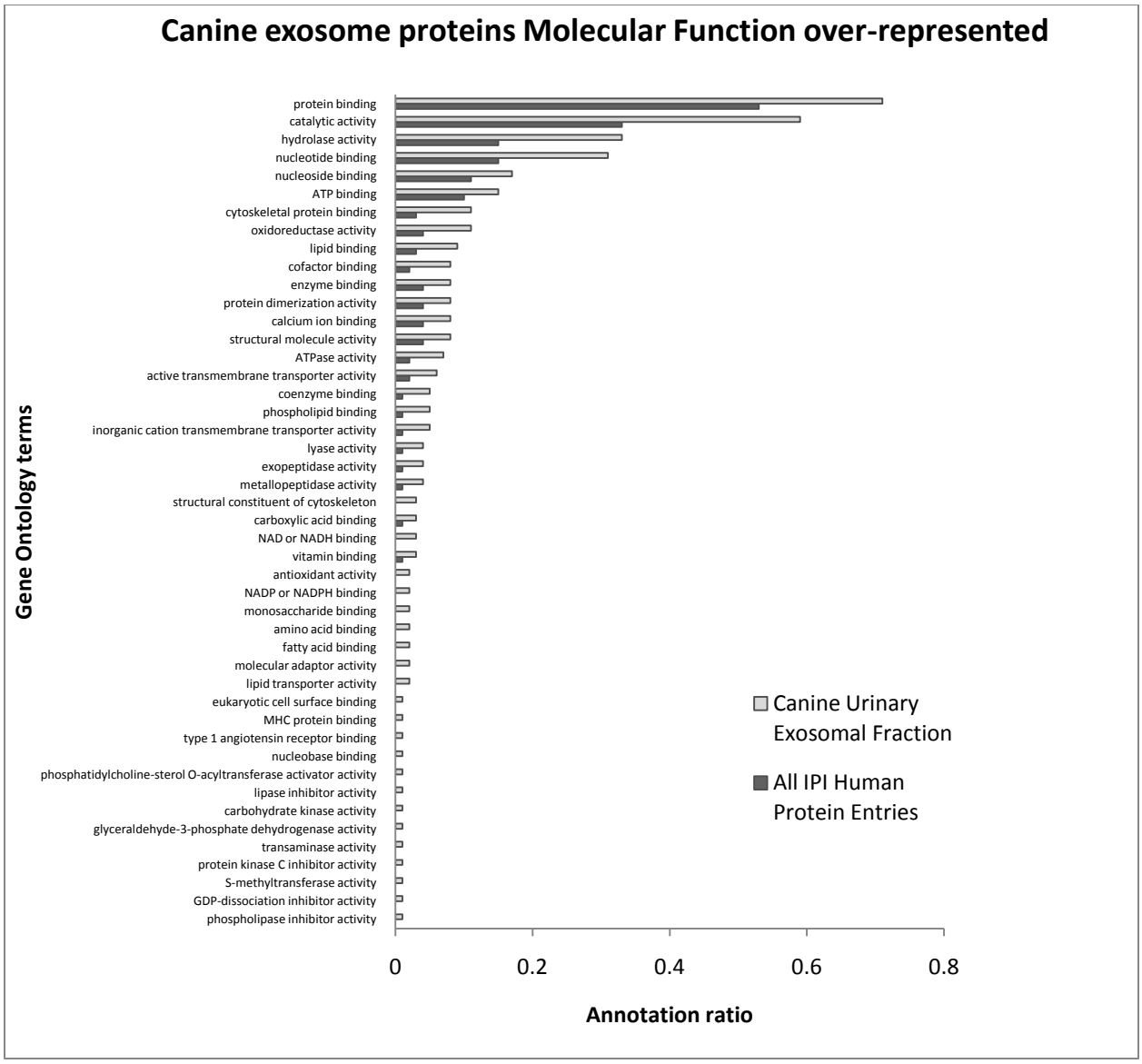

Figure 3.6. GO Molecular Function terms that are significantly over-represented ($P < 0.001$) in the canine urinary exosomal fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

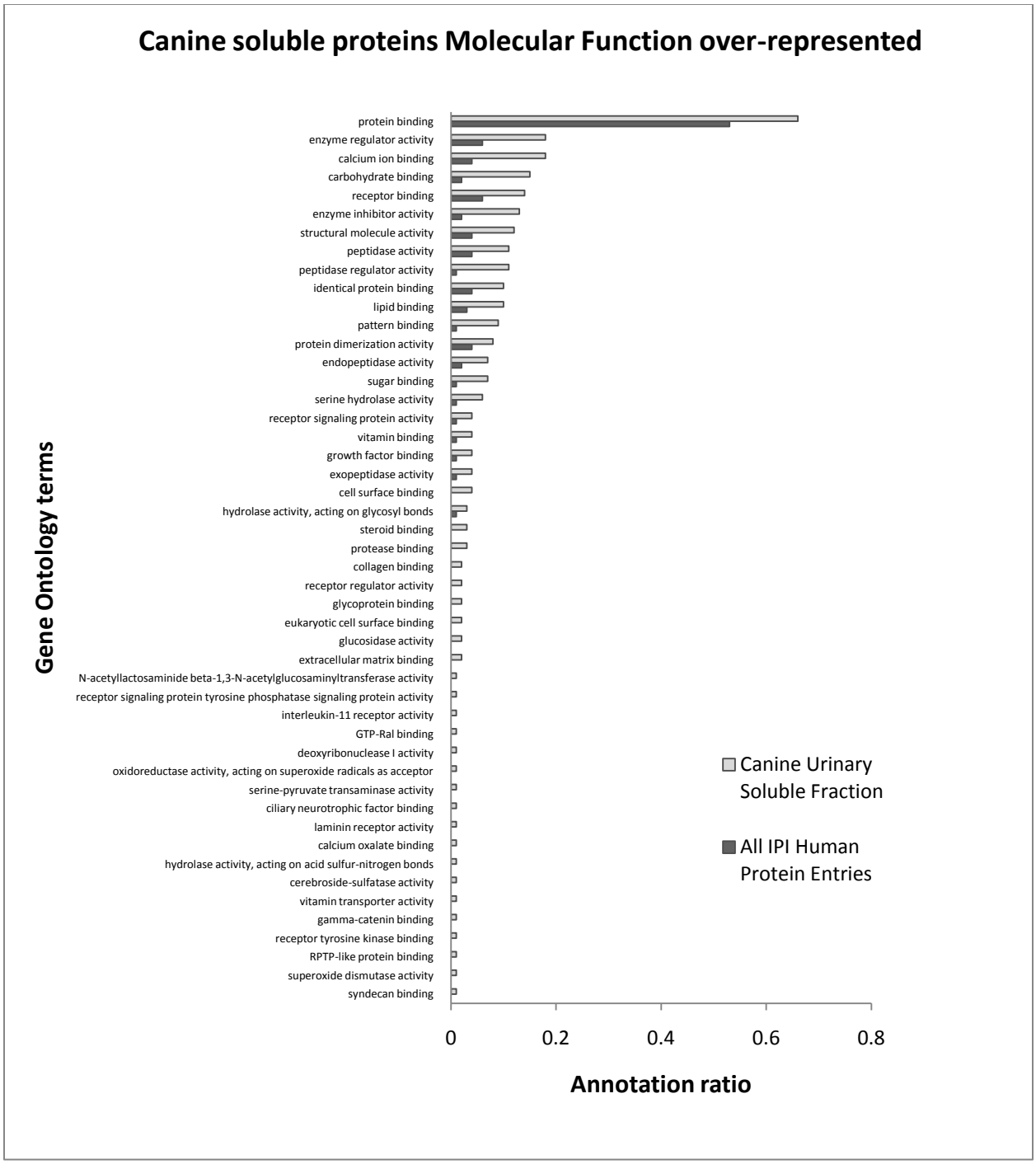

Figure 3.7. GO Molecular Function terms that are significantly over-represented ($P < 0.001$) in the canine urinary soluble fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

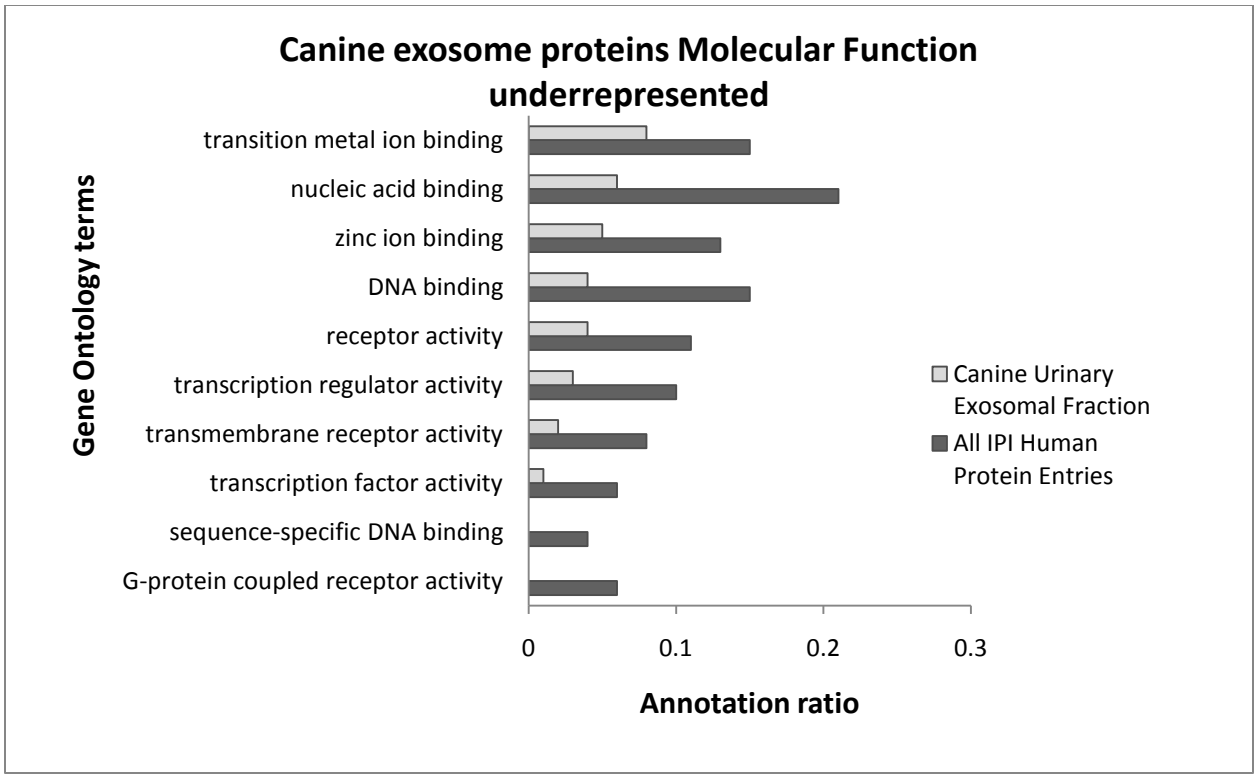

Figure 3.8. All GO Molecular Function terms that are significantly under-represented ($P < 0.001$) in the canine exosomal fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2

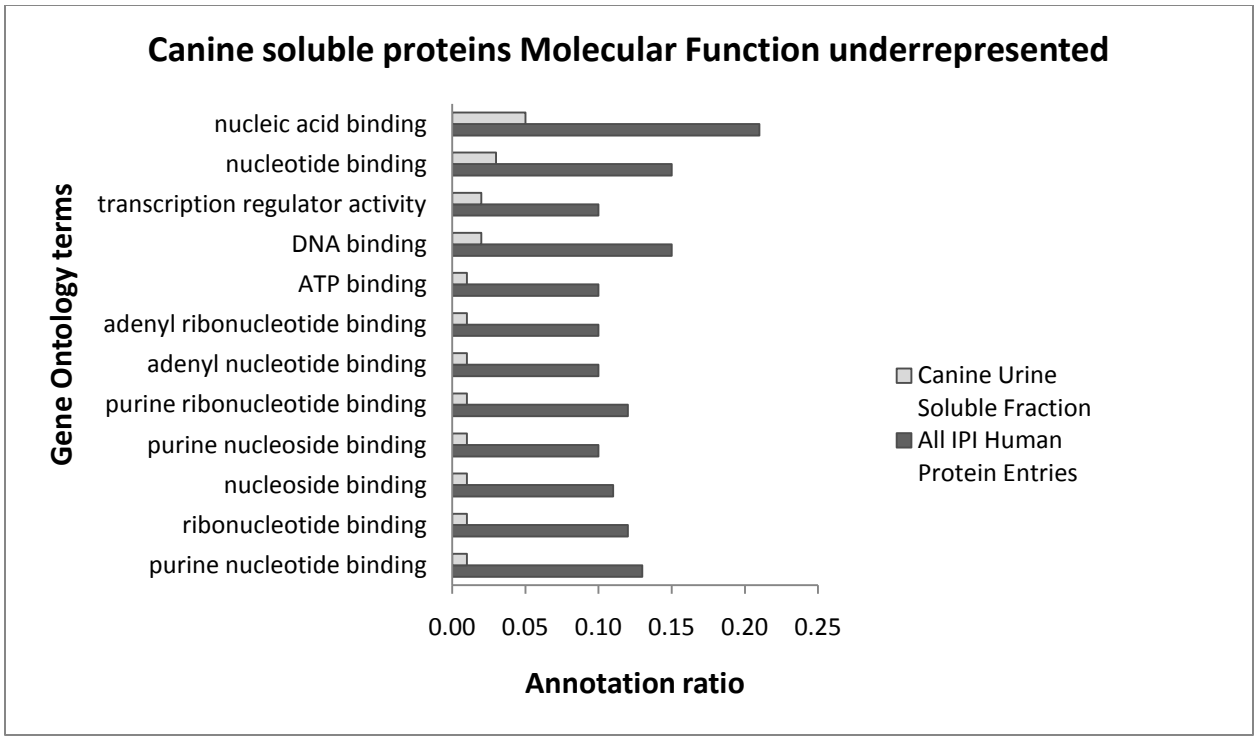

Figure 3.9. All GO Molecular Function terms that are significantly under-represented ($P < 0.001$) in the canine soluble fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

