Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet

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DOKTORSKI RAD

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Mentor:
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# PROTEOMIC APPROACH FOR BIOMARKER DISCOVERY IN DOGS WITH KIDNEY AND HEART DYSFUNCTION

DOCTORAL DISSERTATION

Supervisor:
Prof. Vladimir Mrljak, PhD, *Professor emeritus*

Zagreb, 2026.

Ovaj doktorski rad izrađen je u Laboratoriju za proteomiku na Veterinarskom fakultetu Sveučilišta u Zagrebu pod vodstvom prof. dr. sc. Vladimira Mrljaka u sklopu projekta Hrvatske zaklade za znanost naziva *Proteomski pristup u otkrivanju ranih biomarkera bolesti bubrega i srca u pasa* (BioDog, IP-2013-11-4135). Doktorski rad izrađen je u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu.

ZAHVALE

Ponajprije zahvaljujem svom mentoru prof. dr. sc. Vladimiru Mrljaku na strpljenju, vodstvu, podršci i svim stručnim savjetima tijekom izrade ovog doktorskog rada. Zahvalna sam što me poticao na usavršavanje, učenje novih istraživačkih metoda te pisanje originalnih znanstvenih radova od samog početka studija i tako omogućio izradu ovog doktorskog rada prema skandinavskom modelu.

Nadalje, hvala kolegama s Klinike za unutarnje bolesti Veterinarskog fakulteta Sveučilišta u Zagrebu koji su pridonijeli ovom radu, osobito prikupljanjem potrebnih uzoraka za istraživanja. Osobito hvala tadašnjim suradnicima iz Laboratorija za proteomiku; Josipa, Anita, Asier, Nicolas i Blanka, bez vaše stručnosti i nesebičnog dijeljenja znanja ne bi bilo ni rezultata ovog rada.

Hvala profesorima s inozemnih sveučilišta na suradnji, profesoru Mangeshu Bhide-u, Davidu Eckersallu i Richardu Burchmore-u koji su pokazali izrazitu gostoljubivost prilikom istraživačkih boravaka na njihovim sveučilištima te poticali provođenje zajedničkih istraživanja.

Hvala mojoj obitelji na podršci tijekom izrade ovog rada, ponajprije suprugu Davoru koji je preuzimao sve obveze na sebe kako bih se mogla posvetiti pisanju, pri čemu mu to nije predstavljalo nikakav problem. Pravi si junak. Hvala i mojoj djeci, Tomi, Evi, Andriji i Viti, što su me motivirala da im dam primjer kako je dobro završiti nešto vrijedno što si započeo.

Hvala i mom tati što me uvijek poticao na obrazovanje i vjerovao u moje sposobnosti, znam da bi sada bio neizmjerno ponosan.

Non mihi, Domine, sed Tibi sit gloria.

INFORMACIJE O MENTORU

Dr. sc. Vladimir Mrljak, redoviti je profesor u trajnom zvanju na Klinici za unutarnje bolesti Veterinarskog fakulteta Sveučilišta u Zagrebu. Doktorirao je 1995. godine s temom „Promet bakra, cinka i željeza u različitim razdobljima reproduktivnog ciklusa krmača". Sudjelovao je u svim oblicima nastave na integriranom preddiplomskom i diplomskom studiju veterinarske medicine. Za studente veterinarske medicine napisao je program iz više kolegija koji su dio integriranog preddiplomskog i diplomskog studija na Veterinarskom fakultetu. Profesor Mrljak sudjeluje u poslijediplomskoj nastavi na doktorskom studiju „Veterinarske znanosti" gdje je napisao program za tri kolegija doktorskog studija. Bio je mentor na 13 doktorskih radova, među kojima se ističu dva dvojna doktorata između Sveučilišta u Zagrebu i University of Glasgow te Sveučilišta u Zagrebu i University of Bonn.

Profesor Mrljak organizirao je grupu istraživača koja tijekom više od 18 godina istražuje protozojske bolesti. Rezultat tih aktivnosti je 10 obranjenih doktorskih radova te jedan magistarski rad, odnosno više desetaka radova objavljenih u relevantnim bazama podataka. Zadnjih deset godina profesor Mrljak je usmjerio pažnju s babezioze prema primjeni postgenomskih tehnologija, naročito proteomike i metabolomike, na otkrivanje novih biomarkera unutarnjih bolesti, te time postavio temelje razvoju kliničke proteomike i metabolomike u veterinarskoj medicini u Hrvatskoj. Bio je voditelj na 15 domaćih i stranih projekata. Među dobivenim projektima posebno se ističe ERA Chairs projekt „VetMedZg" ukupne vrijednosti preko 2,9 milijuna eura, u okviru kojeg je poticao istraživanja u molekularnoj medicini na Veterinarskom fakultetu Sveučilišta u Zagrebu, osobito u primjeni proteomike i metabolomike u veterinarskoj medicini. U okviru projekta osnovan je Laboratorij za proteomiku, opremljen s različitim instrumentima za molekularna istraživanja, među kojima se posebno ističe spektrometar masa Orbitrap tehnologije (MS/MS) spregnut s tekućinskim kromatografima (nanoLC i UHPLC). Zajedno sa svojim kolegama iz inozemstva bio je gost urednik u više znanstvenih časopisa.

Profesor Mrljak aktivni je znanstvenik koji trenutno vodi jedan HORIZON MSCA projekt, dva projekta NextGenerationEU i 1 istraživački projekt Hrvatske zaklade za znanost.

Do sada je objavio više od 290 znanstvenih i stručnih radova (CROSBI 2024), među kojima valja posebno istaknuti 146 znanstvenih radova indeksiranih u citatnoj bazi podataka SCOPUS. Radovi profesora Mrljaka citirani su preko 2380 puta, h-indeks 26 (SCOPUS), odnosno preko 3860 puta, h-indeks 32 (Google Scholar). Profesor Mrljak dobitnik je više priznanja Sveučilišta u Zagrebu te Veterinarskog fakulteta Sveučilišta u Zagrebu. Godine 2023. Sveučilište u Zagrebu dodijelilo je profesoru Mrljaku počasno zvanje *Professor emeritus*.

Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet

Biološki odsjek

Doktorski rad

# PROTEOMSKI PRISTUP U OTKRIVANJU BIOMARKERA U PASA S POREMEĆAJEM FUNKCIJE BUBREGA I SRCA

PETRA BILIĆ

U pasa diljem svijeta i u Hrvatskoj često se javlja babezioza, bolest prenosiva putem krpelja. Psi invadirani vrstom *Babesia canis* podložni su teškim komplikacijama poput poremećaja funkcije bubrega što povećava vjerojatnost smrtnog ishoda. Tradicionalni dijagnostički parametri pokazuju ograničenja u ranom otkriću poremećaja funkcije bubrega zbog niske osjetljivosti. U pasa se često javljaju i srčana oboljenja, najčešće idiopatska dilatativna kardiomiopatija (iDKM) i kronična bolest srčanih zalistaka (KBSZ), čija se dijagnostika temelji na ultrazvuku. Cilj ovog istraživanja bio je visokoprotočnom proteomskom analizom otkriti nove biomarkere u serumu i/ili urinu za ranu dijagnostiku poremećaja funkcije bubrega u babeziozi te za navedena srčana oboljenja u pasa. Proteomskom analizom otkrivene su značajno promijenjene razine 58 proteina u serumu i 259 proteina u urinu pasa s poremećajem funkcije bubrega u babeziozi, pri čemu se kao ključni biomarkeri ističu serumski leucinom-bogati alfa-2-glikoprotein 1 i alfa-1-kiseli glikoprotein te urinarni lipokalin udružen s neutrofilnom gelatinazom i jetreni protein koji veže masne kiseline. Korištenjem komercijalnih testova, utvrđene su povišene razine urinarnog albumina, imunoglobulina, molekule bubrežne ozljede-1, retinol-vezujućeg proteina i N-acetil-glukozaminidaze u pasa s poremećajem funkcije bubrega u babeziozi. U pasa s iDKM-om, proteomskom analizom otkriveno je 12 serumskih proteina sa značajno promijenjenom razinom, pri čemu je protein povezan s mikrofibrilima 4 validiran kao potencijalni biomarker. U pasa s KBSZ-om utvrđeno je 15 serumskih proteina sa značajno promijenjenom razinom, pri čemu adiponektin i apolipoprotein B-100 imaju potencijal relevantnih biomarkera. Uz otkriće novih biomarkera, ovaj rad doprinosi pronalasku bioloških mehanizama koji imaju ulogu u poremećaju funkcije bubrega u babeziozi te u istraživanim srčanim bolestima u pasa.

(126 stranica / 26 slika / 17 tablica / 231 literaturnih navoda / jezik izvornika hrvatski)

Ključne riječi: proteomika, biomarker, pas, babezioza, poremećaj funkcije bubrega, idiopatska dilatativna kardiomiopatija, kronična bolest srčanih zalistaka

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Doctoral thesis

# PROTEOMIC APPROACH FOR BIOMARKER DISCOVERY IN DOGS WITH KIDNEY AND HEART DYSFUNCTION

PETRA BILIĆ

Babesiosis is a tick-borne disease prevalent in dogs worldwide and in Croatia. Dogs infected with *B. canis* are prone to severe complications, such as renal dysfunction, which increases the chance of mortal outcome. Traditional diagnostic parameters have limitations in the early detection of kidney dysfunction due to low sensitivity. Cardiac diseases also frequently occur in dogs, most commonly idiopathic dilated cardiomyopathy and chronic valve disease with diagnostics based on ultrasonography. The aim of this study was to use high-throughput proteomic analysis to identify novel serum and/or urinary biomarkers for the early diagnosis of kidney dysfunction in babesiosis and the aforementioned cardiac diseases. Proteomic analysis revealed significantly altered levels of 58 serum proteins and 259 urinary proteins in dogs with renal dysfunction during babesiosis, with key biomarkers being serum leucine-rich alpha-2-glycoprotein 1 and alpha-1-acid glycoprotein, as well as urinary neutrophil gelatinase-associated lipocalin and liver-type fatty acid-binding protein. Using commercial assays, elevated levels of urinary albumin, immunoglobulin, kidney injury molecule-1, retinol-binding protein, and N-acetyl-glucosaminidase were identified in dogs with renal dysfunction during babesiosis. In dogs with idiopathic dilated cardiomyopathy, proteomic analysis identified 12 serum proteins with significantly altered levels, with microfibrillar-associated protein 4 validated as a potential biomarker. In dogs with chronic valve disease, 15 serum proteins with significantly altered levels were identified, with adiponectin and apolipoprotein B-100 showing potential as effective biomarkers. Along with the discovery of new biomarkers, this work contributes to identification of the biological mechanisms underlying renal dysfunction during babesiosis and the investigated cardiac diseases in dogs.