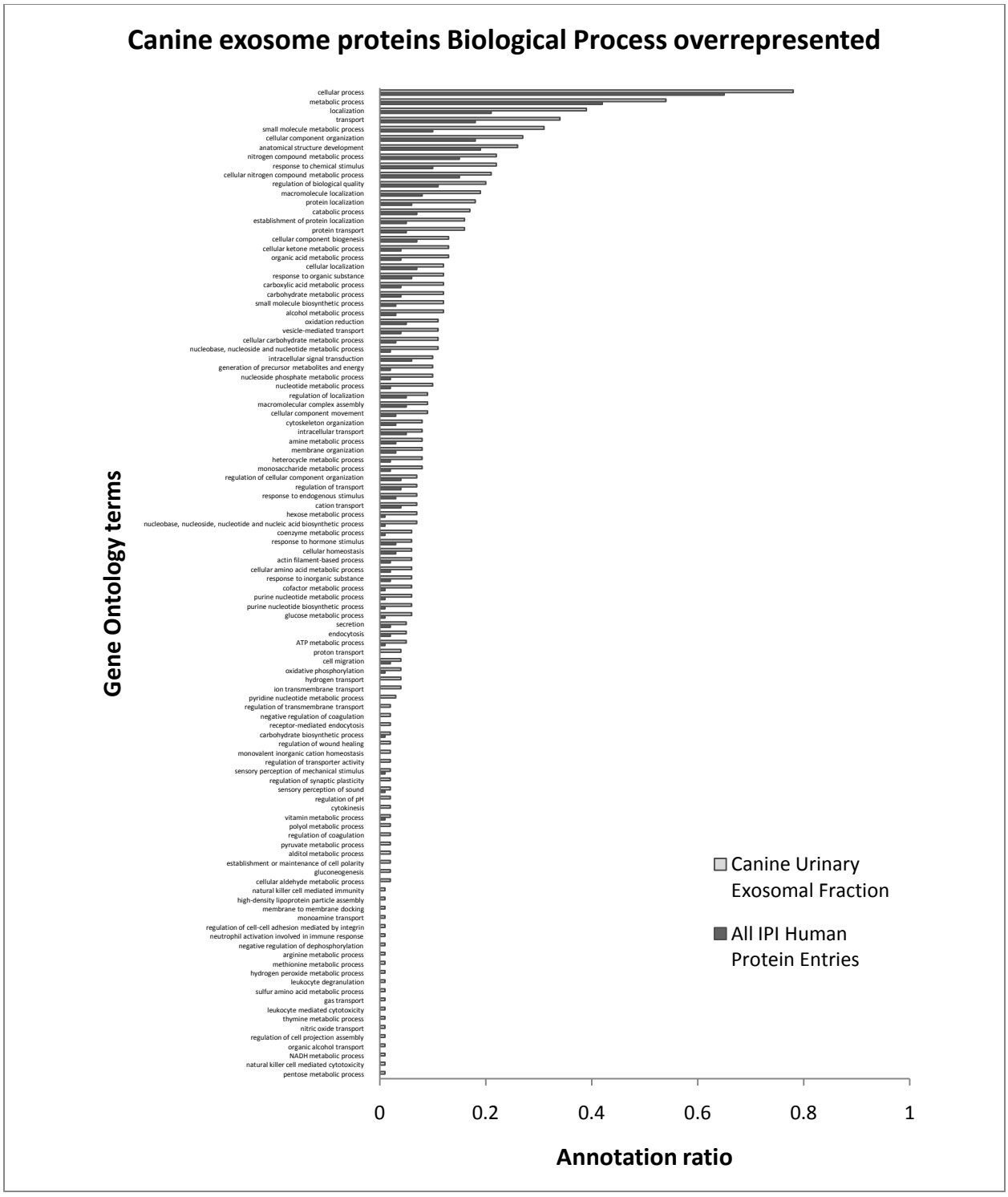

Figure 3.10. GO Biological Process terms that are significantly over-represented ($P < 0.001$) in the canine urinary exosomal fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

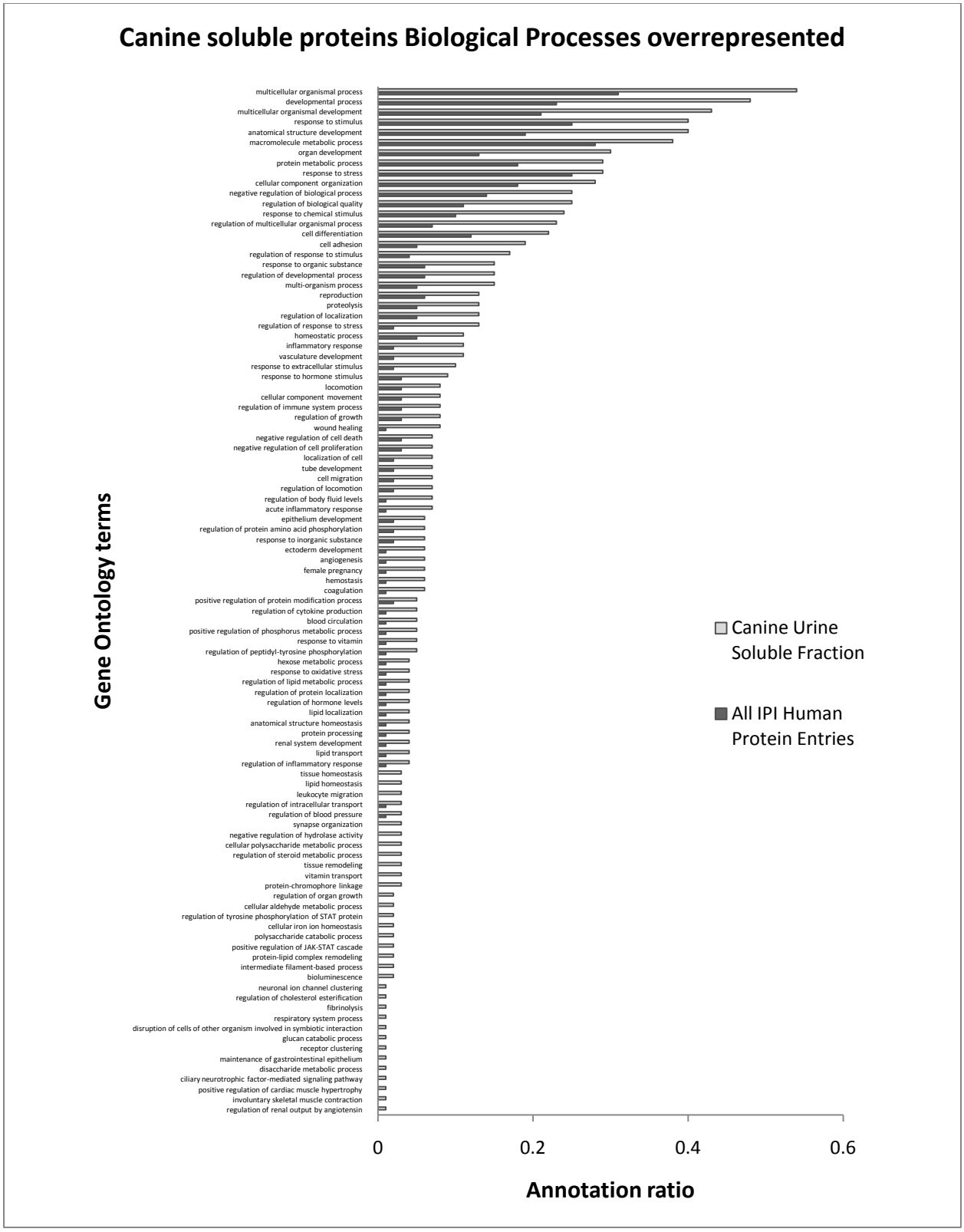
Canine soluble proteins Biological Processes overrepresented
Gene Ontology terms
multicellular organismal process
developmental process
multicellular organismal development
response to stimulus
anatomical structure development
macromolecule metabolic process
organ development
protein metabolic process
response to stress
cellular component organization
negative regulation of biological process
regulation of biological quality
response to chemical stimulus
regulation of multicellular organismal process
cell differentiation
cell adhesion
regulation of response to stimulus
response to organic substance
regulation of developmental process
multi-organism process
reproduction
proteolysis
regulation of localization
regulation of response to stress
homeostatic process
inflammatory response
vasculature development
response to extracellular stimulus
response to hormone stimulus
locomotion
cellular component movement
regulation of immune system process
regulation of growth
wound healing
negative regulation of cell death
negative regulation of cell proliferation
localization of cell
tube development
cell migration
regulation of locomotion
regulation of body fluid levels
acute inflammatory response
epithelium development
regulation of protein amino acid phosphorylation
response to inorganic substance
ectoderm development
angiogenesis
female pregnancy
hemostasis
coagulation
positive regulation of protein modification process
regulation of cytokine production
blood circulation
positive regulation of phosphorus metabolic process
response to vitamin
regulation of peptidyl-tyrosine phosphorylation
hexose metabolic process
response to oxidative stress
regulation of lipid metabolic process
regulation of protein localization
regulation of hormone levels
lipid localization
anatomical structure homeostasis
protein processing
renal system development
lipid transport
regulation of inflammatory response
tissue homeostasis
lipid homeostasis
leukocyte migration
regulation of intracellular transport
regulation of blood pressure
synapse organization
negative regulation of hydrolase activity
cellular polysaccharide metabolic process
regulation of steroid metabolic process
tissue remodeling
vitamin transport
protein-chromophore linkage
regulation of organ growth
cellular aldehyde metabolic process
regulation of tyrosine phosphorylation of STAT protein
cellular iron ion homeostasis
polysaccharide catabolic process
positive regulation of JAK-STAT cascade
protein-lipid complex remodeling
intermediate filament-based process
bioluminescence
neuronal ion channel clustering
regulation of cholesterol esterification
fibrinolysis
respiratory system process
disruption of cells of other organism involved in symbiotic interaction
glucan catabolic process
receptor clustering
maintenance of gastrointestinal epithelium
disaccharide metabolic process
ciliary neurotrophic factor-mediated signaling pathway
positive regulation of cardiac muscle hypertrophy
involuntary skeletal muscle contraction
regulation of renal output by angiotensin
Canine Urine Soluble Fraction
All IPI Human Protein Entries
0
0.2
0.4
0.6
Annotation ratio

Figure 3.11. GO Biological Process terms that are significantly over-represented (P < 0.001) in the canine soluble fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

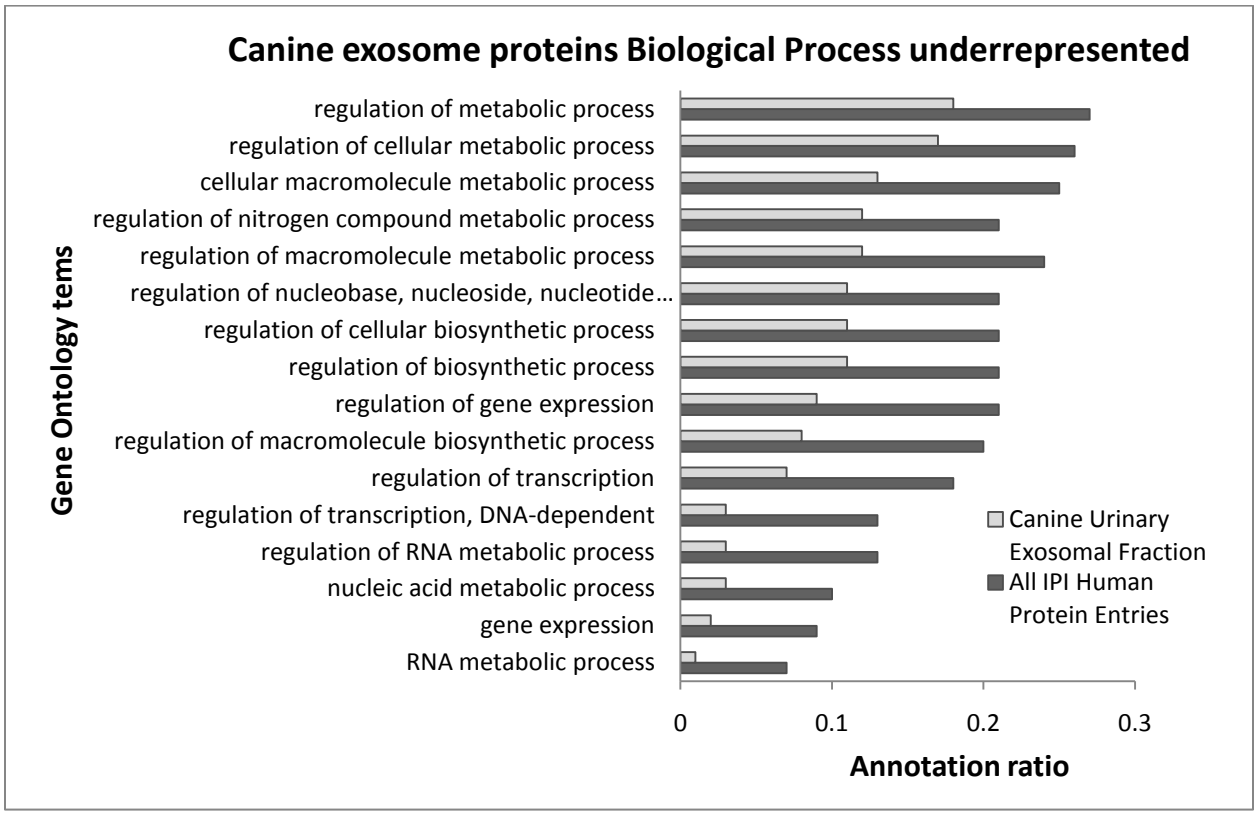

Figure 3.12. All GO Biological Process terms that are significantly under-represented (P < 0.001) in the canine exosomal fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

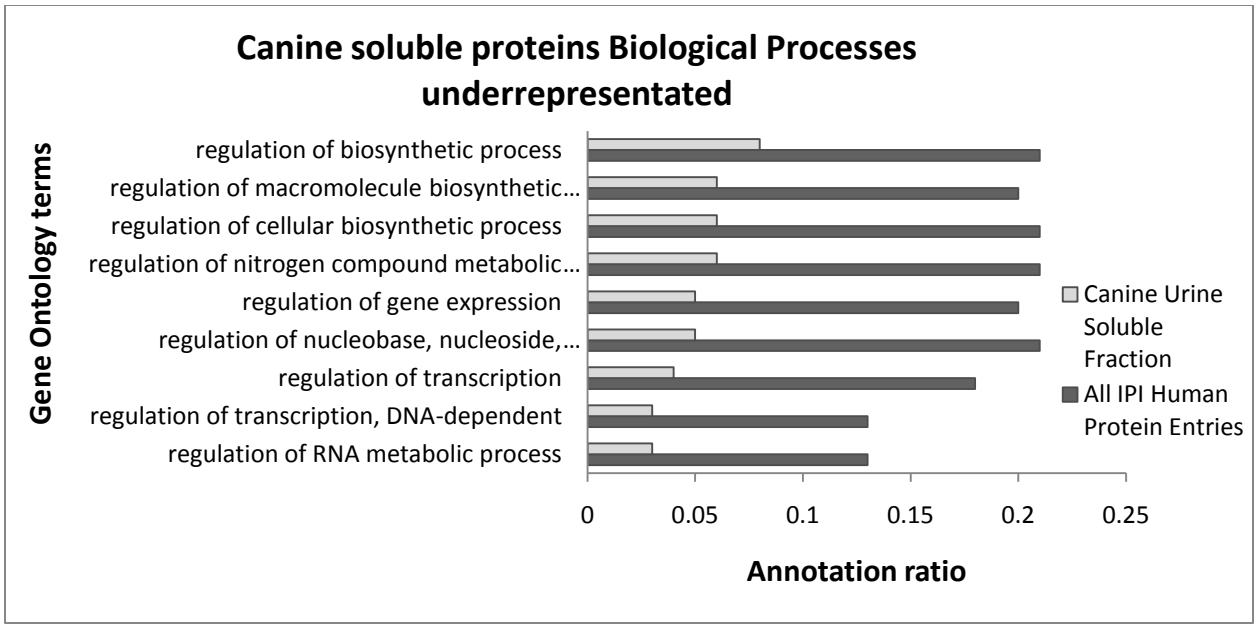

Figure 3.13. All GO Biological Process terms that are significantly under-represented ($P < 0.001$) in the canine soluble fraction in comparison to the total IPI human proteome are displayed here. The annotation ratio is calculated as previously described in Fig. 3.2.

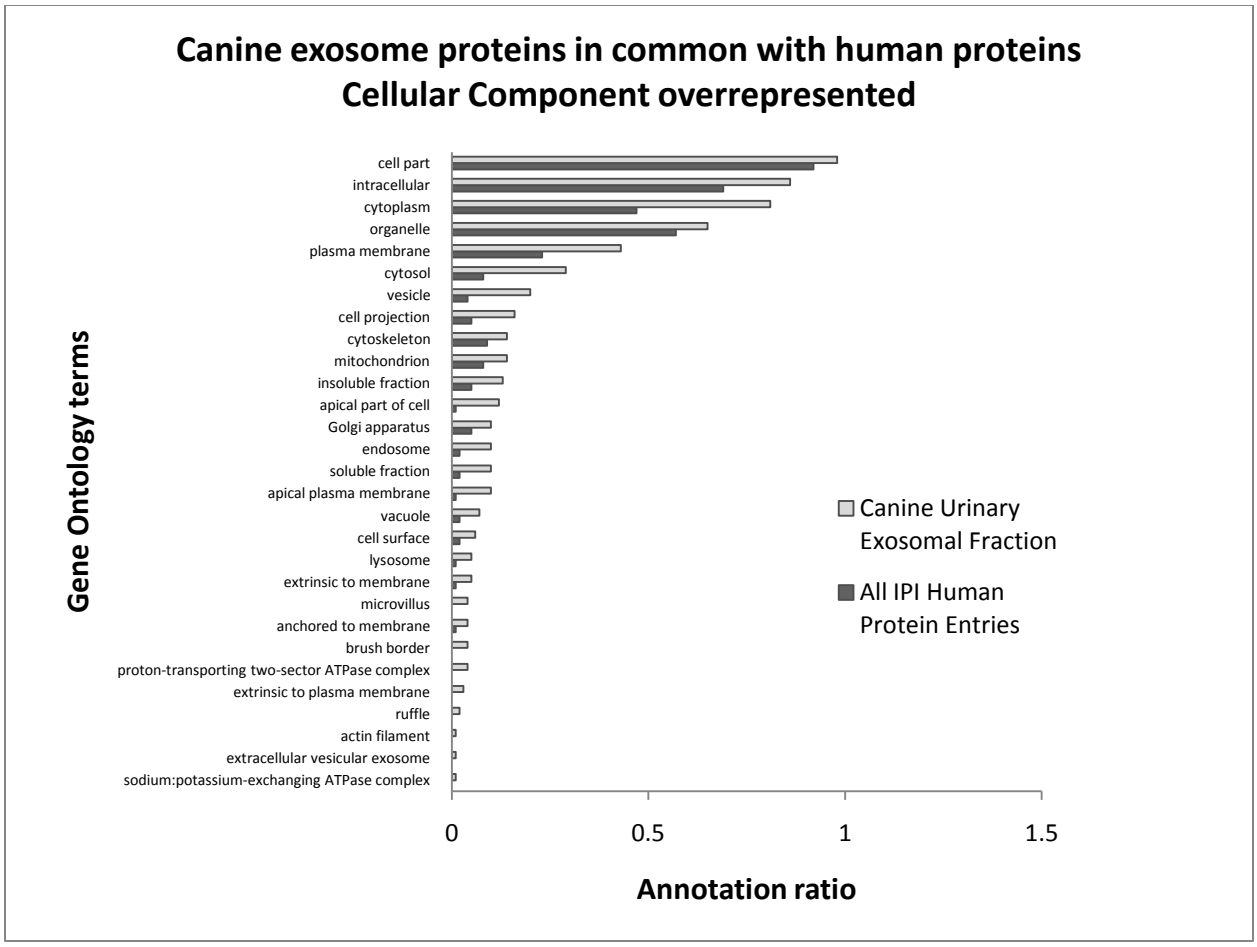

Figure 3.14. A selection of major annotated GO Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine exosomal fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig 2.2.

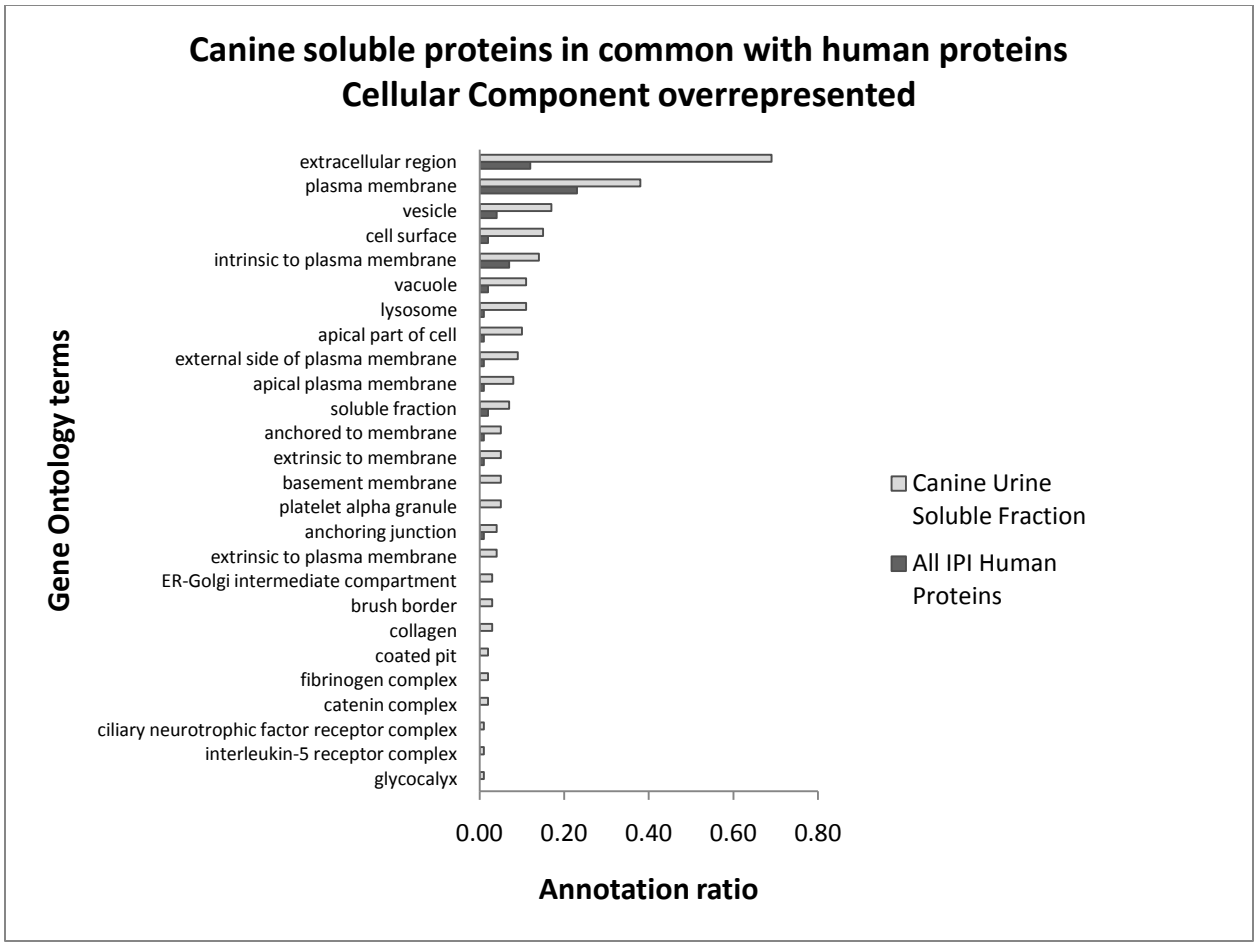

Figure 3.15. A selection of major annotated GO Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine soluble fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

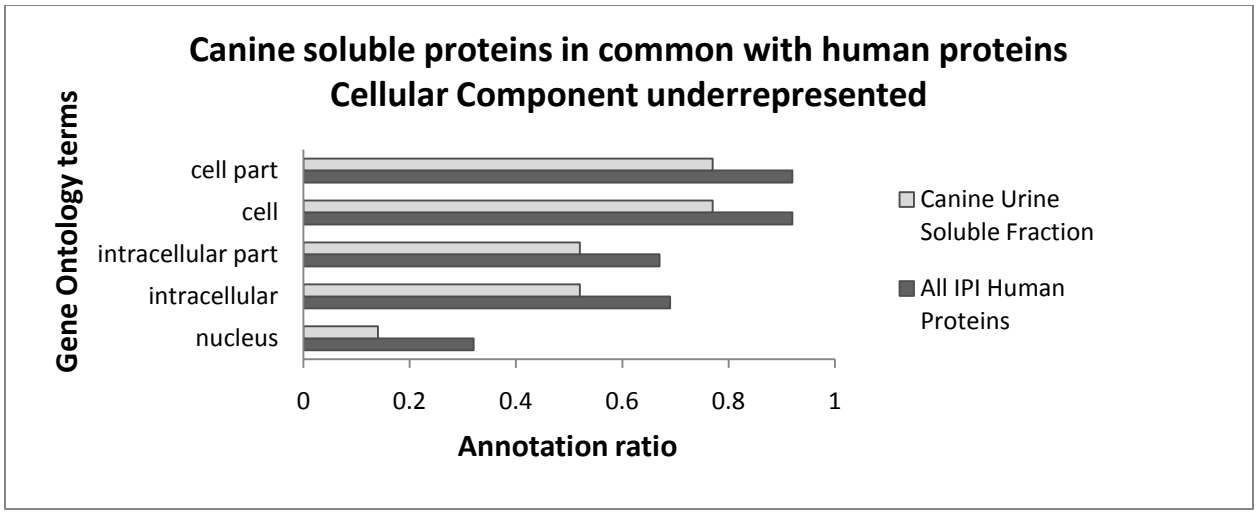

Figure 3.16. All annotated GO Cellular Component terms that are significantly under-represented ($P < 0.001$) in the canine soluble fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