(126 pages / 26 figures / 17 tables / 231 references / original in Croatian)

Keywords: proteomics, biomarker, dog (canine), babesiosis, kidney dysfunction, idiopathic dilated cardiomyopathy, chronic valve disease

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**SADRŽAJ**

# 1. UVOD

## *1.1. Otkriće proteinskih biomarkera*

### *1.1.1. Razvoj proteomike*

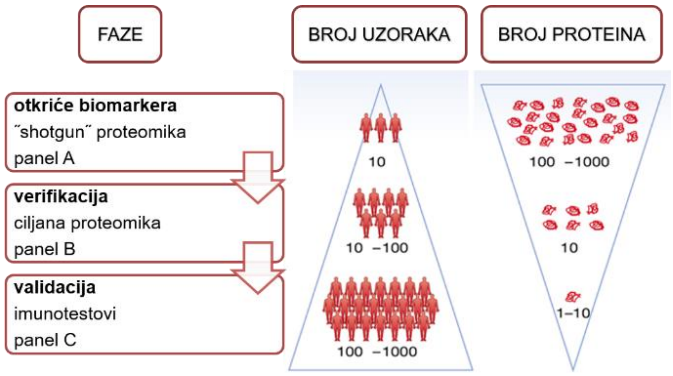
Ljudski genom sekvencioniran je početkom 21. stoljeća, a zatim je uslijedila i karakterizacija genoma drugih vrsta organizama što je omogućilo razvoj post-genomskih tehnologija, poput transkriptomike, proteomike, metabolomike i nutrigenomike. Post-genomske tehnologije se do današnjeg dana intenzivno razvijaju i postaju dostupnije, a omogućuju stvaranje sveobuhvatnih skupova podataka o molekularnim svojstvima u biološkim sustavima kao što su stanice, tkiva i organizmi (Manzoni i sur., 2018). Razvoj post-genomskih tehnologija doveo je do napretka u istraživanjima u biljnim znanostima, znanosti o okolišu te u dijagnostici i liječenju bolesti u ljudi. Sličan interes javio se i u veterinarskoj medicini zbog velikih mogućnosti kliničke primjene (Almeida i sur., 2021).

Posljednjih desetljeća osobito se razvija proteomika koja obuhvaća niz metoda kojima se istražuje proteom, odnosno cjelokupni set proteina organizma, tkiva ili stanica eksprimiran pri određenim okolišnim uvjetima (Ceciliani i sur., 2014). Počeci proteomike sežu u 1970-e i 1980-e godine kada je razvijena osnovna metoda razdvajanja proteina na poliakrilamidnom gelu pomoću dvodimenzionalne (2D) elektroforeze praćena identifikacijom proteina iz gela spektrometrijom masa. Iako metoda korištenjem 2D gel-elektroforeze omogućuje detekciju post-translacijskih modifikacija proteina, ima kao nedostatak poteškoću identifikacije slabije zastupljenih proteina (Gonzalez-Arostegui i sur., 2022). Tijekom zadnja dva desetljeća dolazi do razvoja tehnologija spektrometrije masa koje postaju osjetljivije i novčano dostupnije pa se sve više provode proteomska istraživanja bez upotrebe 2D gel-elektroforeze (takozvana *shotgun*-proteomika). *Shotgun*-proteomska metoda obuhvaća digestiju proteina iz biološkog uzorka, razdvajanje peptida korištenjem tekućinske kromatografije te analizu peptida tandemnim spektrometrom masa što rezultira identifikacijom proteina na temelju usporedbe dobivenih spektara s proteinskim bazama podataka. Ovakva metoda ima veliku analitičku specifičnost i osjetljivost što omogućuje otkrivanje proteina koji su slabije zastupljeni u uzorku (Gonzalez-Arostegui i sur., 2022).

U posljednjem desetljeću dolazi do razvoja ciljane proteomike, kvantitativnih proteomskih metoda, proteomike jedne stanice/organela, interaktomike (proteomike interakcije proteina) te metoda detekcije posttranslacijskih modifikacija proteina (glikozilacije i fosforilacije) uporabom spektrometrije masa (Almeida i sur., 2021). U veterinarskoj znanosti postoje

značajni napredci u upotrebi modernih proteomskih tehnologija u više područja, primjerice u istraživanju mesa, mlijeka i vune, sigurnosti hrane, zoonoza i raznih bolesti životinja. U skladu s tim, opus informacija prikupljen proteomskim istraživanjima korištenjem osnovnih i naprednih proteomskih tehnologija raste u veterinarskoj znanosti (Miller i sur., 2021).

S obzirom na strukturnu i izvršnu funkciju proteina, istraživanjem proteoma otkrivaju se mehanizmi bioloških procesa uključeni u fiziološka i patološka stanja, a uočene razlike između ta dva stanja mogu upućivati na specifične proteinske biološke markere (Perera i sur., 2022). Biološki marker (biomarker) definiran je kao objektivno mjerljivo obilježje koje upućuje na normalan biološki proces, patološki proces ili odgovor na izloženost/intervenciju, uključujući farmakološki odgovor na liječenje (*Biomarkers Definitions Working Group*, 2001). Korištenjem proteomskih metoda mogu se otkriti brojni proteinski biološki markeri s potencijalom za upotrebu u dijagnostici, određivanju stupnja bolesti, prognozi ili ishodu liječenja bolesti u ljudi i životinja (Doherty et al., 2008). Novootkriveni proteinski biomarkeri imaju potencijal pridonijeti bržoj dijagnostici bolesti, učinkovitom praćenju liječenja i ciljanoj terapiji u ljudi i životinja te na taj način utjecati na njihovu dobrobit. Slikom 1. prikazan je shematski proces kojim novootkriveni proteinski biomarkeri mogu dospjeti u kliničku primjenu. Uzimajući u obzir kompleksnost bolesti i heterogenost u populaciji, u kliničkoj primjeni može biti potrebno analizirati panel biomarkera, a ne samo jedan, kako bi se postigla visoka osjetljivost i specifičnost (Qian i sur., 2006).




Slika 1. Shematski proces otkrića novih proteinskih biomarkera. Mala populacija (N=10) analizira se *shotgun*-proteomskom metodom korištenjem spektrometrije masa, što vodi do kvantifikacije mnoštva proteina (panel A). U srednje velikoj populaciji (N=10-100) se manji broj razlikovnih proteinskih biomarkerskih kandidata analizira ciljanim proteomskim metodama (panel B). Naposlijetku, nekoliko odabranih proteina validira se imunotestovima u velikoj populaciji (N=100-1000) te primjenjuje u klinici (panel C). Preuzeto i prilagođeno iz Geyer i sur. (2017).

Dijagnostika bolesti u životinja još uvijek se najvećim dijelom temelji na kliničkom pregledu, radiografiji, ultrazvuku te laboratorijskim testovima, no to zahtjeva stručnost i vremenski period od više dana. Odgoda liječenja dok se ne postavi dijagnoza može rezultirati napredovanjem bolesti u jedinke ili u slučaju zaraznih bolesti njihovo širenje uzrokujući i ekonomske gubitke (Perera i sur., 2022). Iz tog razloga javila se potreba za rutinskim, ranim i osjetljivim biomarkerima za dijagnostiku bolesti u životinja. Iako postoje napredci u korištenju proteomskih metoda u istraživanju zdravlja i bolesti životinja, upotreba proteinskih biomarkera u veterinarskoj medicini još uvijek je većim dijelom u preliminarnom stupnju zbog nedovoljne validacije i manjka standardizacije (Eman i sur., 2025). Rezultate proteomskih analiza koji upućuju na potencijalne biomarkere potrebno je validirati u većoj populaciji imunotestovima, odnosno metodama detekcije proteina specifičnim protutijelima. Dostupnost protutijela specifičnih za detekciju novootkrivenih/slabo karakteriziranih proteina i proteina pseće vrste često je ograničena, a razvoj pouzdanih imunotestova iziskuje dug vremenski period (Perera i sur., 2022).

Proteomskim istraživanjima dolazi se do otkrića vezanih uz bolesti domaćih životinja koja se mogu potencijalno primijeniti u istoj bolesti u ljudi. Psi se često koriste kao modelni organizmi za bolesti koje se u njih prirodno razvijaju radi izloženosti istom okolišu te sličnosti u fiziologiji i anatomiji s čovjekom (Bilić i sur., 2018a). Od velike vrijednosti je stoga istraživanje proteinskih biomarkera u pasa za kardiovaskularne bolesti, starenje te više tipova tumora poput tumora dojke, limfoma, melanoma i osteosarkoma (Dolnicka i sur., 2025).

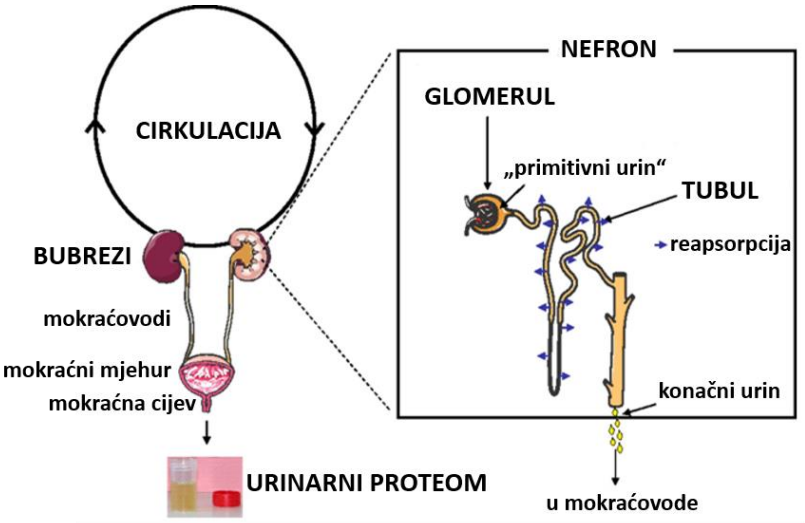
### *1.1.2. Plazma/serum i urin kao izvor proteinskih biomarkera u pasa*

Uzimajući u obzir da proteomi tjelesnih tekućina odražavaju biološke procese u organima i tkivima, njihovo istraživanje ima osobito velik potencijal kao izvor proteinskih biomarkera (Miller i sur., 2014). Plazma, serum, urin i slina su najčešće korišteni uzorci u proteomskim istraživanjima biomarkera za bolesti u ljudi i pasa s obzirom na laku dostupnost te izravnu i neizravnu interakciju s procesima u cijelom tijelu (Gonzalez-Arostegui i sur., 2022). S druge strane, proteomi tjelesnih tekućina poput suza, žući, sjemene, sinovijalne i amnionske tekućine su još uvijek slabo karakterizirani u pasa (Miller i sur., 2020). Pristup *shotgun*-proteomike korišten je dosada u brojnim istraživanjima seruma i urina u potrazi za proteinskim biomarkerima za razne bolesti u pasa (Ahn i sur., 2023). U istraživanju autora Ahn i sur. (2023) detaljno su karakterizirani proteomi seruma i urina pasa stvaranjem spektralne baze podataka korištenjem spektrometrije masa velike rezolucije kako bi se omogućilo pretraživanje

proteinskih baza podataka. Dobivene spektralne baze sadrže 1132 proteinske grupe za serum te 4749 proteinske grupe za urin pasa.