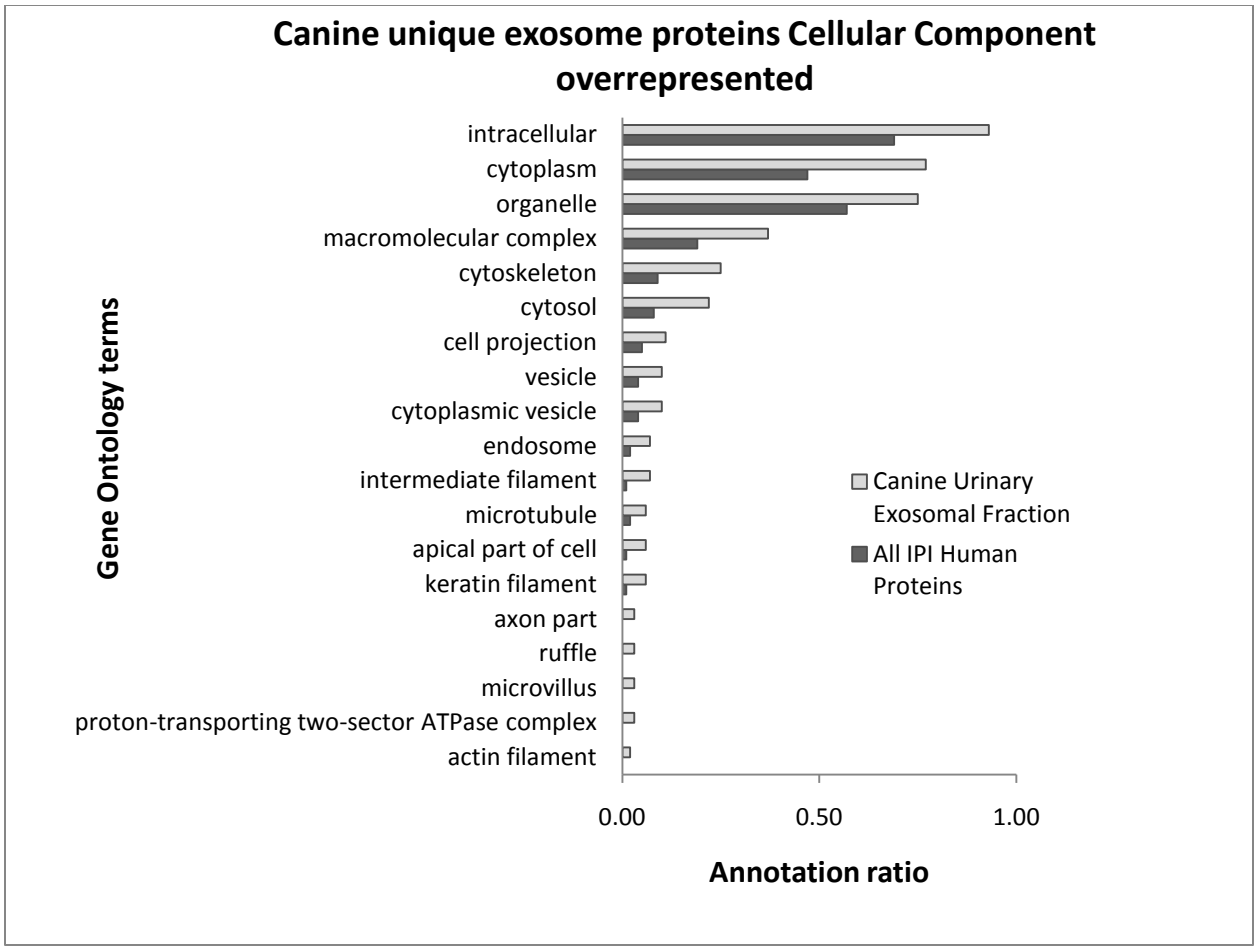

Figure 3.17. A selection of major annotated GO Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine exosomal fraction comprised of proteins that are unique to the canine urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

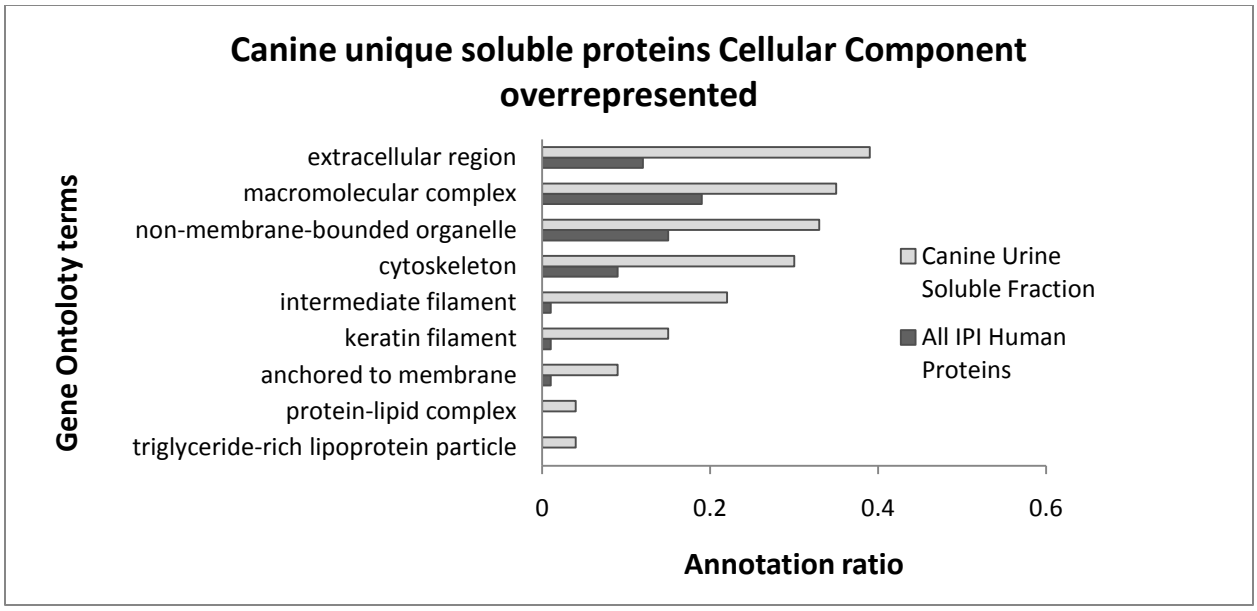

Figure 3.18. A selection of major annotated GO Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine soluble fraction comprised of proteins that are unique to the canine urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

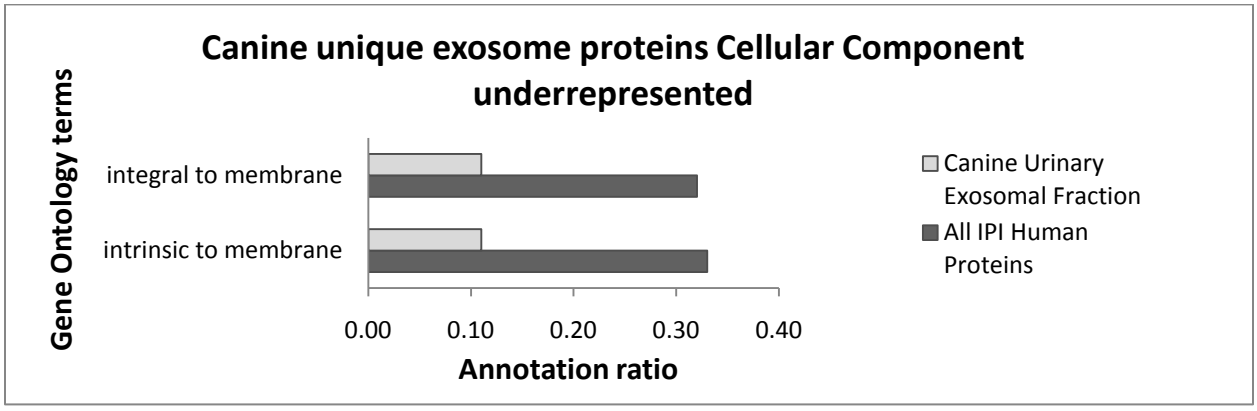

Figure 3.19. All annotated GO Cellular Component terms that are significantly under-represented ($P < 0.001$) in the canine exosomal fraction comprised of proteins that are unique to the canine urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

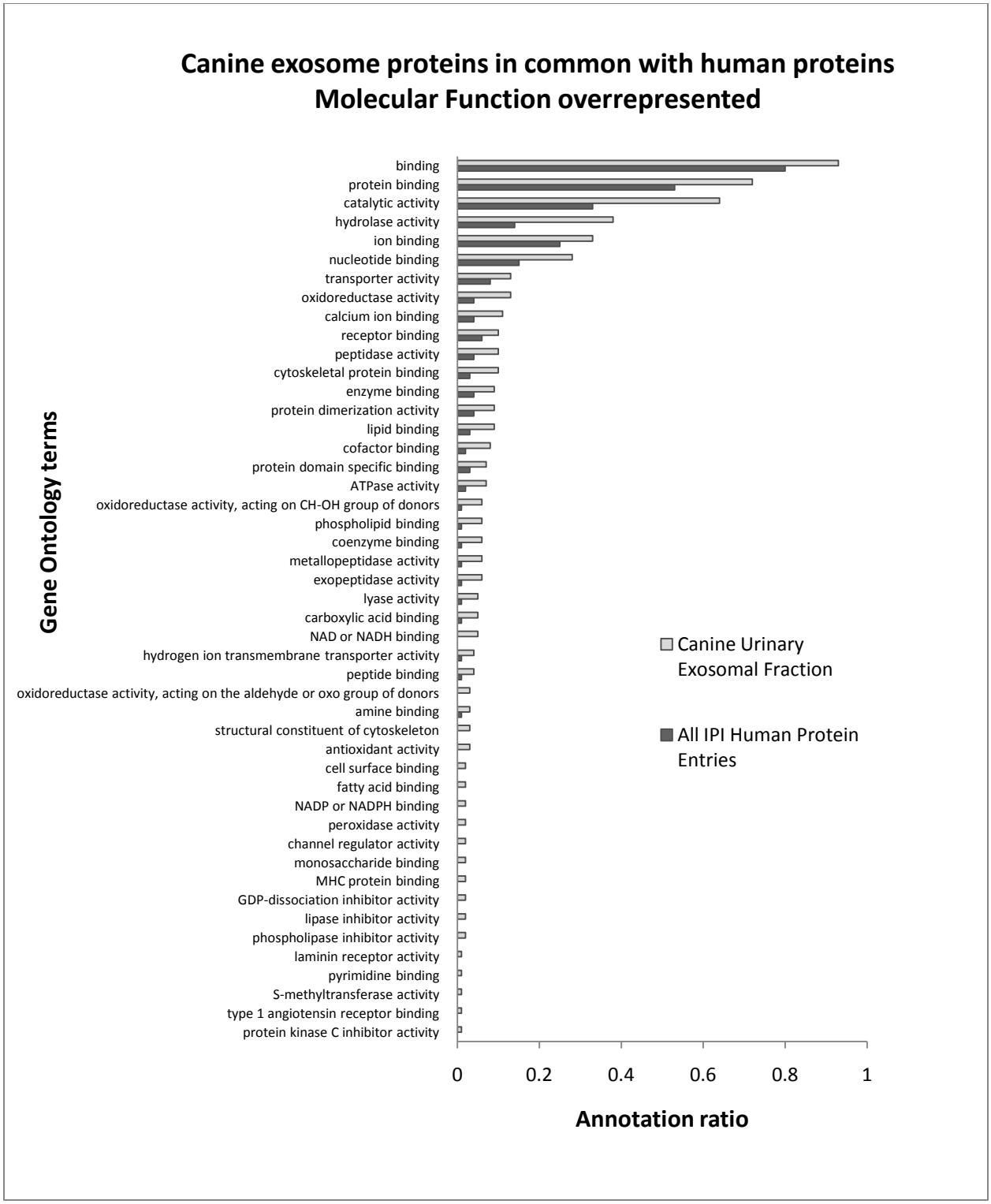

Figure 3.20. A selection of major annotated GO Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine exosomal fraction comprised of

proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

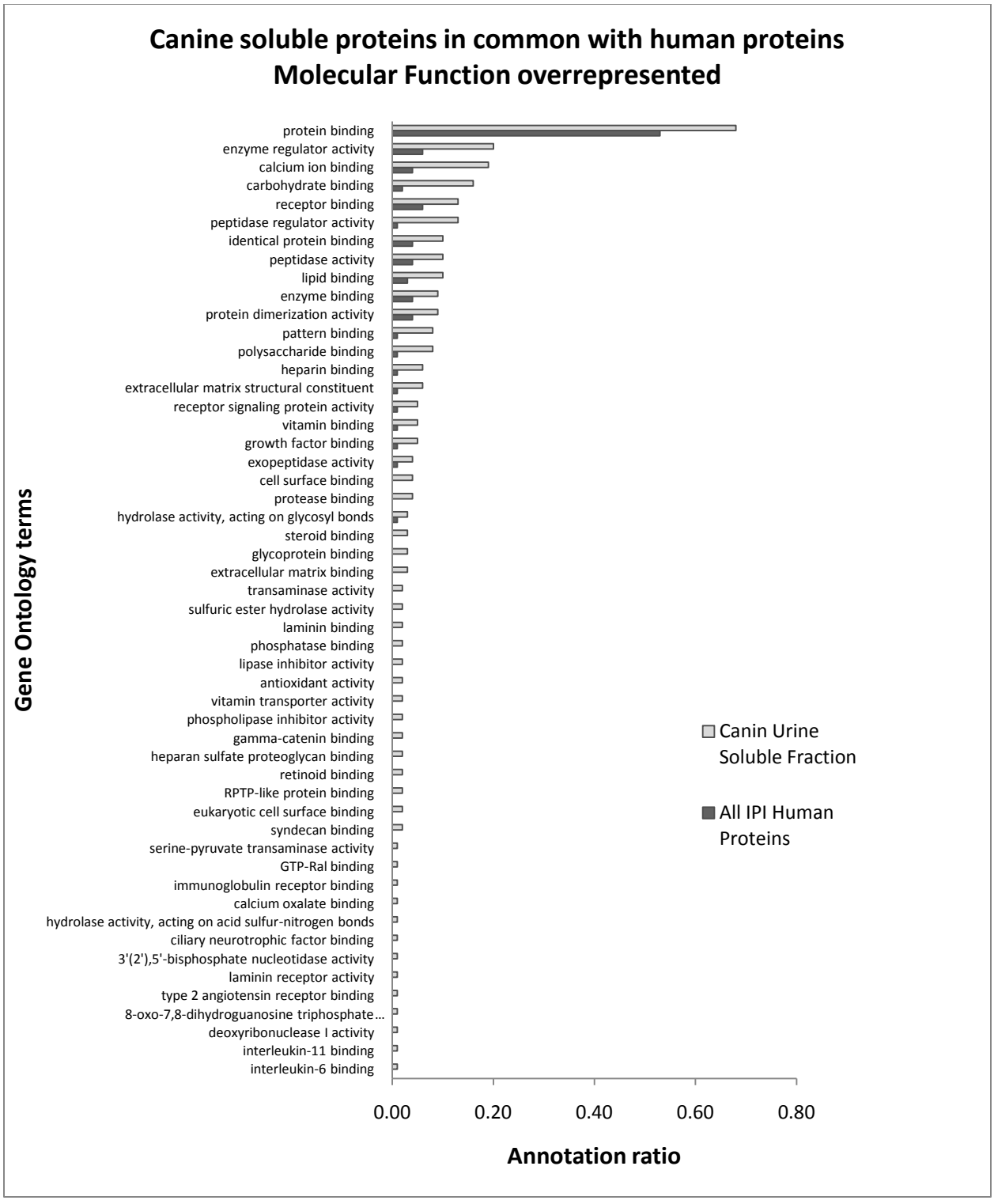

Figure 3.21. A selection of major annotated GO Cellular Component terms that are significantly over-represented ($P < 0.001$) in the canine soluble fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI

human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

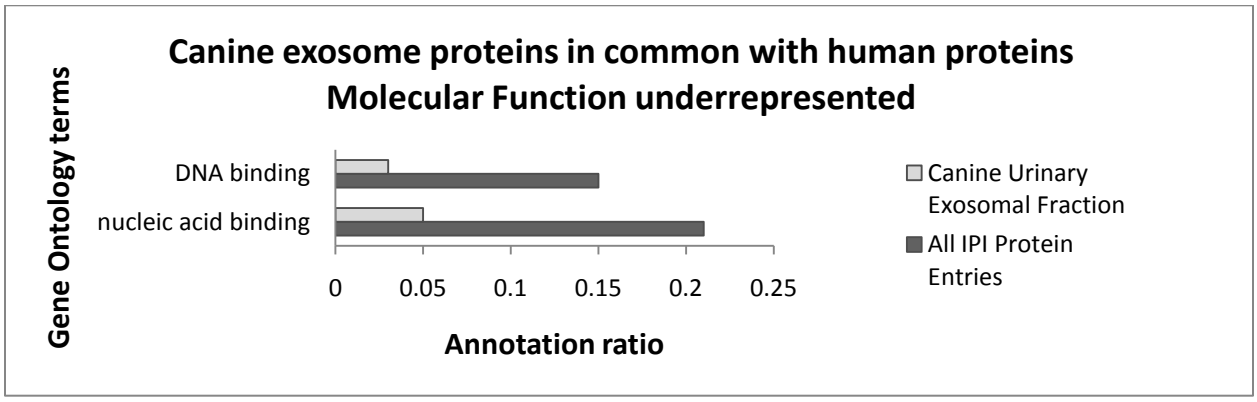

Figure 3.22. All annotated GO Molecular Function terms that are significantly under-represented ($P < 0.001$) in the canine exosomal fraction comprised of proteins found in duplicate within the human urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

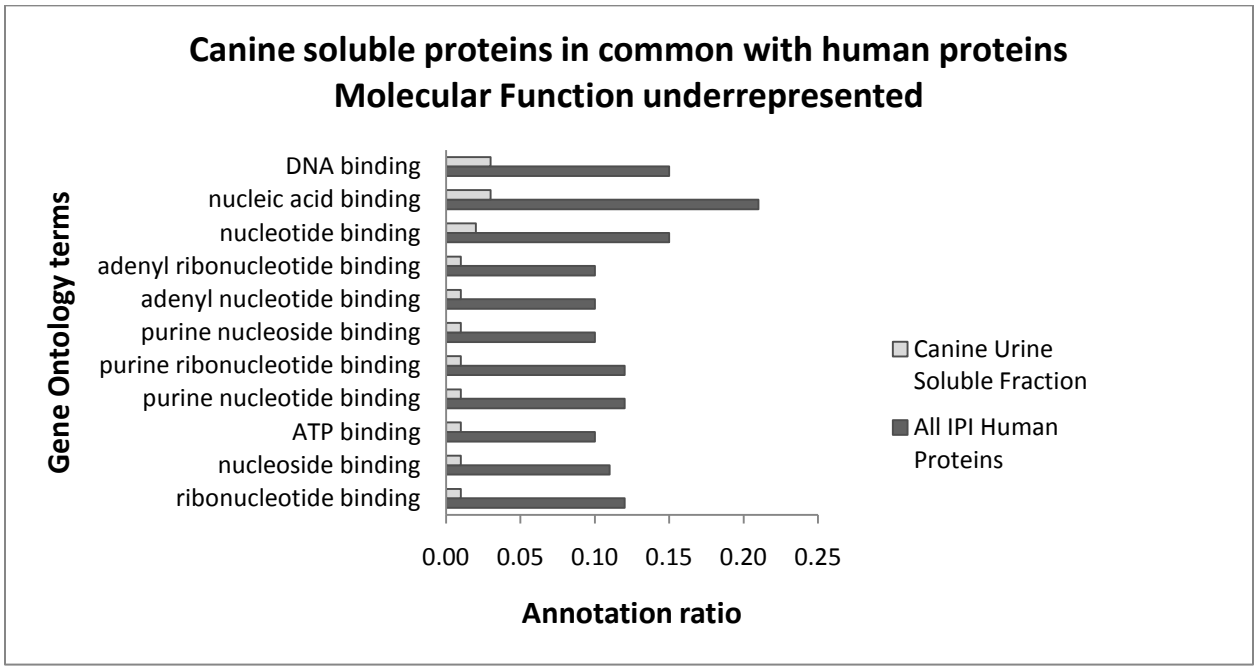

Figure 3.23. All annotated GO Molecular Function terms that are significantly under-represented ($P < 0.001$) in the canine soluble fraction comprised of proteins found in duplicate within the human urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

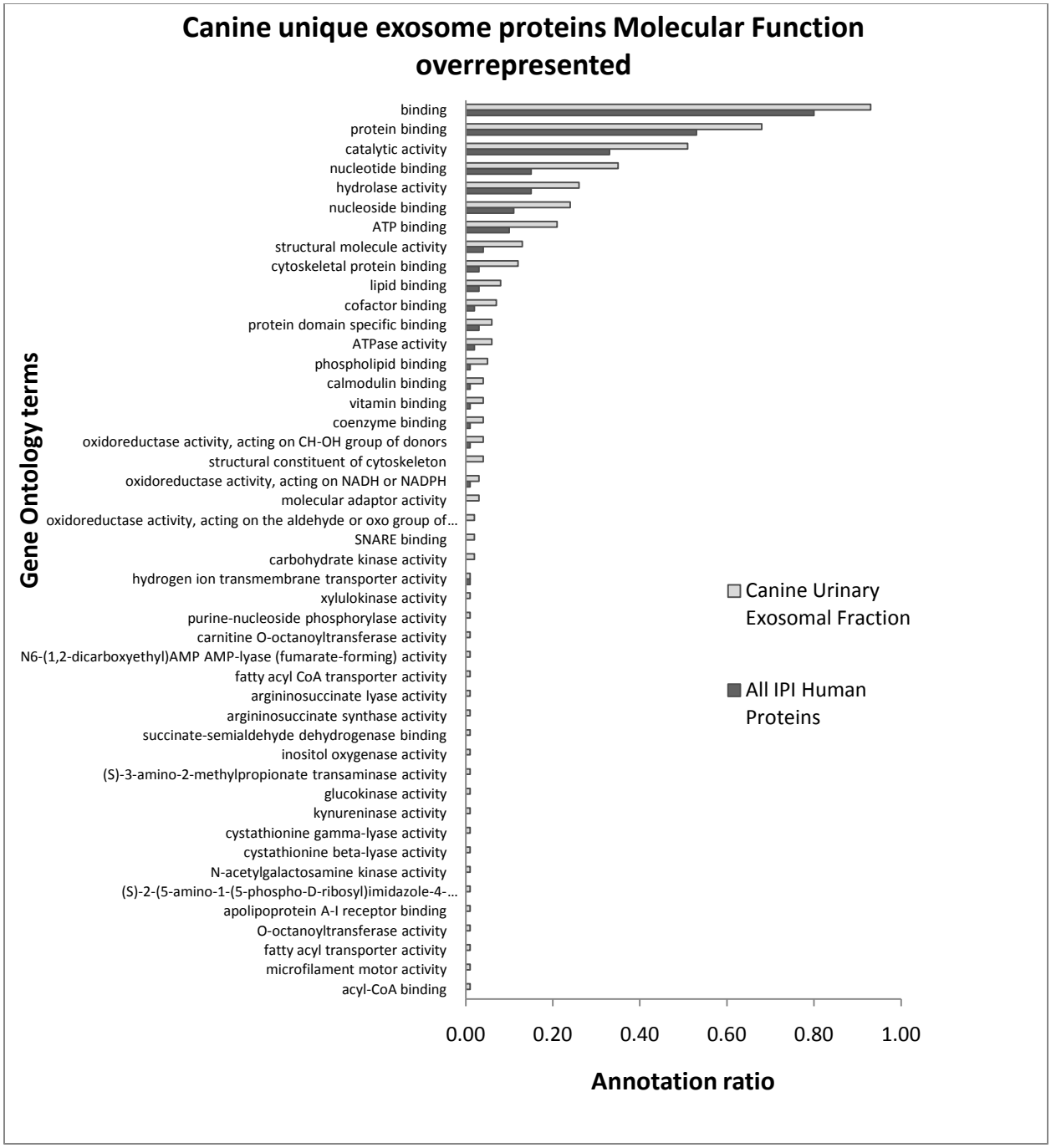

Figure 3.24. A selection of major annotated GO Molecular Function terms that are significantly over-represented ($P < 0.001$) in the canine exosomal fraction comprised of proteins that are unique to the canine urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

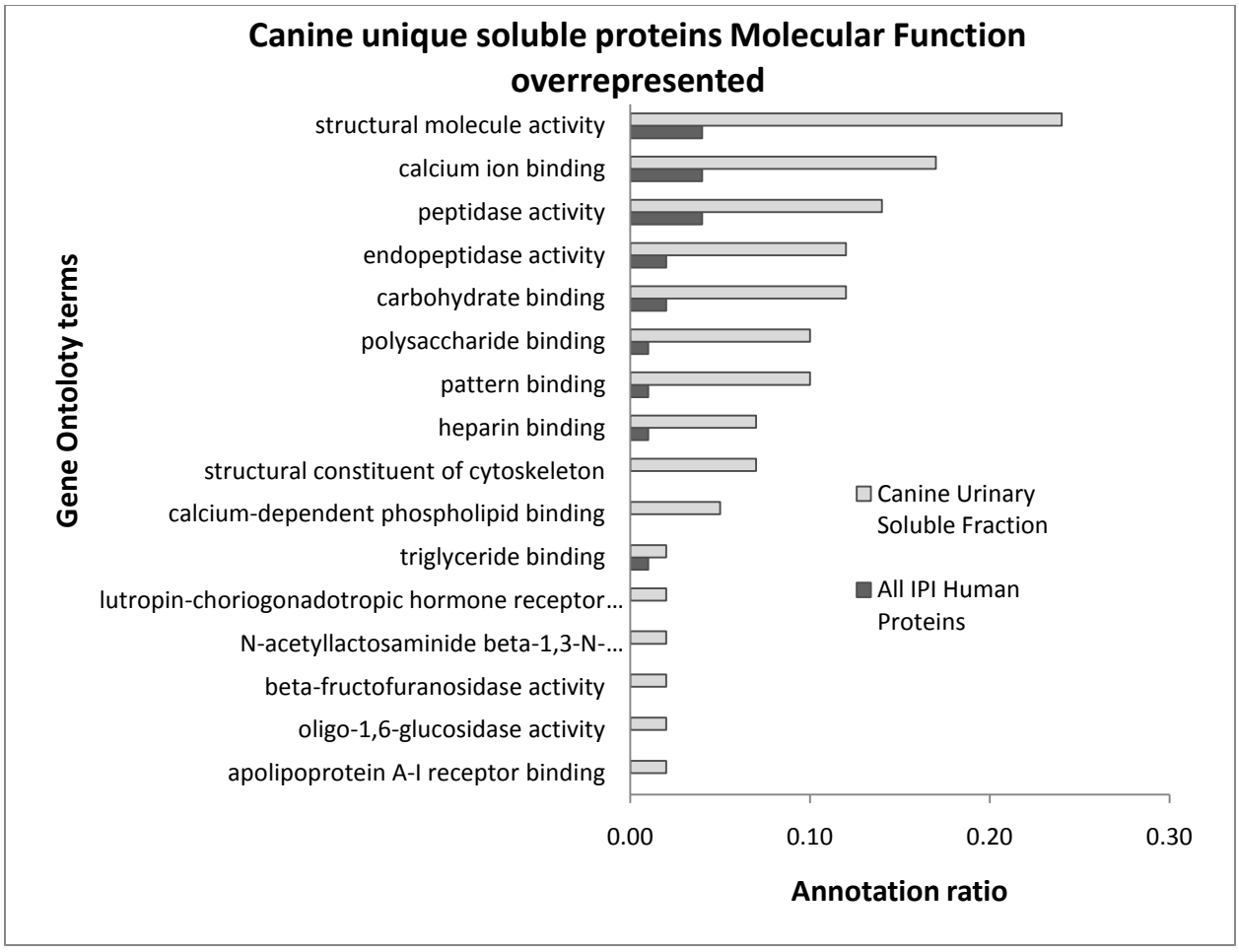

Figure 3.25. A selection of major annotated GO Molecular Function terms that are significantly over-represented ($P < 0.001$) in the canine urinary soluble fraction comprised of proteins that are unique to the canine urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