Proteom plazme/seruma pasa prvotno je karakteriziran pomoću dvodimenzionalne elektroforeze i spektrometrije masa te tada nije pronađen nijedan protein plazme/seruma specifičan samo za pse (Miller i sur., 2014). Pokazano je da različita biološka stanja, poput nutritivnog stanja, upale, gamopatija te bolesti bubrega i srca utječu na koncentracije proteina plazme/seruma u pasa (Bilić i sur., 2018a). Međutim, u plazmi/serumu postoji veliki dinamički raspon u koncentracijama proteina, pri čemu nekoliko vrlo visoko zastupljenih proteina poput albumina dominira u količini, dok su drugi zastupljeni u vrlo niskim koncentracijama, što otežava pronalazak biomarkera specifičnih za različite bolesti. Također, proteom plazme/seruma odražava izrazitu biološku kompleksnost koja proizlazi iz prisutnosti proteina tkiva iz cijelog organizma, post-translacijskih modifikacija poput glikozilacije i postojanja različitih formi eksprimiranih gena. Iz tih razloga je analiza proteoma drugih tjelesnih tekućina koje predstavljaju sadržajniji izvor potencijalnih biomarkera od plazme/seruma, poput urina, cerebrospinalne tekućine i sline, postala od značajnijeg interesa (Miller i sur., 2020).

Urin se smatra idealnim izvorom biomarkera od kliničkog značaja, uzimajući u obzir da se uzorci urina mogu skupljati u više navrata, neinvazivno i u velikoj količini. Također, s obzirom da u zdravih jedinki 70% proteina u urinu potječe iz bubrega i mokraćnih puteva, a 30% proteina potječe iz krvi kao rezultat filtracije u glomerulima (Slika 2), analiza proteoma urina omogućava otkriće proteinskih biomarkera za lokalne bubrežne, ali i sistemske bolesti (Decramer i sur., 2008).

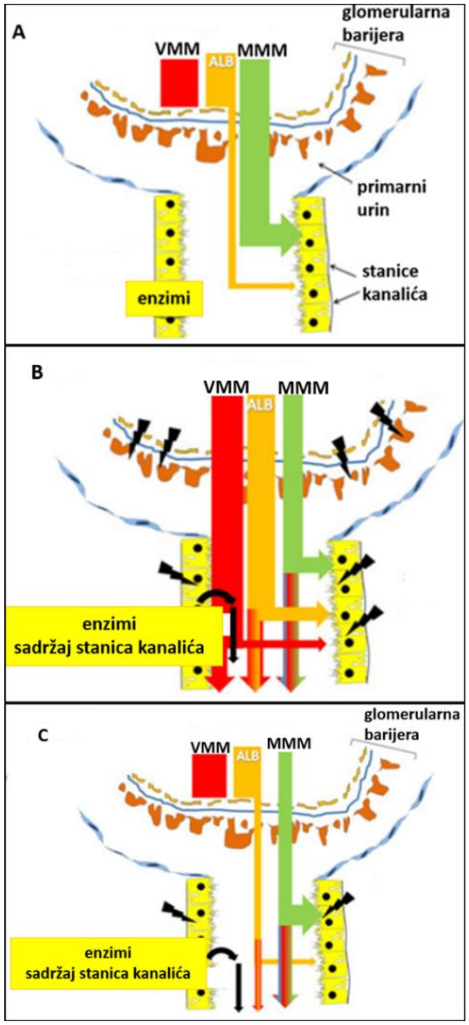



Slika 2. Podrijetlo urinarnog proteoma. Funkcionalne jedinice u bubrezima su nefroni koji se dijele na dva dijela: glomerule gdje se filtrira plazma u takozvani „primitivni urin“ te bubrežne kanaliće gdje se reapsorbira većina (99%) „primitivnog urina“. Ostatak urina izlazi iz bubrega putem mokraćovoda u mokraćni mjehur. Proteom urina u zdrave jedinke čini 70% proteina i peptida podrijetlom iz bubrega i mokraćnih puteva, a preostalih 30% dolazi iz cirkulacije. Preuzeto i prilagođeno iz Decramer i sur. (2008).

Budući da većina proteina iz krvi ne može proći glomerularnu filtracijsku barijeru, a većina onih koji i prođu se reapsorbira u bubrežnim kanalićima, zdravi bubrezi pasa proizvode urin s relativno malenom količinom proteina (Isani i sur., 2018). Urin zdravih pasa sadrži malo visoko zastupljenih proteina, poput albumina, uromodulina i lakih lanaca imunoglobulina te proteina specifičnih za prostatu u odraslih mužjaka (Miller i sur. 2014). U kompletnijoj karakterizaciji proteoma urina u zdravih pasa koristeći spektrometriju masa, otkriveno je 605 različitih proteina iz topive i eksosomalne frakcije (Brandt i sur., 2014).

Poremećaj u radu glomerula i/ili bubrežnih kanalića može dovesti do pojave povećane količine proteina u urinu (proteinurije) različitim mehanizmima koji se ponekad preklapaju (Slika 3). U normalnim fiziološkim uvjetima naboj i veličina proteina iz krvi utječu na njihov prolazak preko glomerularne filtracijske barijere na način da pozitivno nabijeni proteini lakše prolaze barijeru od negativnih, uz nemogućnost prolaska proteina veličine poput albumina ili većih od njega (molekulske mase > 69 kDa) (Slika 3A). Primarni glomerularni poremećaj funkcije (Slika 3B) uslijed kojeg se razvija azotemija (povišenje razine ureje i kreatinina u krvi) vodi do pojave većih količina proteina srednjih i velikih molekulskih masa u urinu, poput albumina i imunoglobulina G. Proteini koji su manji od albumina (male molekulske mase) se slobodno filtriraju kroz glomerule, ali se reapsorbiraju u proksimalnom dijelu bubrežnih

kanalića. Ukoliko postoji primarni ili sekundarni poremećaj funkcije proksimalnog dijela kanalića, onemogućena je potpuna reapsorpcija filtriranih proteina te se u urinu pojavljuju veće količine proteina male molekulske mase (primjerice retinol vezujući protein), uključujući i enzime koji se oslobađaju iz oštećenih stanica kanalića (poput N-acetil-glukozaminidaze, NAG) (Slika 3C). Prolazak proteina kroz filtracijsku barijeru i poremećaj funkcije u radu bubrežnih kanalića može dovesti do progresivnog gubitka bubrežne funkcije (De Loor i sur., 2013).




Slika 3. Prolaz proteina iz krvi preko glomerularne filtracijske barijere, reapsorpcija u stanicama bubrežnih kanalića te lučenje enzima i sadržaja stanica bubrežnih kanalića. A) u fiziološkim uvjetima, B) u patofiziološkim uvjetima primarnog glomerularnog poremećaja funkcije sa sekundarnim poremećajem funkcije proksimalnog dijela kanalića, C) u patofiziološkim uvjetima primarnog poremećaja funkcije proksimalnog dijela kanalića. Kratice: VMM – velika molekulska masa, MMM – mala molekulska masa, ALB – albumin. Preuzeto i prilagođeno iz De Loor i sur. (2013).

### *1.1.3. Metoda kvantifikacije proteina pomoću izobarnih privjesaka i LC-MS/MS-a*

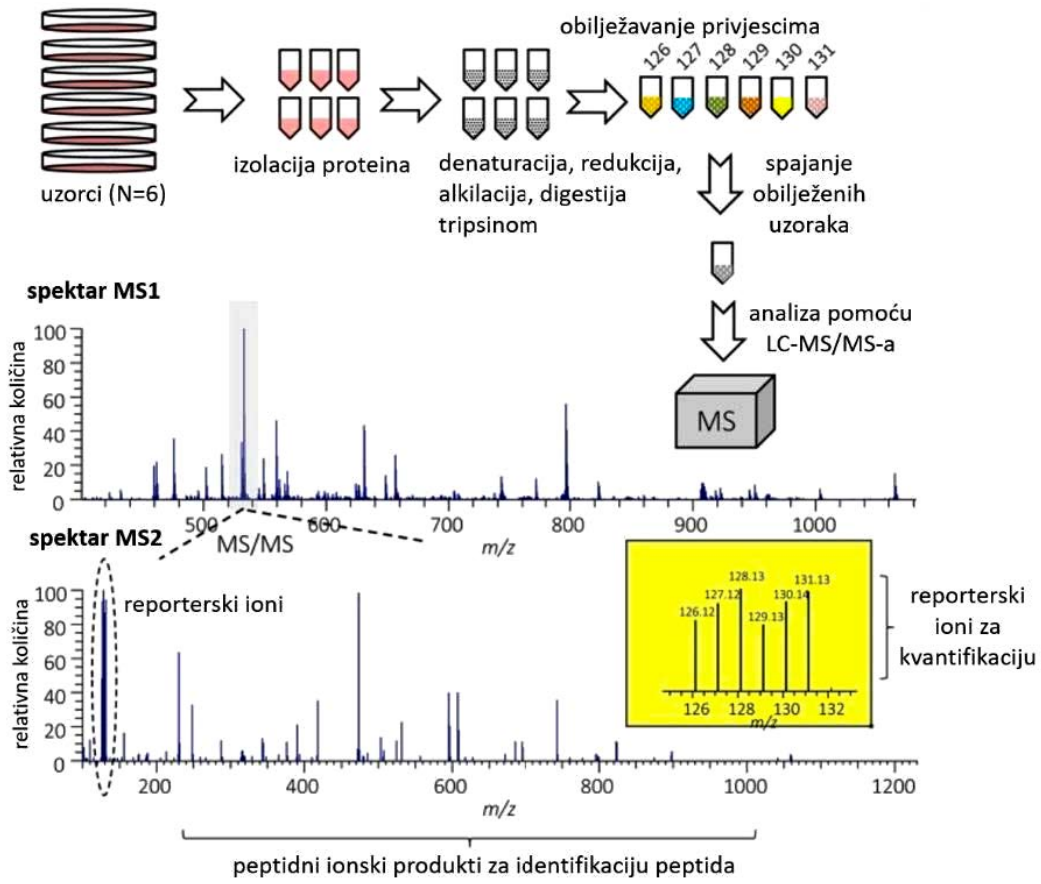
Metoda koja je korištena u proteomskim istraživanjima uključenima u ovaj rad naziva se vezani sustav tekućinske kromatografije i tandemne spektrometrije masa (engl. *liquid chromatography-tandem mass spectrometry*, LC-MS/MS). LC-MS/MS je efikasna metoda pomoću koje se mogu identificirati i kvantificirati proteini u kompleksnim biološkim uzorcima (Cindrić i sur., 2009).

U metodi LC-MS/MS se pomoću tekućinske kromatografije peptidi prvo razdvajaju na temelju veličine i naboja, a zatim se frakcije neutralnih molekula peptida ioniziraju i dalje razdvajaju u spektrometru masa na temelju omjera mase i naboja (m/z) čime nastaju spektri MS1. Nadalje, izazivanjem fragmentacije peptida prekursora razdvojenih na temelju m/z, dobivaju se spektri ionskih produkata MS2 koji se uspoređuju s bazama podataka u svrhu identifikacije proteina u početnom uzorku (van Vliet, 2014).

Kako bi se utvrdila relativna količina proteina između različitih bioloških uzoraka koja ovisi o biološkom stanju, može se koristiti obilježavanje proteina pomoću izobarnih privjesaka prije analize uzoraka s metodom LC-MS/MS. Glavna prednost metode relativne kvantifikacije pomoću izobarnih privjesaka je mogućnost istovremene višestruke analize proteinskih uzoraka, s obzirom da se svaki uzorak obilježava drugačijom varijantom izobarnog privjeska, a zatim se uzorci spajaju u jedan i u isto vrijeme analiziraju pomoću metode LC-MS/MS. Budući da su različite varijante privjesaka izobarne (jednake mase), isti peptidi iz različitih uzoraka obilježeni s različitim privjescima imaju isto vrijeme zadržavanja na kromatografskoj koloni te se pojavljuju kao jedan pik iste vrijednosti m/z u spektru MS1. Nadalje, fragmentacijom obilježenih peptidnih prekursornih iona u MS2, nastaju pikovi reporterskih iona (od privjesaka) te pikovi peptidnih ionskih produkata (od peptida). Relativna kvantifikacija se postiže direktnom korelacijom relativnog intenziteta reporterskih iona, a identifikacija proteina na temelju pikova peptidnih ionskih produkata. Budući da svaki peptid može biti obilježen izobarnim privjeskom, identifikacijom više od jednog peptida koji pripadaju nekom proteinu povećava se pouzdanost kvantifikacije i identifikacije (Rauniyar i Yates, 2014).

U slučaju kada se analizira više eksperimentalnih setova, potrebno je uključiti referentni uzorak (interni standard) u svaki set, što omogućuje normalizaciju podataka i usporedbu bioloških replika između eksperimentalnih setova. Interni standard može biti skupni uzorak pripremljen miješanjem malih alikvota s istom količinom proteina iz različitih pojedinačnih

uzoraka (Zhou i sur., 2012). Slikom 4 shematski je prikazana metoda relativne kvantifikacije proteina pomoću izobarnih privjesaka i LC-MS/MS-a.




Slika 4. Shema eksperimenta korištenjem metode relativne kvantifikacije proteina pomoću izobarnih privjesaka i LC-MS/MS-a. U slučaju šesterostruke analize s izobarnim privjescima TMT (engl. *tandem mass tag*), svaki od šest uzoraka u setu se obilježava drugačijim izobarnim privjeskom te se alikvoti iste količine uzoraka spajaju u jedan. Uzorci peptida obilježeni izobarnim TMT privjescima analiziraju se LC-MS/MS-om. U spektru MS1, peptidi iste aminokiselinske sekvence iz različitih uzoraka pojavljuju se kao pojedinačni nerazdvojeni prekursorski ioni. Nakon fragmentacije prekursorskog iona u spektru MS2, pojavljuje se šest reporterskih iona različitih m/z od 126 do 131 te peptidni ionski produkti pomoću kojih se određeni peptid kvantificira i identificira istovremeno u šest uzoraka. Preuzeto i prilagođeno iz Rauniyar i Yates (2014).

### *1.2. Poremećaji funkcije bubrega i srca u pasa*

#### *1.2.1. Babezioza u pasa*

Babezioza je jedna od najznačajnijih bolesti pasa koja se prenosi putem krpelja s osobitim globalno-ekonomskim i kliničkim značajem diljem svijeta te jedna od najčešćih bolesti pasa u Hrvatskoj (Cacciò i sur., 2002; Mrljak i sur., 2017). Babezioza je uzrokovana intraeritrocitnim protozoarnim parazitima iz roda *Babesia* kojima domaćini mogu biti različiti kralješnjaci, uključujući domaće i divlje životinje, te ljudi (Bilić i sur., 2018b). Vrste roda *Babesia* su filogenetski povezane s vrstama roda *Plasmodium* koje uzrokuju malariju u ljudi te s njima dijele mnoge strukturne i funkcionalne značajke (Lau, 2009), što čini istraživanje babezioze potencijalno primjenjivim i u ljudskoj medicini. Vrste roda *Babesia* koje invadiraju pse su tradicionalno klasificirane na temelju morfologije intraeritrocitnog stadija na velike (2,5-5,0 μm) i male (1,0-2,5 μm) oblike (Beugnet i Moreau, 2015). Tako je u pasa identificirana babezioza uzrokovana s pet velikih vrsta roda *Babesia* (*B. canis*, *B. rossi*, *B. vogeli*, *B. sp (Coco)*, *B. caballi*) i tri male vrste (*B. gibsoni*, *B. conradae*, *B. vulpes*) koje se prenose određenom vrstom krpelja vektora te imaju specifičnu geografsku raspodjelu (Bilić i sur., 2018b; Abdoli i sur., 2024).

Geografska raspodjela vrsta roda *Babesia* u Europi raznolika je i ovisi o prisutnosti domaćina i krpelja vektora (Matijatko i sur., 2012). Pojavnost babezioze u pasa vezana je uz sezonsku aktivnost krpelja vektora, pri čemu su umjerene temperature i kišno vrijeme u proljeće i jesen idealni uvjeti za širenje krpelja (Leschnik i sur., 2008). Vrsta *B. canis* je glavni uzročnik babezioze pasa u Europi, koja je detektirana u njenom središnjem, istočnom i sjevernom dijelu, što je povezano s rasprostranjenosti njenog glavnog vektora krpelja vrste *Dermacentor reticulatus* (Karbowiak, 2014). Utvrđena prevalencija babezioze uzrokovane s *B. canis* u pasa u Hrvatskoj na temelju imunoloških ispitivanja bila je 20,0% (Mrljak i sur., 2017), a na temelju molekularnih metoda 3,4% (Beck i sur., 2009). Pozitivni imunološki rezultati u detekciji *B. canis* su posljedica prethodnog kontakta i akutne infekcije, dok se molekularnim metodama utvrđuje samo trenutačna infekcija.

Sve vrste *Babesia* prenose se slinom u krv domaćina prilikom hranjenja krpelja, gdje sporozoiti izravno invadiraju crvene krvne stanice u kojima se diferenciraju u trofozoite i dijele binarnom fizijom stvarajući merozoite koji izlaze iz eritrocita pritom ih uništavajući. Ciklus umnažanja se nastavlja do smrti domaćina ili dok liječenje i/ili imunosni odgovor domaćina spriječe dijeljenje parazita (Uilenberg, 2006). Vrlo rijetko se *Babesia* prenosi bez vektora,

primjerice transfuzijom zaražene krvi psa donora ili ugrizom tijekom borbe pasa (Köster i sur., 2015).

U patogenezi babezioze prevladavaju dva glavna mehanizma – hemoliza te imunosni odgovor domaćina na parazitemiju (Schetters i sur., 1997). Razvoj anemije u babeziozi nije posljedica samo raspadanja eritrocita zbog direktnog utjecaja parazita na eritrocitnu membranu, nego i uklanjanja invadiranih eritrocita u slezeni i imunosnog odgovora u vidu uništavanja eritrocita posredstvom anti-eritrocitnih protutijela i aktivacijom komplementa (Vannier i sur., 2015). Ostale kliničke značajke uključuju leukopeniju, trombocitopeniju, aktivaciju koagulacijskog sustava, fibrinolizu i aktivaciju endotela (Barić Rafaj i sur., 2009; Kuleš i sur., 2017). Infekcija s *B. canis* može imati blagi, nekompliciran tijek sa simptomima poput povišene tjelesne temperature, smanjenog apetita, letargije, slabosti, dehidracije i žutice (Furlanello i sur., 2005). Međutim, moguć je i razvoj kompliciranog oblika bolesti gdje nastale komplikacije nisu samo posljedica hemolize, već i imunosnog odgovora domaćina (Welzl i sur., 2001).

Patogeneza babezioze uvelike ovisi o intenzitetu imunosnog odgovora, odnosno o stvaranju proupalnih citokina i posljedične aktivacije odgovora akutne faze upale (Reyers i sur., 1998). Pokazano je da u pasa invadiranih s *B. canis* dolazi do aktivacije odgovora akutne faze upale pri čemu rastu koncentracije pozitivnih proteina akutne faze, poput C-reaktivnog proteina (CRP-a) i serumskog proteina amiloida A, a smanjuju se koncentracije negativnih proteina akutne faze (Matijatko i sur., 2010; Kuleš i sur., 2014; Kuleš i sur., 2016). Iako su citokini korisni u obrambenom odgovoru domaćina, njihovo pretjerano oslobađanje može biti štetno za domaćina, uzrokujući oštećenje tkiva i organa (Borghetti i sur., 2009). U jednog dijela pasa s kompliciranom babeziozom razvija se sindrom sistemskog upalnog odgovora (engl. *systemic inflammatory response syndrome*, SIRS) koji je posljedica pretjeranog oslobađanja upalnih posrednika u krvotok, a povezan je s većom stopom smrtnosti (Reyers i sur., 1998). Također, u kompliciranim slučajevima može doći do razvoja sindroma višestrukog poremećaja funkcije organa (engl. *multiple organ dysfunction syndrom*, MODS) kao rezultata izravne ozljede organa uslijed hipoksije ili kao posljedica pretjeranog upalnog odgovora domaćina (SIRS-a) pri čemu dolazi do deregulacije proupalnih i protuupalnih mehanizama te posljedične samodestruktivne upale (Goris i sur., 1985). Najčešće komplikacije u MODS-u u pasa oboljelih od babezioze su akutna ozljeda bubrega (AOB), cerebralna babezioza, hepatopatija, akutni respiratorni distres sindrom, pankreatitis i kardiopatije, pri čemu je pokazano da je broj organa uključenih u MODS povezan sa smrtnošću (Welzl i sur., 2001).