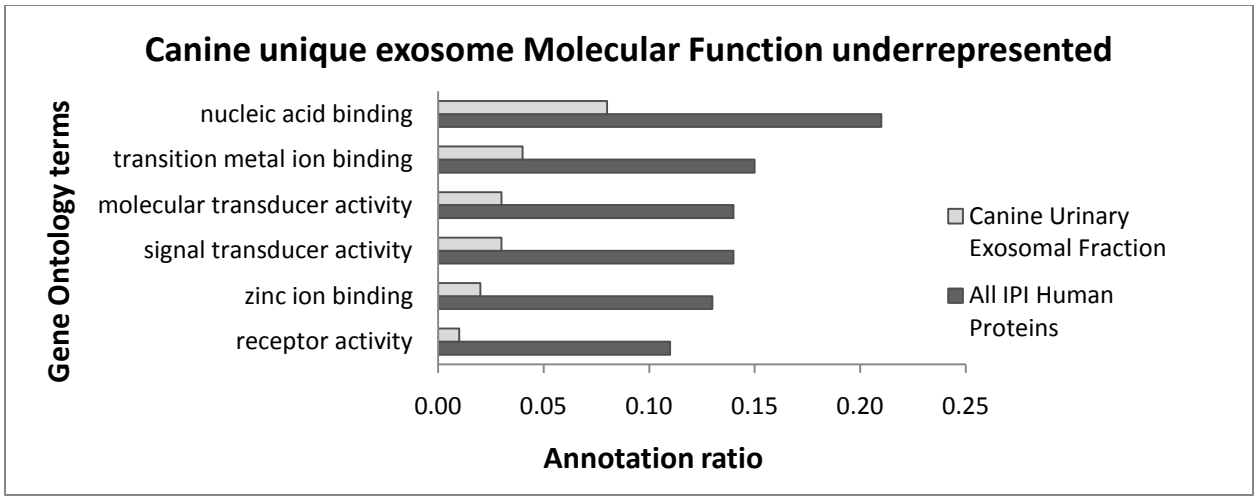

Figure 3.26. All annotated GO Molecular Function terms that are significantly under-represented ($P < 0.001$) in the canine exosomal fraction comprised of proteins that are unique to the canine urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

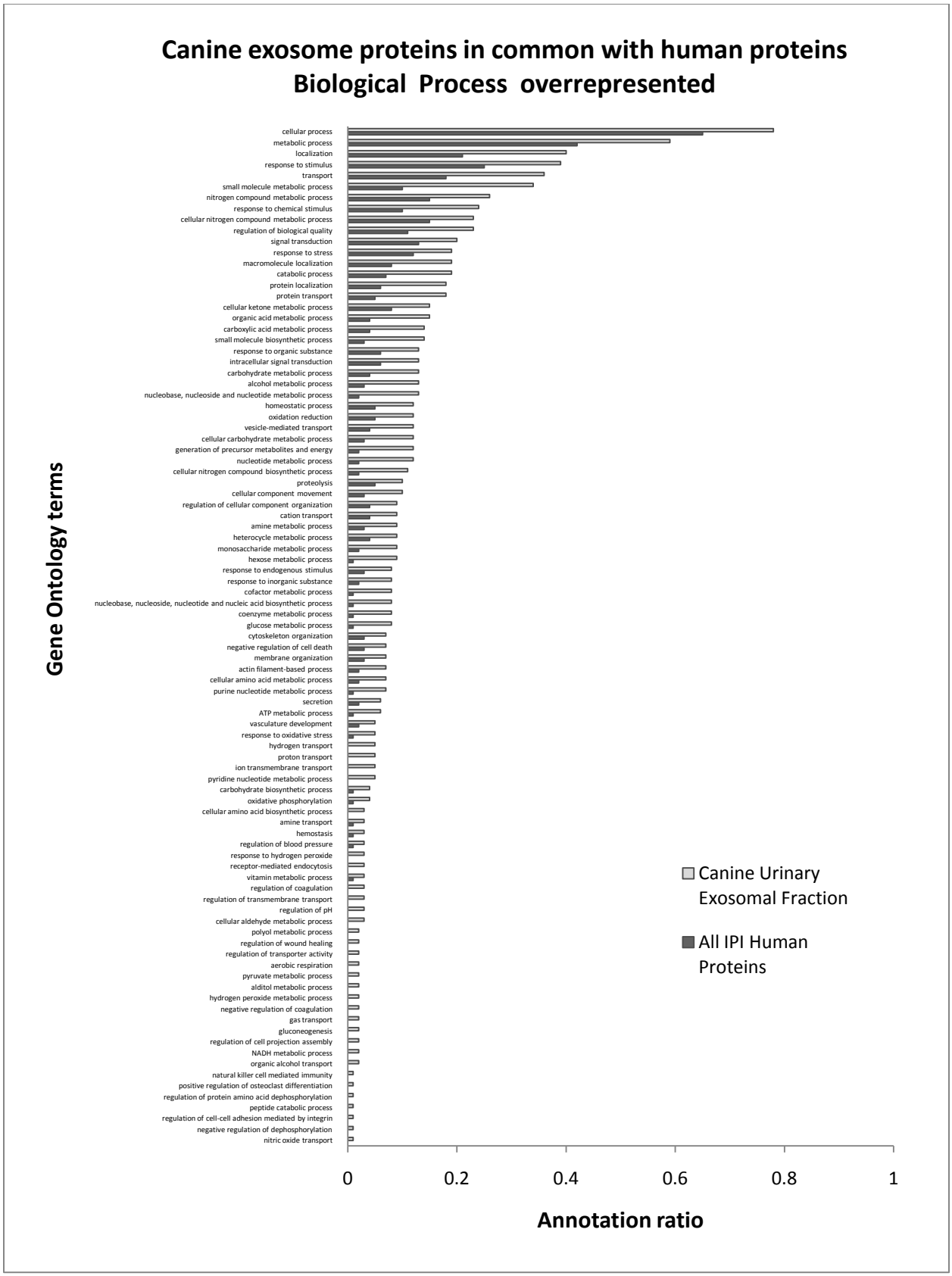
Canine exosome proteins in common with human proteins
Biological Process overrepresented
Gene Ontology terms
cellular process
metabolic process
localization
response to stimulus
transport
small molecule metabolic process
nitrogen compound metabolic process
response to chemical stimulus
cellular nitrogen compound metabolic process
regulation of biological quality
signal transduction
response to stress
macromolecule localization
catabolic process
protein localization
protein transport
cellular ketone metabolic process
organic acid metabolic process
carboxylic acid metabolic process
small molecule biosynthetic process
response to organic substance
intracellular signal transduction
carbohydrate metabolic process
alcohol metabolic process
nucleobase, nucleoside and nucleotide metabolic process
homeostatic process
oxidation reduction
vesicle-mediated transport
cellular carbohydrate metabolic process
generation of precursor metabolites and energy
nucleotide metabolic process
cellular nitrogen compound biosynthetic process
proteolysis
cellular component movement
regulation of cellular component organization
cation transport
amine metabolic process
heterocycle metabolic process
monosaccharide metabolic process
hexose metabolic process
response to endogenous stimulus
response to inorganic substance
cofactor metabolic process
nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process
coenzyme metabolic process
glucose metabolic process
cytoskeleton organization
negative regulation of cell death
membrane organization
actin filament-based process
cellular amino acid metabolic process
purine nucleotide metabolic process
secretion
ATP metabolic process
vasculature development
response to oxidative stress
hydrogen transport
proton transport
ion transmembrane transport
pyridine nucleotide metabolic process
carbohydrate biosynthetic process
oxidative phosphorylation
cellular amino acid biosynthetic process
amine transport
hemostasis
regulation of blood pressure
response to hydrogen peroxide
receptor-mediated endocytosis
vitamin metabolic process
regulation of coagulation
regulation of transmembrane transport
regulation of pH
cellular aldehyde metabolic process
polyol metabolic process
regulation of wound healing
regulation of transporter activity
aerobic respiration
pyruvate metabolic process
alditol metabolic process
hydrogen peroxide metabolic process
negative regulation of coagulation
gas transport
gluconeogenesis
regulation of cell projection assembly
NADH metabolic process
organic alcohol transport
natural killer cell mediated immunity
positive regulation of osteoclast differentiation
regulation of protein amino acid dephosphorylation
peptide catabolic process
regulation of cell-cell adhesion mediated by integrin
negative regulation of dephosphorylation
nitric oxide transport
0
0.2
0.4
0.6
0.8
1
Annotation ratio
Canine Urinary Exosomal Fraction
All IPI Human Proteins

Figure 3.27. A selection of major annotated GO Biological Process terms that are significantly over-represented ($P < 0.001$) in the canine exosomal fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

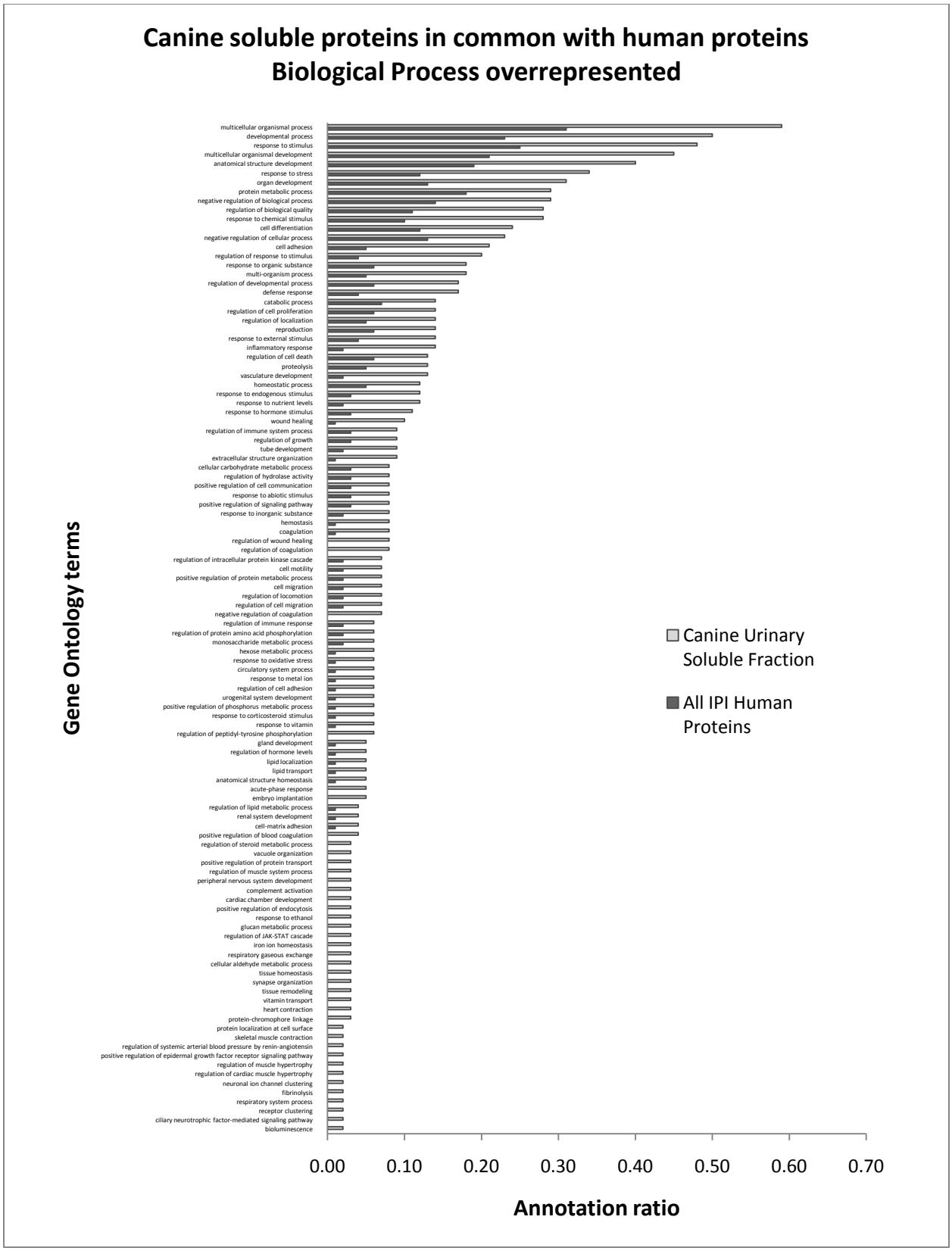
Canine soluble proteins in common with human proteins
Biological Process overrepresented
Gene Ontology terms
multicellular organismal process
developmental process
response to stimulus
multicellular organismal development
anatomical structure development
response to stress
organ development
protein metabolic process
negative regulation of biological process
regulation of biological quality
response to chemical stimulus
cell differentiation
negative regulation of cellular process
cell adhesion
regulation of response to stimulus
response to organic substance
multi-organism process
regulation of developmental process
defense response
catabolic process
regulation of cell proliferation
regulation of localization
reproduction
response to external stimulus
inflammatory response
regulation of cell death
proteolysis
vasculature development
homeostatic process
response to endogenous stimulus
response to nutrient levels
response to hormone stimulus
wound healing
regulation of immune system process
regulation of growth
tube development
extracellular structure organization
cellular carbohydrate metabolic process
regulation of hydrolase activity
positive regulation of cell communication
response to abiotic stimulus
positive regulation of signaling pathway
response to inorganic substance
hemostasis
coagulation
regulation of wound healing
regulation of coagulation
regulation of intracellular protein kinase cascade
cell motility
positive regulation of protein metabolic process
cell migration
regulation of locomotion
regulation of cell migration
negative regulation of coagulation
regulation of immune response
regulation of protein amino acid phosphorylation
monosaccharide metabolic process
hexose metabolic process
response to oxidative stress
circulatory system process
response to metal ion
regulation of cell adhesion
urogenital system development
positive regulation of phosphorus metabolic process
response to corticosteroid stimulus
response to vitamin
regulation of peptidyl-tyrosine phosphorylation
gland development
regulation of hormone levels
lipid localization
lipid transport
anatomical structure homeostasis
acute-phase response
embryo implantation
regulation of lipid metabolic process
renal system development
cell-matrix adhesion
positive regulation of blood coagulation
regulation of steroid metabolic process
vacuole organization
positive regulation of protein transport
regulation of muscle system process
peripheral nervous system development
complement activation
cardiac chamber development
positive regulation of endocytosis
response to ethanol
glucan metabolic process
regulation of JAK-STAT cascade
iron ion homeostasis
respiratory gaseous exchange
cellular aldehyde metabolic process
tissue homeostasis
synapse organization
tissue remodeling
vitamin transport
heart contraction
protein-chromophore linkage
protein localization at cell surface
skeletal muscle contraction
regulation of systemic arterial blood pressure by renin-angiotensin
positive regulation of epidermal growth factor receptor signaling pathway
regulation of muscle hypertrophy
regulation of cardiac muscle hypertrophy
neuronal ion channel clustering
fibrinolysis
respiratory system process
receptor clustering
ciliary neurotrophic factor-mediated signaling pathway
bioluminescence
Canine Urinary Soluble Fraction
All IPI Human Proteins
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
Annotation ratio