Dijagnoza babezioze postavlja se na temelju kliničkih simptoma, pregleda razmaza krvi pomoću mikroskopa te testiranjem koje uključuje imunološke i molekularne metode. Iako je mikroskopski pregled razmaza krvi najjednostavniji test, nedostaci su niska osjetljivost i nemogućnost utvrđivanja vrste *Babesia* (Miró i sur., 2015). Imunološki testovi poput testa indirektne imunofluorescije i imunoenzimske metode ELISA koriste se u detekciji babezioze u svrhu nadzora i istraživanja, no nedostaci su niska specifičnost zbog križne reaktivnosti između vrsta *Babesia*, nemogućnost razlikovanja akutnih od kroničnih infekcija te moguće lažno negativni rezultati s obzirom da je potrebno 8 do 10 dana da se razviju protutijela prilikom akutne infekcije (Solano-Gallego i Baneth, 2011). Lančana reakcija polimerazom (PCR, *polymerase chain reaction*) ima veću osjetljivost i specifičnost u detekciji babezioze od ostalih metoda, omogućavajući detekciju infekcije u pasa s niskom parazitemijom i razlikovanje između vrsta *Babesia*. Uspostavljeno je više protokola testova PCR, među kojima je i protokol pomoću kojeg se mogu razlikovati vrste *B. canis*, *B. rossi* i *B. vogeli* korištenjem specifično dizajniranih početnica u jednom PCR testu (Duarte i sur., 2008).

Liječenje babezioze u pasa provodi se primjenom antiprotozoalnih i antimikrobnih lijekova te potpornom terapijom u kompliciranim slučajevima. Antiprotozoalni lijek imidokarb-dipropionat primijenjen u jednoj dozi od 6 mg/kg intramuskularno ili subkutano je visoko učinkovit tretman za invaziju pasa s *B. canis* koji u nekompliciranim slučajevima omogućuje značajno poboljšanje u prvih 24-48 sati, no mogući su i relapsi (Schoeman, 2009).

#### *1.2.1.1. Poremećaj funkcije bubrega u babeziozi pasa*

Akutna ozljeda bubrega (AOB), obilježena anurijom ili oligurijom usprkos prikladnoj rehidraciji, prepoznata je komplikacija u babeziozi pasa čija prevalencija varira u literaturi ovisno o ispitivanim populacijama, pristranosti u odabiru uzoraka i različitim kriterijima za definiciju AOB-a (Defauw i sur., 2018). Jedan od najčešće korištenih parametara u dijagnozi poremećaja funkcije bubrega je azotemija, koja predstavlja povišene razine ureje i kreatinina u krvi do kojih dolazi zbog smanjene glomerularne filtracije u bubrezima. U literaturi prevalencija azotemije u babeziozi pasa varira od 0% do 36% ako se uzmu u obzir sve vrste roda *Babesia* (Defauw i sur., 2018). U istraživanju autora Matijatko i sur. (2010), u 90% pasa s MODS-om u kompliciranoj infekciji s *B. canis* utvrđen je poremećaj funkcije bubrega, na temelju kriterija koncentracije serumskog kreatinina veće od 180 μmol/L. Iako prerenalni uzroci koji dovode do povećane koncentracije serumskog kreatinina ne mogu biti isključeni u svim slučajevima, autopsija koja je napravljena u dijela pasa u tom istraživanju otkrila je distrofiju parenhima i/ili akutnu tubularnu nekrozu u bubrezima. Pokazano je da poremećaj

funkcije rada bubrega (definiran s koncentracijom serumskog kreatinina većom od 150 μmol/L) povećava vjerojatnost smrtnog ishoda i do 5 puta u odnosu na druge komplikacije u pasa invadiranih s *B.canis* (Welzl et al., 2001). U babeziozi je ipak češće blaže oštećenje bubrega, nego AOB, što je utvrđeno detekcijom enzima i proteina u urinu (enzimurija i proteinurija) te abnormalnim nalazom sedimenta urina (prisustvo epitelnih stanica bubrežnih kanalića) (Lobetti i Jacobson, 2001).

Uzroci oštećenja bubrega u babeziozi su nerazjašnjeni, a neki od predloženih mehanizama su povišene koncentracije hemoglobina u krvi (hemoglobinemija), renalna hipoksija uzrokovana anemijom i niskim krvnim tlakom i/ili sistemski upalni odgovor (Defauw i sur., 2012). Dugo se smatralo da mehanički i toksični učinci hemoglobina na bubrežne kanaliće uzrokuju AOB u babeziozi (Jacobson i Clark, 1994). Međutim, pokazano je da pokusna infuzija zdravih pasa s hemoglobinom nije rezultirala značajnom bubrežnom bolesti (Lobetti i sur., 1996). Smatra se da bi hipoksija u tkivu bubrega uzrokovana niskim sistemskim tlakom mogla biti glavnim uzrokom oštećenja bubrega u babeziozi. Naime, u pasa s babeziozom invadiranih s *B. canis* nije utvrđena korelacija između anemije i razvoja azotemije (Zygner i Wedrychowicz, 2009), dok je nađena negativna korelacija između krvnog tlaka i azotemije upućujući na zaključak da je sniženi krvni tlak glavni faktor koji utječe na razvoj azotemije (Zygner i Gójska-Zygner, 2014). Također, histopatološki nalazi akutne tubularne nekroze u bubrezima pasa s bubrežnom bolesti invadiranih s *B. canis* upućuju na hipoksičnu ozljedu (Máthé i sur., 2007) s obzirom da su stanice proksimalnog dijela bubrežnih kanalića osobito osjetljive na manjak kisika zbog intenzivne metaboličke aktivnosti (Wang i sur., 2022). Smatra se da do oštećenja bubrega može doći i posredstvom djelovanja citokina uslijed sistemskog upalnog odgovora (Jacobson i Clark, 1994). Primjerice, u pasa invadiranih s *B.canis* utvrđeno je na temelju mjerenih parametara da dolazi do povećanja serumske koncentracije proupalnog citokina tumor nekrotizirajućeg faktora alfa, koji potiče razvoj vazodilatacije i hipotenzije te mogućeg posljedičnog oštećenja bubrega zbog hipoksije u tkivu bubrega (Zygner i sur., 2014).

Kako bi se ispitala funkcija bubrega u pasa, rutinski se koristi kreatinin u plazmi ili serumu kao indirektni marker brzine glomerularne filtracije (GFR) te analiza urina (specifična težina urina, sediment, proteinurija) (Nabity, 2018).

Kreatinin u serumu potječe iz mišića te se slobodno filtrira u glomerulima nefrona sa zanemarivom sekrecijom i reapsorpcijom u bubrezima te metabolizmom izvan bubrega (Finco i sur., 1991). Pokazano je da je koncentracija plazmatskog ili serumskog kreatinina negativno

korelirana s GFR-om (Finch, 2014). Ipak, serumski kreatinin ima ograničenja kao marker oštećenja bubrega s obzirom da različiti faktori utječu na njegovu koncentraciju te je neosjetljiv u otkrivanju rane bolesti (Braun i sur., 2003). Nemasna tjelesna masa je faktor koji utječe na osnovnu koncentraciju serumskog kreatinina u pasa (Hall i sur., 2015), ali je pokazano da utjecaj imaju i dob, vrijeme obroka i korištene analitičke metode mjerenja (Braun i sur., 2003). Također, do razvoja azotemije, odnosno porasta serumskog kreatinina iznad referentnih vrijednosti dolazi tek kad je funkcija bubrega smanjena za 65-75%, odnosno 48-72 sata od početka bubrežne ozljede čineći kreatinin neosjetljivim markerom za otkrivanje ranog oštećenja bubrega (Braun i sur., 2003; Cianciolo i sur., 2016).

Pored navedenih nedostataka, postoje dodatna ograničenja u primjeni serumskog kreatinina kao markera za poremećaj rada bubrega u babeziozi zbog prisutnosti hemolize. Pokazano je da povećane koncentracije bilirubina i slobodnog hemoglobina u serumu pasa s babeziozom mogu dovesti do interferencije u mjerenju serumskog kreatinina kad se koriste uobičajene metode mjerenja (de Scally i sur., 2004). Također, u eksperimentalnoj infekciji s *B. canis* zabilježene su smanjene koncentracije kreatinina u plazmi, što je moguće uzrokovano povećanjem volumena plazme nakon oboljenja (Schetters i sur., 2009).

Proteinurija, odnosno povećano izlučivanje proteina urinom, upućuje na oštećenje glomerula i/ili bubrežnih kanalića, iako može biti posljedica i drugih pre- i postrenalnih poremećaja (Harley i Langston, 2012). Proteinurija je najčešće korišten marker oštećenja i poremećaja rada bubrega ako se mogu isključiti pre- i postrenalni uzroci (Nabity, 2018). Mehanizmi nastanka proteinurije uzrokovani poremećajem rada bubrega opisani su u potpoglavlju 1.1.2. Budući da je pokazano da bubrežne bolesti popraćene proteinurijom napreduju brže od onih gdje proteinurija nije prisutna te da smanjenje proteinurije usporava upalne procese u bubrežnim bolestima pasa, smatra se da proteinurija sama doprinosi već postojećem oštećenju bubrega (Harley i Langston, 2012). Moguće je da je oštećenje bubrežnih kanalića i intersticija bubrega u pacijenata s proteinurijom potaknuto direktnim djelovanjem filtriranih proteina na stanice kanalića ili aktivacijom komplementa i kemoatraktanata, što vodi do stvaranja posrednika upale te tako pridonosi fibrozi (Perico i sur., 2005).

Standardni test za proteinuriju jest omjer ukupnih proteina i kreatinina u mokraći (engl. *Urine Protein to Creatinine Ratio*, UPCR) mjeren u jednom uzorku mokraće koji ima dobru korelaciju s 24-satnom kvantifikacijom proteina u urinu (Monroe i sur., 1989). Prema smjernicama udruženja IRIS (engl. *International Renal Interest Society*) u pasa nije prisutna proteinurija ako je UPCR manji od 0,2, granična proteinurija postoji ako je UPCR od 0,2 do

0,5, a značajna proteinurija je prisutna ako je UPCR veći od 0,5 (IRIS, 2023). U pasa s azotemijom, UPCR veći od 1 povezan je s većim rizikom od smrtnog ishoda (Jacob i sur., 2005). Iako je UPCR bitan biomarker u utvrđivanju proteinurije bubrežnog podrijetla, njegovi nedostaci su niska specifičnost u određivanju mjesta i stupnja oštećenja bubrega (Cianciolo i sur., 2016).