Figure 3.28. A selection of major annotated GO Biological Process terms that are significantly over-represented (P < 0.001) in the canine urinary soluble fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

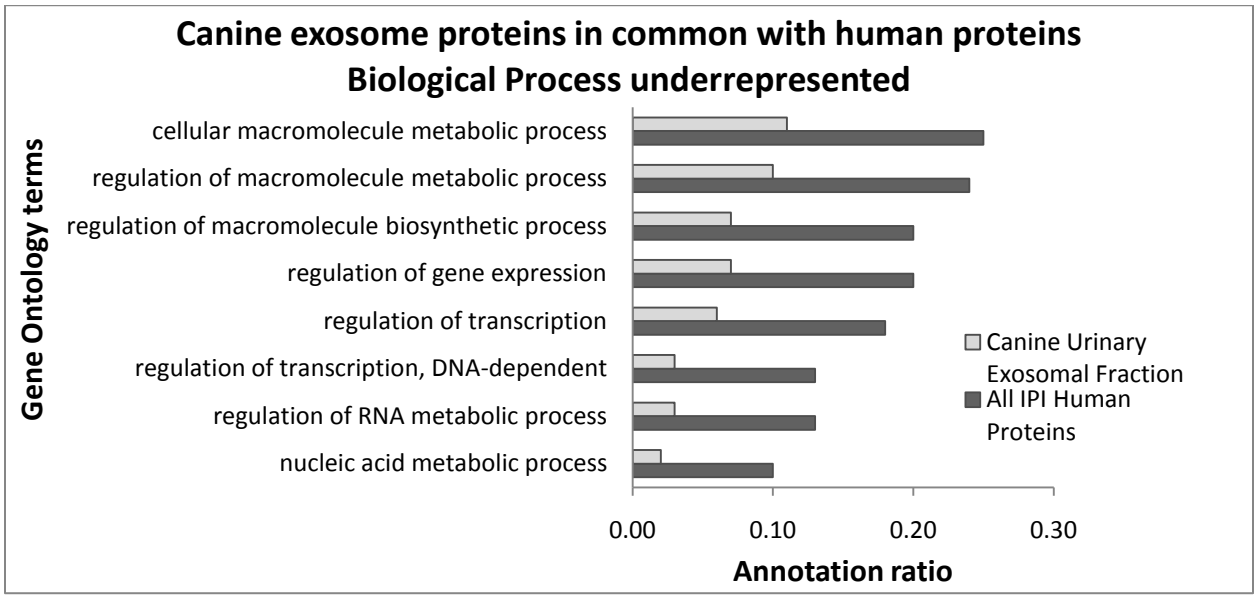

Figure 3.29. All annotated GO Biological Process terms that are significantly under-represented (P < 0.001) in the canine exosomal fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

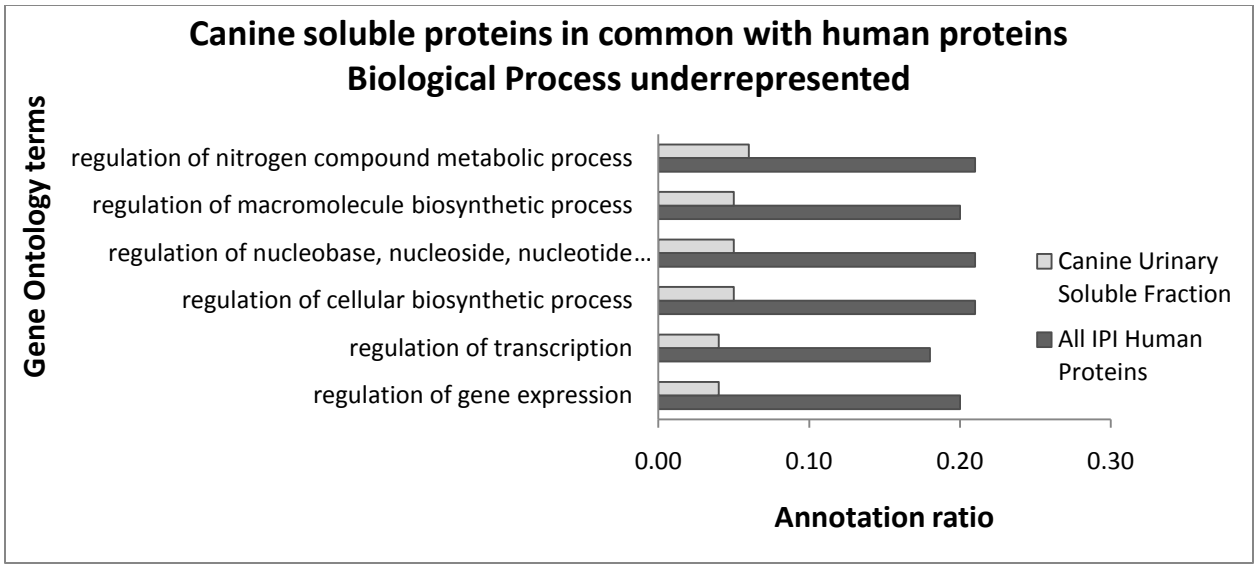

Figure 3.30. All annotated GO Biological Process terms that are significantly under-represented ($P < 0.001$) in the canine urinary soluble fraction comprised of proteins found in duplicate within the human urine proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

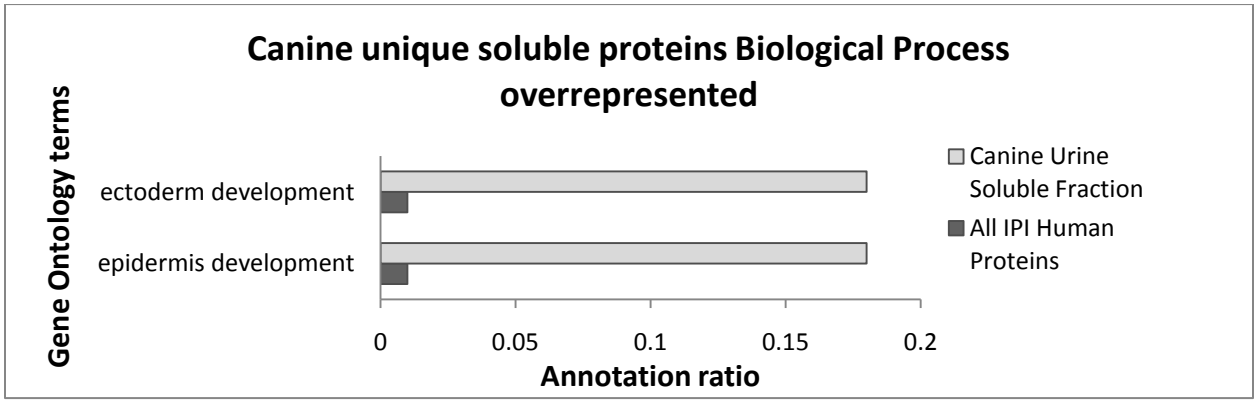

Figure 3.31. All annotated GO Biological Process terms that are significantly over-represented ($P < 0.001$) in the canine urine soluble fraction comprised of proteins unique to the canine urinary proteome are compared to the total IPI human proteome here. The annotation ratio is calculated as previously described in Fig. 3.2.

Table 3.1. Sixty proteins associated with various human diseases, and found in human urine, were also identified in normal canine urine. Many of the diseases listed below are currently thought to be unique to humans, however several of these human diseases have a canine counterpart.

URINARY PROTEINS RELATED TO HUMAN DISEASES DISCOVERED IN NORMAL CANINE URINE			
GENE NAME	**PROTEIN NAME**	**RELATED HUMAN DISEASE**	**CANINE URINARY SOLUBLE (S) V. EXOSOME (EX) FRACTION**
ACE	**Angiotensin 1-coverting enzyme isoform 1**	**Hypertension**	**S & EX**
ACE 2	**Angiotensin 1-coverting enzyme 2**	**Hypertension**	**S & EX**
ACY1	**Aminoacylase 1**	**Aminoacylase 1 deficiency**	**EX**
AHCY	**S-adenosylhomocysteine**	**Hypermethioninemia**	**EX**
ALB	**Albumin precursor**	**Dysalbuminemic hyperthyroxinemia, hyperthyroxinemia - dysalbuminemic analbuminemia bisalbuminemia**	**S & EX**
ALDOA	**[Fructose-bisphosphate] aldolase A (aka "lung cancer antigen")**	**Aldolase deficiency of red cells; myopathy & hemolytic anemia**	**EX**
AMN	**Amnionless protein precursor**	**Megaloblastic anemia 1**	**EX**
APOA 1	**Apolipoprotein A-1 precursor**	**Primary hypoalphalipoproteinemia**	**EX**
AQP 1	**Aquaporin 1**	**AQP1 deficiency, Colton-Null**	**EX**
ARL6	**ADP-ribosylation factor-(like) 6**	**Bardet-Biedl syndrome 3**	**EX**

ASL	**Argininosucinate lyase isoform 3; [also got isoform 1]**	**Argoninosuccinic aciduria**	**EX**
ASS1	**Argininosucinate synthetase 1 - [we got 3]**	**Citrullinemia**	**EX**
ATP6V0A4	**ATPase, H^+ transporting, lysosomal V0 subunit a4**	**Renal tubular acidosis, distal, autosomal recessive**	**EX**
ATP6V1B1	**Atpase, H+ transporting, lysosomal 56/58 kDa, V1 subunit B1**	**Renal tubular acidosis, distal, w/ progressive deafness**	**EX**
B4GALT1	**UDP-Gal:βGlcNac β 1,4-galactosyltransferase 1, membrane bound form**	**Congenital disorder of glycosylation type IId**	**S**
CA2	**Carbonic anhydrase II**	**Autosomal recessive syndrome of osteopetrosis with renal tubular acidosis**	**EX**
CA4	**Carbonic anhydrase IV precursor**	**Proximal renal tubular acidosis**	**EX**
CHMP2B	**Chromatin modifying protein 2B**	**Frontotemporal dementia, chromosome 3-linked**	**EX**
COL6A3	**α 3 type VI collagen (isoform 5 precursor)**	**Ullrich congenital muscular dystrophy**	**S & EX**
CRYM	**Crystalline, μ isoform (1), [we got 3]**	**Autosomal dominant nonsyndromic deafness**	**EX**
CTH	**Cystathionase isoform (2), [we got 1]**	**Cystathioninuria**	**EX**
CTSA	**Cathepsin A (precursor)**	**Galactosialidosis**	**S**
CTSD	**Cathepsin D preproprotein**	**Papillon-Lefevre syndrome**	**S & EX**
DDAH1	***Dimethylarginine dimethylaminohydrolase 1***	***Hypertension***	***EX***
DNM2	**Dynamin 2 (isoform 4), [we got isoform 2 isoform 22]**	**Charcot-Marie-Tooth neuropathy**	**EX**
DYSF	**Dysferlin**	**Miyoshi myopathy**	**EX**
DPYS	**Dihydropyrimidinase [isoform 1]**	**Dihydropyrimidinuria**	**EX**

EFEMP1	**EGF-containing fibulin-like extracellular matrix**	**Doyne Honeycomb retinal dystrophy**	**S**
ELA2	**Elastase (2), neutrophil preprotein**	**Cyclic hematopoiesis**	**S**
ENPEP	***Glutamyl aminopeptidase (aminopeptidase A)***	***Hypertension***	**S & EX**
FBP1	**Fructose-1, 6-bisphosphatase 1**	**Fructose-1, 6-bisphosphatase deficiency**	**EX**
FGA	**Fibrinogen, α (polypeptide isoform α-E) preprotein**	**Renal amyloidosis, Dysfibrinogenemia**	**S**
FTL	**Ferritin, light (polypeptide), [chain]**	**Iron overload, autosomal dominant**	**EX**
GAA	**[Lysosome] (Acid) α-glucosidase preprotein**	**Infantile-onset glycogen storage disease Type II**	**S**
GPI	**Glucose [6-]phosphate isomerase**	**Chronic hemolytic anemia duet to GPI deficiency**	**EX**
GSS	**Glutathione synthetase**	**Glutathione synthetase deficiency**	**EX**
HPD	**4-Hydroxyphenylpyruvate dioxygenase**	**Tyrosinemia type III**	**EX**
HPGD	***Hydroxyprostaglandin dehydrogenase 15-(NAD)*; [carbonyl reductase]**	**Hypertension**	**EX**
KLK1	**Kallikrein 1 preprotein**	**Decreased urinary activity of kallikrein**	**S**
LGALS3	**Galectin 3 [binding precursor]**	**Lymphocyte function-associated antigen 1**	**S & EX**
LRRK2	**[Ig superfamily containing] Leucine rich repeat (kinase 2)**	**Parkinson disease**	**S**
LYZ	**Lysozyme precursor**	**Familial visceral amyloidosis**	**S**
MME	***Membrane metallo-endopeptidase (neprilysin)***	***Hypertension***	**EX**
MYH14	**Myosin[-6] (heavy chain 14) isoform 1; we also got calmodulin 1**	**Autosomal dominant nonsyndromic sensorineural deafness**	**EX**

NDRG1	**N-myc downstream regulated gene 1**	**Charcot-Marie-Tooth disease type 4D**	**EX**
NEB	**Nebulin [related anchoring protein isoform S isoform 2]**	**Nemalin myopathy**	**EX**
PARK7	**DJ-1 protein [isoform 1]**	**Parkinson disease**	**EX**
PHGDH	**[D-3] Phosphoglycerate dehydrogenase**	**Phosphoglycerate dehydrogenase deficiency**	**EX**
PKLR	**Pyruvate kinase (liver, and RBC isoform) - [we got isozymes M1/M2]**	**Pyruvate kinase deficiency**	**EX**
PRKCH	**Protein kinase C (ή) [& caseine kinase substrate in neurons 3 isoforms]**	**Cerebral infarction**	**EX**
PROM1	**Prominin 1 [precursor]**	**Autosomal recessive retinal degeneration**	**EX**
RAB3GAP1	**(RAB3) [RAS or Rho] GTPase-activating protein**	**Warburg micro syndrome**	**EX**
RBP4	**Retinol-binding protein 4, plasma precursor**	**Retinal-binding protein deficiency**	**S**
RDX	**Radixin [-moesin binding phosphoprotein 50]**	**Autosomal recessive deafness**	**EX**
SCL3A1	***Solute carrier family 3, member 1***	***Cystinuria***	**EX**
SCL12A3	***Solute carrier family 12, member 3***	***Gitelman syndrome***	**EX**
TF	**Transferrin**	**Alzheimer disease**	**S**
TPP1	**Tripeptidyl-peptidase 1 preprotein**	**Ceroid lipofusinosis neuronal 2**	**EX**
TSG101	**Tumor susceptibility gene 101**	**Breast Cancer**	**EX**
UMOD	***Uromodulin precursor***	***Medullary cystic kidney disease-2, Familial juvenile hypruricemic nephropathy***	**S & EX**

Table 3.2. Several known urinary biomarkers for renal disease, as well as biomarkers in various stages of development, are listed below. A brief description of the protein and its utility in diagnosing renal disease is also given. It is noted whether the protein was found in the soluble or exosomal urinary fraction of our study. The (*) denotes proteins included in a panel of seven total proteins that have recently been approved by the FDA for testing nephrotoxicity in drug development.[2] The (+) denotes novel urinary biomarkers discovered via proteomic methods.[96]

URINE BIOMARKERS and CANDIDATE BIOMARKERS FOR RENAL DISEASE FOUND IN NORMAL CANINE URINE			
PROTEIN	**DESCRIPTION**	**RENAL DISEASE**	**CANINE URINARY SOLUBLE V. EXOSOME FRACTION**
Albumin (ALB)*	Comprises half of the blood serum protein, where ALB functions as a major carrier protein. It is synthesized by the liver	Small amounts (<3-20 mg/dL) are excreted in normal human urine. Increased urine concentrations typically indicate glomerular damage, but can also indicate proximal tubular damage in humans and canines. Microalbuminuria may indicate early/subclinical renal disease in dogs.	Both soluble and exosomal fractions
Clusterin (CLU)*	Involved in cell death, tumor progression and neurodegenerative disorders, but actual functional role is unknown. CLU is thought to play an anti-apoptotic role in kidney injury[78]	Urinary CLU is thought to be renal specific and indicates general renal injury. Gene over expression occurs with glomerular, tubular and renal papillary damage. Recent toxicological study showed high diagnostic power for proximal tubular damage.[23]	Soluble fraction
Epidermal growth factor (EGF)	EGF is a potent mitogenic factor that is involved in growth, proliferation and differentiation of numerous cell types.	Thought to be involved in the regenerative process following acute renal injury in humans. Increased amounts in urine may indicate severity and show potential to predict recovery.[52]	Both soluble and exosomal fractions

Tamm-Horsfall Protein (THP, UMOD)	THP is the most abundant protein in urine and is expressed on the epithelium of the thick ascending limb. Multiple hypothesized functions include providing bacterial defense for the urinary tract and inhibiting calcium oxalate crystal formation.	Decreased urinary excretion has been linked to decreased GFR and chronic renal disease, as well as heritable diseases involving mutations of THP genes and leading to medullary cystic kidney disease and familial juvenile hyperuricemic nephropathy.[108]	Both soluble and exosomal fractions
Aquaporin 1 (AQP1)	AQP1 is an integral membrane protein located in the proximal convoluted tubule and distal thin limb epithelium (among other tissues). It is a water channel protein key to maintaining renal water balance.	AQP1 has been shown to be increased in the urine of humans with renal neoplasms and is down regulated following ischemic reperfusion injury to the kidneys.[65,88]	Exosomal fraction
Hemopexin (HPX)	HPX is a high affinity binder of heme and an acute phase protein. This protein may protect cells from oxidative stress.	As a protein with a molecular weight of 82 kDa, excessive amounts of this protein in the urine indicates glomerular disease. This protein, in conjunction with other urinary biomarkers, was recently used to help distinguish different types of glomerular damage.[105]	Soluble fraction
Retinol binding protein 4 (RBP4)	RBP4 is a carrier for retinol in blood.	This protein has been used in human medicine to diagnose proximal tubular dysfunction. The utility of this protein for this purpose is being explored in canines.[57]	Soluble fraction
Immunoglobulin-γ (IgG)	IgG is an antibody molecule with two antigen binding sites and is primarily involved in secondary immune responses.	As a higher molecular weight protein, urine IgG has been used to document glomerular damage.	Both soluble and exosomal fractions
γ-glutamyltransferase (GGT, GGT1)	GGT catalyzes the transfer of glutamyl moiety of glutathione to amino acids and dipeptide acceptors and is localized to the brush border of proximal tubular epithelial cells.	Increases in urinary GGT has been seen in dogs, rats and humans with proximal tubular (PT) damage.[15,19]	Exosomal fraction
Lysozyme (LYZ, aka neurominidase)	LYZ's substrate is bacterial cell wall peptidoglycan. It is present in lysosomes of many tissues and secretions, where it acts as an antibacterial agent.	LYZ has been used in toxicology studies to document generalized renal disease (although the PT reabsorbs the protein from glomerular filtrate).[15,29,95]	Soluble fraction

Angiotensinogen (AGT)	Urine AGT comes from the kidney and is a precursor to angiotensin I, which is responsible for generating angiotensin II, which regulates systemic blood pressure.	Increased urinary AGT has been linked to the development of hypertension and is a reported marker of chronic kidney disease (CKD) and in patients with IgA nephropathy.[41]	Soluble fraction
Podocalyxin (PODXL)	PODXL is a component of the glomerular podocyte and can be found in both the soluble and exosomal fractions of urine.[32]	Small amounts of PODXL can be found in urine from healthy individuals. The presence of increased amounts of urine PODXL is associated with glomerular disease, especially that seen with diabetic nephropathy, lupus nephritis and IgA nephropathy in humans.	Exosomal fraction
Fetuin-A (aka alpha-2-HS-glycoprotein, AHSG)+	AHSG is synthesized by hepatocytes and is a negative acute phase protein which suppresses TNF-α and suppresses ectopic calcification.[71,81]	AHSG has recently been shown to be a marker for acute renal injury (ARI), however the functional role in ARI is unknown. The protein may be secreted from PT cells.[116]	Soluble fraction
Ubiquitin A-52 ribosomal protein fusion product 1 (UBA52)+	UBA52 is a cytoplasmic and nuclear protein that plays a major role in targeting proteins for degradation, maintains chromatin structure and regulates gene expression and the stress response. In the kidney, UBA52 is localized exclusively to renal tubules.	An increased amount of urinary UBA52 appears to be a specific marker for diabetic nephropathy in humans.[24]	Exosomal fraction

CHAPTER 4

FUTURE DIRECTIONS

As we showed that AQP1, AQP2, calbindin D-28k and THP are reliable markers for the PCT, CD, DCT and TAL, respectively, in the dog, cat and mouse species, the next logical step is to explore the renal expression of these proteins in various disease states and after specific toxic insults. If IHC is shown to consistently highlight proteins in their respective locations, these renal tubular markers would make an excellent tool for toxicological studies, insuring that pathologists accurately localize renal tubular damage. One may also be able to, subjectively, use alterations in intensity of IHC to determine if renal membrane proteins are up- or down-regulated in renal tissue under certain circumstances. Of course, a better way to quantify such changes may be through alternative immunoassays, such as ELISA, or more recently developed proteomic quantification via mass spectrometry. Additionally, looking for changes in urine excretion of these proteins under different pathophysiological conditions and comparing urine excretion to histological kidney lesions could lead to the use of AQP1, AQP2, calbindin D-28k and THP as urinary markers for specific renal tubular damage. Ideally, a panel of urinary biomarkers, possibly including these renal membrane proteins, could provide valuable information about focal renal tubular damage, even potentially negating the need for renal biopsies in some situations.

As GST-α had variable expression amongst individual canines, it does not appear to be as reliable and consistent a marker for proximal tubular damage in the dog. GST-α and its alternative isoform GST-π (a marker of the DCT) have been utilized in many human disease states and rat models to identify and monitor PCT and DCT damage, respectively. Exploring the distribution of all GST isoforms in our domestic species could open interesting avenues of discovery.[17,33,37,115] Even if the distribution and utility of GSTs in dog remain too variable amongst individuals, this will be useful information to have when planning toxicological or other renal studies. GST-α may prove to be a more consistent marker for PCT damage in cats, although additional exploration of other GST isoforms in feline renal tissue is warranted.

The location of GST-α has been well established in rats, making our exclusive glomerular localization of the protein in mice somewhat surprising.[17] If GST-α expression is so variable between rats and the closely related mouse, this is also important information for researchers to be aware of when designing studies and should be explored further. It may be that GST-α could be an excellent marker for glomerular damage in mice.

As the proteomic evaluation of canine urine successfully identified 500 proteins, further exploration of this field is also warranted. Alternative techniques could be developed and employed to increase the protein yield of mass spectrometry evaluation of canine urine. Specifically, further attempts could be made to optimize the depletion of THP (via repetition of DTT step or development of alternative methods), as well as

remove albumin from urine, which could increase the yield of urinary proteins present in low concentrations.

Recently, capillary electrophoresis (CE), followed by MALDI-MS, have become leading techniques in protein separation and identification in many human studies and models of disease. Recent human urine proteomic studies employed CE and obtained protein yields of 1500-2000.[1,49] Exploring this technique in canine urine would likely improve our protein yield, as would using a higher mass accuracy and more sensitive mass spectrometer.

Proteomic analysis of urine from dogs with various types of renal disease is the next logical step in the field. As subtle variables such as age, diet and medications can alter the urinary proteome, initial studies of renal disease may be best suited to a strictly controlled, disease model situation. Once urinary protein variations can be established in a study population, assays may be developed that can be experimentally employed in the diagnoses and monitoring of veterinary patients with naturally occurring renal diseases. Proteomic quantification of specific urinary proteins via selective reaction monitoring (SRM) or immunoassays, could also be utilized to evaluate the diagnostic and monitoring utility of expected markers of renal disease identified in our present study (such as fetuin-A and ubiquitin A-52) in a population of patient dogs.

Biomarkers of renal disease are not the only important protein biomarkers that can be found in urine. Various studies could be developed to examine the urinary proteomes

of dogs with a variety of diseases, including cardiovascular disease, liver disease and different neoplasms, in an attempt to find valuable biomarkers for these diseases in urine. As we have already identified various urinary proteins associated with hypertension and other human diseases in canine urine (Table 3.1), various panels for the identification and quantification of these urinary proteins could be developed and applied to animal models in order to assess the diagnostic and monitoring utility of these proteins. Development of such panels could prove essential to many projects with far reaching implications into the realm of human medicine. These techniques could literally be employed to analyze urine from dogs in zero-gravity environments to help study the effects of physiological changes due to long term exposure to that environment. Ultimately, in this new and exciting field of canine urinary proteomics, the sky is the limit.

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