Uzimajući u obzir da su tradicionalni markeri (serumski kreatinin i UPCR) nespecifični i neosjetljivi markeri za rano otkriće oštećenja bubrega te ne ukazuju na mjesto (glomeruli, proksimalni ili distalni dio bubrežnih kanalića) i stupanj oštećenja bubrega, postoji velik interes u istraživanju novih biomarkera u serumu i urinu koji bi omogućili ranu i preciznu detekciju ozljede bubrega. Nekoliko novih proteinskih biomarkera za oštećenje bubrega ispitano je u urinu pasa pomoću kolorimetrijskih, spektrofotometrijskih i imunoloških metoda (Cobrin i sur., 2013). Kao potencijalni biomarkeri u urinu pasa za oštećenje bubrega predloženi su određeni proteini velikih molekulskih masa kao što je imunoglobulin G (IgG), srednjih molekulskih masa poput albumina (ALB), malih molekulskih masa poput urinarnog retinol-vezujućeg proteina (engl. *Retinol Binding Protein*, RBP) i enzima NAG te proteina koje proizvode bubrežni kanalići kao što su uromodulin i molekula bubrežne ozljede 1 (Cobrin i sur., 2013). Ipak, nijedan od navedenih biomarkera još nije ušao u rutinsku kliničku upotrebu.

### *1.2.2. Idiopatska dilatativna kardiomiopatija u pasa*

Idiopatska dilatativna kardiomiopatija (iDKM) predstavlja poremećaj rada srčanog mišića nepoznatog uzroka karakteriziran smanjenom kontraktilnosti, proširenjem lijevog ili oba ventrikula te smanjenom sistoličkom funkcijom (Richardson i sur., 1996). Europsko kardiološko društvo te društvo AHA (engl. *American Heart Association*) su definirali DKM kao prisutnost proširenja lijevog ventrikula te poremećaja funkcije kontraktilnosti uz odsutnost abnormalnih uvjeta opterećenja i teških bolesti koronarnih arterija (Myers i sur., 2024). Kako se napredovanjem bolesti srčani mišić proširuje, on postaje slabiji i manje učinkovit u cirkulaciji krvi što vodi do nakupljanja tekućine u tijelu te naposlijetku i kongestivnog zatajenja srca (Dukes-McEwan i sur., 2003).

iDKM napreduje kroz tri faze gdje se prve dvije nazivaju predkliničkim DKM-om koji ima duže trajanje, a treća faza jest klinička bolest (O'Grady i O'Sullivan, 2004). U prvoj fazi ne postoje promjene u morfologiji i električnoj aktivnosti srca niti klinički znakovi bolesti, dok u drugoj fazi bolesti te promjene nastupaju, no i dalje bez kliničkih znakova. Od velike važnosti je detekcija predkliničke bolesti kako bi se omogućila rana intervencija s terapijom i praćenjem

pacijenta sa ciljem odgode razvoja kliničke bolesti (Davis i sur., 2024). U trećoj fazi bolesti primjetni su klinički znakovi poput otežanog disanja, kašlja, gubitka težine, kratkotrajni gubitak svijesti, aritmija te kongestivno zatajenje srca što nosi lošu prognozu (Dukes-McEwan i sur., 2003).

Prema sustavu ISACHC (engl. *International Small Animal Cardiac Health Council*), iDKM se može klasificirati u jedan od 5 razreda (Nelson i Couto, 2013):

1) Ia: asimptomatska bolest bez povećanja srca

2) Ib: asimptomatska bolest uz povećanje srca

3) II: blago do umjereno kongestivno zatajenje srca (simptomi pri tjelesnom naporu)

4) IIIa: uznapredovalo kongestivno zatajenje srca uz kućnu terapiju

5) IIIb: uznapredovalo kongestivno zatajenje srca uz hospitalizaciju

iDKM je druga najčešća bolest srca u pasa s prevalencijom od 0,5-1,4% u općoj populaciji te se učestalije javlja u velikim, a rjeđe u srednjim, malim i križanim pasminama (Tidholm i sur., 2001). Prevalencija pasa s DKM-om raste s dobi te češće pogađa mužjake (Sisson i Thomas, 1995). Pasmine u kojih se iDKM javlja s većom učestalosti su doberman, bokser, irski vučji hrt, njemačka doga, njemački ovčar, labrador, newfoundlander i engleski koker španijel (Tidholm i sur., 2001). U istraživanjima u kojima se ispitivala prisutnost iDKM-a u pasa bez kliničkih znakova bolesti prevalencija je bila znatno veća (na primjer 63,2% u dobermana, 24,2% u irskih hrtova te 17,6% u newfoundlander-a) (Dukes-McEwan i sur., 2003).

Ukoliko je uzrok DKM-a poznat, poput genetskih faktora, nedostatka hranjivih tvari, metaboličkih poremećaja, bolesti imunosnog sustava, infektivnih bolesti ili otrovanja, bolest se ne smatra idiopatskom (Dutton i López-Alvarez, 2018). U ljudi su najčešće prepoznati uzroci DKM-a genetski, imunosni i toksični faktori. U pasa uzrokom može biti genetika, nedostatak taurina i karnitina te otrovanje, a postoje i dokazi za uključenost imunosnih reakcija u nastanak bolesti, poput pronalaska povišenja serumske koncentracije proupalnih proteina (CRP-a i citokina) (O'Grady i O'Sullivan, 2004). U nekih pasmina koje su zastupljenije među oboljelima, poput dobermana i boksera, pronađeni su geni koji utječu na nastanak bolesti (Shen i sur., 2022). Posljednjih godina javili su se slučajevi DKM-a u pasmina pasa koje nisu genetički predisponirane, a povezani su s prehranom bez žitarica bogatom mahunarkama (graškom, lećom i slanutkom) te krumpirima i batatom. Mnogi psi su pokazali kliničko poboljšanje uz

promjenu prehrane i terapiju, ukazujući da je ovaj oblik DKM-a reverzibilan (Mornard i sur., 2025).

Patofiziološki mehanizmi iDKM-a su većim dijelom nepoznati, iako je prepoznato da dolazi do strukturnih i funkcionalnih promjena srčanog mišića, odnosno remodeliranja miokarda, kao posljedice oštećenja kardiomiocita te posljedične upalne reakcije. Nakon upale slijedi faza popravka, odnosno cijeljenja tkiva pri čemu dolazi do fibroze, odnosno diferencijacije fibroblasta i odlaganja izvanstaničnog matriksa (Gasparini i sur., 2020). Patogeneza razvoja zatajenja srca u pasa s iDKM-om uključuje aktivaciju neurohumoralnog sustava i upale posredovane imunosnim sustavom koja dovodi do pogoršanja stanja oboljelog psa. Zatajenje srca je uzrokovano apoptozom kardiomiocita, remodeliranjem tkiva lijevog ventrikula, sistoličkim i dijastoličkim poremećajem funkcije i aritmijama (Vatnikov i sur., 2019).

Histološki nalazi srčanog tkiva pasa s iDKM-om ukazuju na postojanje dva različita oblika bolesti, što uključuje degenerativni oblik s infiltracijom lipida te oblik s pojavom oslabljenih valovitih srčanih mišićnih vlakana. U degenerativnom obliku s infiltracijom lipida pronalaze se lizirani miociti, degeneracija srčanih vlakana, atrofija miocita te izrazita fibroza s infiltracijom lipida. U obliku sa oslabljenim valovitim srčanim vlaknima pronalaze se atrofirana, stanjena i valovita srčana vlakna koja su specifičan nalaz za ranu fazu iDKM-a (Tidholm i Jonsson, 2005).

Dijagnoza iDKM-a se postavlja na temelju ultrazvuka srca, dok ostale metode pridonose dijagnostici bolesti. Funkcija srca se uobičajeno ispituje pomoću elektrokardiografije, radiografije i ultrazvuka, a koristi se i određivanje razine serumskih markera - srčanih troponina te natriuretskih peptida (Cruz i sur., 2012). Otprije poznati proteini koji imaju povišene koncentracije u serumu ljudi i pasa s iDKM-om su srčani troponin I (cTnI) i N-terminalni prohormon B-tipa natrijuretskog peptida (NT-proBNP) (Spratt i sur., 2005; Oyama i sur., 2008). Iako se navedeni proteini koriste kao biomarkeri koji mogu pridonijeti dijagnozi DKM-a, njihova uporaba je ograničena zbog nespecifičnosti budući da se javljaju i u drugim bolestima srca i bubrega (Fonfara i sur., 2010).

cTnI je protein specifično eksprimiran u kardiomiocitima koji se uslijed ozljede srčanog mišića otpušta u krv gdje postiže najveću koncentraciju 12-24h nakon oštećenja srca, a ostaje prisutan i danima (Fonfara i sur., 2010). U ljudi se cTnI koristi kao biomarker akutnog srčanog infarkta (Langhorn i Willesen, 2016). U pasa s DKM-om su serumske koncentracije cTnI-a bile

značajno povišene u odnosu na zdrave (Spratt i sur., 2005), ali i u pasa bez simptoma u kojih se DKM razvio unutar 1,5 godine od prvog pregleda (Wess i sur., 2010). Budući da je ovaj protein nađen povišen u serumu pasa s oštećenjem bubrega i drugim sistemskim bolestima koje isključuju oštećenje srca (Porciello i sur., 2008), cTnI se ne može koristiti kao biomarker specifičan za iDKM u pasa.

Prepoznati markeri srčanih bolesti su i natrijuretski peptidi, koji se u obliku prohormona konstitutivno proizvode u kardiomiocitima, a uslijed povećanja volumena krvi i rastezanja srčanog mišića, dolazi do njihove povećane ekspresije te se otpuštaju u krv gdje ih proteaze cijepaju u N-terminalne i C-terminalne fragmente. C-terminalni fragmenti potiču vazodilataciju i diurezu u bubrezima kako bi se snizio krvni tlak, no nisu stabilni, dok N-terminalni fragmenti (poput NT-proBNP-a) imaju veću stabilnost te se mogu koristiti kao biomarkeri srčanih oboljenja (Oyama, 2013). U pasa s DKM-om nađene su povišene koncentracije NT-proBNP-a u plazmi u odnosu na zdrave, a također i u pasa bez simptoma u kojih se DKM razvio unutar 1,5 godine od uzimanja uzoraka (Wess i sur., 2011). Međutim, pokazano je da na koncentraciju NT-proBNP-a utječe i poremećaj funkcije bubrega, sistemska hipertenzija, infektivne bolesti i lijekovi koji potiču diurezu čineći ovaj biomarker nespecifičnim za DKM (Oyama i Singletary, 2010).

### *1.2.3. Kronična bolest srčanih zalistaka u pasa*

Kronična bolest srčanih zalistaka (KBSZ) najčešća je bolest srca u pasa javljajući se u više od 70% slučajeva srčanih bolesti (Borgarelli i Haggstrom, 2010). Ovu bolest karakterizira progresivna degeneracija mitralnih zalistaka (u 62% slučajeva), a ponekad i oba atrioventrikularna zaliska – mitralnih i trikuspidalnih (u 33% slučajeva) te rijetko aortnih zalistaka (1%) (Buchanan, 1977). U KBSZ-u dolazi do histopatoloških promjena u mitralnim srčanim zaliscima što vodi do njihove malformacije i biomehaničkog poremećaja funkcije. Stoga se bolest manifestira pojavom mitralne regurgitacije, odnosno vraćanja krvi u lijevi atrij za vrijeme sistole ventrikula. U daljnjem stadiju bolesti, povećanje volumena krvi u srcu uzrokuje remodeliranje lijevog atrija i ventrikula te kongestivno zatajenje srca (Fox, 2012). Klinički znakovi bolesti uključuju kašalj, otežano disanje, nemogućnost fizičke aktivnosti i gubitak težine (Olsen i sur., 2010).

Psi oboljeli od KBSZ-a mogu se svrstati u jedan od 4 stupnja prema Američkom društvu za veterinarsku internu medicinu (engl. *American College of Veterinary Internal Medicine*, ACVIM) (Keene i sur., 2019):

1) Stupanj A: asimptomatski psi koji su predisponirani za razvoj bolesti
2) Stupanj B: prisutan šum na srcu uslijed mitralne regurgitacije te strukturne promjene zalistaka, bez kliničkih znakova zatajenja srca
3) Stupanj C: klinički znakovi zatajenja srca
4) Stupanj D: krajnja faza bolesti s kliničkim znakovima zatajenja srca u kojoj lijekovi više ne pomažu

Prevalencija KBSZ-a se razlikuje između pasmina, a najčešća je u pasmina male i srednje veličine (<20 kg), a rjeđa u velikih pasmina (Häggström i sur., 2009). Bolest osobito pogađa pasmine poput *Cavalier King Charles* španijela, bul terijera i jazavčara te se javlja češće u mužjaka (Suh i sur., 2016). U malih pasa bolest uglavnom napreduje sporije, pri čemu se u većine javlja šum mitralne regurgitacije i godinama prije zatajenja srca, dok u većih pasa bolest obično brže napreduje (Atkins i sur., 2009). Prevalencija KBSZ-a znatno raste s dobi u malih pasmina te je utvrđeno da 85% malih pasa u dobi do 13 godina imaju neki nalaz oštećenja zalistaka, što ne znači da nužno razviju kliničke znakove zatajenja srca (Borgarelli i sur., 2008). Pokazano je da je KBSZ prisutna u oko 50% *Cavalier King Charles* španijela u dobi od 6-7 godina (Häggström i sur., 1992) i u oko 50% jazavčara u dobi od 10 godina (Pedersen i sur., 1996).

Uzroci KBSZ-a su većim dijelom nepoznati, iako je u nekih pasmina pokazano da genetski faktori i starenje utječu na nastanak bolesti (Borgarelli i sur., 2008). Istraživanja su pokazala da u predisponiranih vrsta poput *Cavalier King Charles* španijela postoje geni koji utječu na nastanak bolesti (Madsen i sur., 2011). Nekoliko gena i regulatornih puteva su potencijalno uključeni u patogenezu bolesti, a važnu ulogu imaju proteini uključeni u remodeliranje izvanstaničnog matriksa poput matriks metaloproteinaza i njihovih inhibitora, kao i signalni put koji uključuje transformirajući faktor rasta beta (Lewicki i sur., 2025).

Patogenezu KBSZ-a karakteriziraju stalne promjene u staničnim komponentama te izvanstaničnom matriksu mitralnih zalistaka, poput promjena u sastavu kolagena i kolagenskih vlakana te nakupljanju glikozaminoglikana, što vodi do zadebljanja, deformacije i poremećaja funkcije mitralnog zaliska (Aupperle i Disatian, 2012). Čest nalaz u pasa s KBSZ-om je prolaps mitralnog zaliska, odnosno izvrtanje listića mitralnog zaliska u lijevi atrij tijekom sistole, što dovodi do regurgitacije i s napredovanjem bolesti vodi do remodeliranja atrija i ventrikula te njihovog poremećaja funkcije (Sargent i sur., 2015). Smatra se da patogenezi bolesti pridonose

i neurohormonski i imunosni sustav s utjecajem na napredovanje poremećaja funkcije miokarda, no ti su mehanizmi većim dijelom nerazjašnjeni (Aupperle i Disatian, 2012).

Dijagnoza KBSZ-a postavlja se na temelju kliničkog pregleda, elektrokardiografije, radiografije i ultrazvuka (Suh i sur., 2016). Od koristi može biti i određivanje razine serumskih markera troponina i natrijuretskih peptida. U pasa s KBSZ-om nađene su povišene serumske koncentracije cTnI-a (Ljungvall i sur., 2010) i NT-proBNP-a (Wolf i sur., 2013) te je pokazano da ovi markeri mogu biti korisni u prognozi smrtnog ishoda (Mattin i sur., 2019). Međutim, kao što je navedeno u prethodnom potpoglavlju, ovi markeri nisu specifični samo za srčana oboljenja.

### *1.3. Ciljevi i hipoteze rada*

Glavni cilj istraživanja ovog rada je pomoću proteomskog pristupa otkriti proteine prisutne u tjelesnim tekućinama pasa (serumu i/ili urinu) koji bi mogli biti od značaja kao biomarkeri u dijagnostici:

1) poremećaja funkcije bubrega u babeziozi
2) poremećaja funkcije srca u idiopatskoj dilatativnoj kardiomiopatiji
3) poremećaja funkcije srca u kroničnoj bolesti srčanih zalistaka.

Hipoteza ovog istraživanja je da postoje razlike između proteoma seruma i urina pasa s poremećajem funkcije bubrega invadiranih s protozoonom *Babesia canis* i proteoma seruma i urina zdravih pasa koje upućuju na poremećaj u radu bubrega. Također, hipoteza je da postoje razlike između proteoma seruma pasa s idiopatskom dilatativnom kardiomiopatijom, odnosno pasa s kroničnom bolesti srčanih zalistaka, i zdravih pasa koje odražavaju sistemske promjene uslijed poremećaja funkcije srca.

Postojeći biomarkeri koji mogu biti od koristi u dijagnostici navedenih bolesti imaju ograničenja, poput nespecifičnosti te neosjetljivosti, odnosno kasne pojave u tijeku bolesti. S obzirom da postoji potreba za novim specifičnim i osjetljivim biomarkerima s dijagnostičkim i/ili prognostičkim značajem koji bi se uveli u rutinsku kliničku uporabu u navedenim bolestima, ovo istraživanje može pridonijeti njihovom otkriću. Također, uzimajući u obzir da su patofiziološki mehanizmi u poremećaju funkcije bubrega u babeziozi pasa, odnosno poremećaju funkcije srca u idiopatskoj dilatativnoj kardiomiopatiji pasa i kroničnoj bolesti srčanih zalistaka uvelike nerazjašnjeni, ovim radom identificirani proteini od značaja mogu pridonijeti njihovom boljem razumijevanju.

Proteomski pristup u istraživanju navedenih bolesti slabije je zastupljen u literaturi, što predstavlja originalan pristup temi rada. Naime, pregledom literature utvrđeno je da je u ovom istraživanju navedenih bolesti prvi put korištena visokoprotočna kvantitativna proteomska metoda pomoću izobarnih privjesaka (TMT) i LC-MS/MS-a uz bioinformatičku analizu rezultata što omogućuje istovremeno otkrivanje velikog broja potencijalnih biomarkera te bioloških puteva uključenih u razvoj bolesti.

## 2. RASPRAVA

U posljednjem desetljeću, a osobito posljednjih godina, došlo je do provedbe većeg broja proteomskih istraživanja tjelesnih tekućina pasa (poput seruma, sline i urina) u potrazi za potencijalnim biomarkerima koji pridonose osjetljivoj i specifičnoj dijagnostici različitih bolesti te pridonose njihovom boljem razumijevanju (Gonzalez-Arostegui i sur., 2022; Ahn i sur., 2023). Psi predstavljaju najraširenije vrstu mesojeda, kojih prema podacima iz 2017. godine ima oko 210 milijuna u 10 država s najvećim brojem pasa (Miller i sur., 2020). Ovaj podatak potvrđuje društvenu važnost psa za čovjeka te čini istraživanje bolesti pasa potrebnim u svrhu poboljšanja liječenja bolesti, odnosno produženja životnog vijeka pasa. Također, uzimajući u obzir da ljudi i psi dijele sličnu anatomiju i fiziologiju te žive u sličnim okolišnim uvjetima, psi služe i kao modelni organizmi u istraživanjima čiji rezultati mogu biti primjenjivi i za čovjeka (Bilić i sur., 2018a).

### *2.1. Poremećaj funkcije bubrega u babeziozi pasa*

Uzimajući u obzir čestu pojavnost poremećaja funkcije bubrega u babeziozi pasa, što u slučaju akutne ozljede bubrega (AOB) može nositi visoku stopu smrtnosti od 50% (Máthé i sur., 2006), od velike je važnosti rana dijagnostika smanjene funkcije bubrega u pasa s babeziozom. S obzirom da su postojeći biomarkeri poput serumskog kreatinina i UPCR-a neosjetljivi i nespecifični za mjesto oštećenja bubrega, postoji potreba za novim biomarkerima koji mogu rano detektirati poremećaj funkcije bubrega te ukazivati na mjesto ozljede kako bi liječenje bilo što učinkovitije. S obzirom da postoji potreba za pronalaskom prikladnih biomarkera za rano otkrivanje oštećenja bubrega u pasa s babeziozom, visokoprotočne proteomske metode daju prednost otkrivanja velikog broja potencijalnih markera koji imaju značenje u dijagnostici, određivanju stupnja ili prognozi bolesti te pridonose razumijevanju patofizioloških procesa. Proteomski pristup u istraživanju poremećaja funkcije bubrega u pasa relativno je slabo zastupljen, a najčešće korištena metoda je 2D gel-elektroforeza uz spektrometriju masa (Nabity i sur., 2011; Palviainen i sur., 2012; Hormaeche i sur., 2014; Bracha i sur., 2014), što ima potencijal za otkrivanje manjeg broja biomarkera.

Ovom doktorskom radu priložena su dva objavljena znanstvena članka u kojima je ispitan poremećaj funkcije bubrega u babeziozi pasa u uzorcima seruma i urina; i to u jednom istraživanju uporabom proteomske metode (Prilog 1), a u drugom korištenjem komercijalno dostupnih testova (Prilog 2). Nadalje, priloženi su i neobjavljeni rezultati preliminarnog proteomskog istraživanja dobiveni ispitivanjem manjeg broja uzoraka pomoću tekućinske

kromatografije i tandemne spektrometrije masa gdje je uspoređen proteom urina pasa s babeziozom (N=6) i zdravih pasa (N=6), a prikazani su Prilogom 3 (Tablica 1).

U proteomskom istraživanju koje je sastavni dio ovog rada potvrđena je hipoteza da postoje razlike između proteoma seruma i urina pasa s poremećenom funkcijom bubrega invadiranih s protozoonom *Babesia canis* i proteoma seruma i urina zdravih pasa koje upućuju na poremećaj u radu bubrega (Prilog 1). U objavljenom istraživanju pasa s poremećajem funkcije bubrega u babeziozi po prvi put je korištena kvantitativna visokoprotočna proteomska metoda uz bioinformatičku analizu gdje su analizirani uzorci seruma i urina istih pasa kako bi se dobio kompletniji uvid u patološke promjene u kontekstu ove bolesti. S obzirom da su psi s babeziozom bili podijeljeni u skupine prema stupnju poremećaja funkcije bubrega (skupina A, B i C), dobivene razlike u proteomu seruma i urina između skupina omogućile su otkriće potencijalnih ranih proteinskih biomarkera koji se javljaju i prije povećanja koncentracije serumskog kreatinina (azotemije).

U istraživanjima priloženim ovom radu nađeno je mnoštvo potencijalnih serumskih i urinarnih biomarkera koji upućuju na poremećaj funkcije bubrega u babeziozi na razini glomerula, proksimalnih i distalnih kanalića nefrona, od kojih bi neki mogli služiti kao rani i specifični biomarkeri za AOB i prije razvoja azotemije.

### *2.1.1. Proteinski serumski biomarkeri*

Serumski proteini od značaja otkriveni u pasa s poremećajem rada bubrega u babeziozi u odnosu na zdrave većinom odražavaju sistemske patofiziološke procese povezane s babeziozom i imunosnim odgovorom domaćina, a proteini prisutni u urinu u povećanoj količini upućuju i na sistemske i lokalne procese u bubrezima. Iz tog razloga su serumski proteini sa značajno promijenjenom razinom u pasa s babeziozom manje indikativni za poremećaj rada bubrega nego urinarni uzimajući u obzir sistemske promjene u hemostazi i upalnom odgovoru koji su pod utjecajem raznih posrednika i puteva aktiviranih prisustvom parazita. U proteomskom istraživanju uključenom u ovaj rad nađena je povećana razina nekoliko serumskih proteina koji sudjeluju u imunosnom odgovoru domaćina poput različitih komponenti komplementa te proteina akutne faze upale u pasa s babeziozom. Bioinformatička analiza serumskih proteina sa značajno promijenjenom razinom uputila je na uključenost akutnog upalnog odgovora i regulacije koagulacije krvi, što je u skladu i s prethodnim istraživanjima promjena serumskog proteoma u pasa invadiranih s *B. canis* u odnosu na zdrave (Kuleš i sur., 2014; Kuleš i sur., 2016). Ipak, nekoliko serumskih proteina u pasa s babeziozom,

čije su povećane razine nađene i u serumu i urinu pasa s babeziozom u odnosu na zdrave, moglo bi biti od značaja u kontekstu poremećaja rada bubrega, poput leucinom-bogatog alfa-2-glikoproteina 1 (engl. *Leucine-rich alpha-2-glycoprotein 1*, LRG1) i alfa-1-kiselog glikoproteina (engl. *Alpha-1 acid glycoprotein*, AGP).

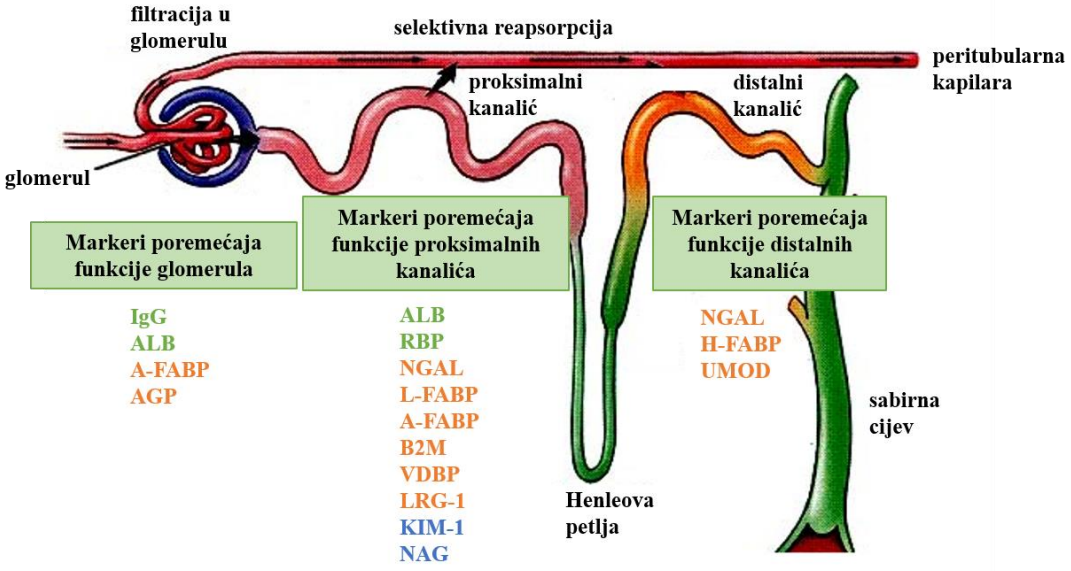
U proteomskom istraživanju uključenom u ovaj rad nađena je povećana razina leucinom-bogatog alfa-2-glikoproteina 1 (LRG1-a) u serumu i urinu pasa s babeziozom u svih skupina u odnosu na zdrave, kao i u urinu pasa s babeziozom u odnosu na zdrave u preliminarnom istraživanju. LRG-1 je serumski glikoprotein molekulske mase 50 kDa koji se proizvodi u sklopu odgovora akutne faze upale u jetri i neutrofilima, ali i drugim tipovima stanica posredstvom proupalnih citokina. LRG-1 je prepoznat kao serumski biomarker za upalne bolesti poput reumatoidnog artritisa i upalne bolesti crijeva, no i za dijabetes i neke tipove raka. Također, povećane količine LRG1-a nađene su u serumu, urinu i/ili tkivu bubrega u različitim oboljenjima bubrega, poput dijabetičke nefropatije, AOB-a, IgA nefropatije, bubrežne fibroze i kronične bolesti bubrega (Chen i sur., 2024). Na mišjem modelu je pokazano da tijekom oštećenja kanalića nefrona proupalni citokin interleukin-1 beta inducira ekspresiju LRG1-a u stanicama kanalića što povećava koncentraciju LRG-1-a u urinu (Lee i sur., 2018). Povećana ekspresija LRG-1-a u urinu mogla bi upućivati na upalu, hipoksiju i infiltraciju imunosnih stanica tijekom AOB-a, a inhibicijom LRG-1-a bi se moglo postići usporavanje napredovanja bubrežnih bolesti povezanih s upalom (Chen i sur., 2024). U proteomskom istraživanju pasa s lišmaniozom koji su imali poremećaj funkcije bubrega u urinu je nađena povećana količina LRG-1-a (Gonzalez i sur., 2022). U kontekstu babezioze povećane razine ovog biomarkera u serumu i urinu bi mogle biti povezane i s imunosnim odgovorom domaćina na invaziju s parazitom, kao i s poremećajem u radu bubrega.

Alfa-1-kiseli glikoprotein (AGP) je još jedan glikoprotein od značaja detektiran u našem radu s povećanom razinom u serumu i urinu pasa s babeziozom u svih skupina u odnosu na zdrave, kao i u urinu pasa s babeziozom u preliminarnom istraživanju. Povećana serumska koncentracija u skupina B i C pasa s babeziozom u odnosu na zdrave pse potvrđena je i metodom ELISA. AGP je glikoprotein s molekulskom masom od 43 kDa s velikim udjelom ugljikohidrata (45%) koji kao dio odgovora akutne faze upale djeluje protuupalno s aktivnostima usmjerenim protiv neutrofila, komplementa i aktivacije makrofaga kako bi modulirao imunosni odgovor. U pasa je detektiran u serumu kao protein akutne faze upale s umjerenim porastom (2-10x) u bolestima poput babezioze i erlihioze (Cerón i sur., 2005). U ljudi s različitim bubrežnim oboljenjima utvrđene su povećane urinarne koncentracije AGP-a,

kao i u proteinuriji povezanoj s upalom (Magid i sur., 2005). U istraživanju autora Talks i sur. (2018) pokazano je da je urinarni AGP osjetljiviji marker za ozljedu glomerularne filtracijske barijere od albumina u zdravih ljudi izloženih hipoksiji u uvjetima visoke nadmorske visine. Povećanje razine AGP-a u serumu i urinu pasa s babeziozom utvrđeno u našem istraživanju odraz je odgovora akutne faze upale, ali moguće i poremećaja funkcije bubrega.

### *2.1.2. Proteinski urinarni biomarkeri*

Rezultati našeg proteomskog istraživanja biomarkera u urinu pasa s babeziozom nadopunjuju se s rezultatima drugog istraživanja uključenog u ovaj rad gdje je ispitan poremećaj rada bubrega u pasa s babeziozom koristeći komercijalno dostupne testove za odabrane urinarne biomarkere s dijagnostičkim značajem za AOB u ljudi koji upućuju na poremećaj funkcije glomerula i/ili kanalića nefrona (Prilog 2). S obzirom da su psi s babeziozom bili podijeljeni u skupine prema stupnju oštećenja bubrega (skupine A, B i C) kao i u proteomskom radu, dobivene razlike omogućile su otkriće potencijalnih ranih proteinskih biomarkera. Slikom 5. predočeni su objedinjeni rezultati dvaju članaka uključenih u ovaj rad koji prikazuju potencijalne proteinske biomarkere u urinu za poremećaj funkcije bubrega u pasa s babeziozom invadiranih s *B. canis*, a koji su otkriveni korištenjem proteomske metode, komercijalnih testova ili oboje.




Slika 5. Shematski prikaz nefrona s pregledom potencijalnih urinarnih proteinskih biomarkera za poremećaj funkcije bubrega u pasa s babeziozom otkrivenih u priloženim radovima. Proteini otkriveni u oba objavljena rada označeni su zelenom bojom, proteini otkriveni proteomskim istraživanjem narančastom bojom, a proteini otkriveni komercijalnim metodama plavom bojom. Kratice: IgG = imunoglobulin gama, ALB = albumin, A-FABP/L-FABP/H-FABP = adipocitni/jetreni/srčani protein koji veže masne kiseline, AGP = alfa-1-kiseli glikoprotein, RBP = retinol-vezujući protein, NGAL = lipokalin udružen s neutrofilnom gelatinazom, B2M = beta-2-mikroglobulin, VDBP = vitamin D-vezujući protein, LRG-1 = leucinom-bogati alfa-2-glikoprotein 1, KIM-1 = molekula bubrežne ozljede-1, NAG = N-acetil-glukozaminidaza, UMOD = uromodulin. Slika nefrona kao predloška preuzeta iz Mader (1991).

Razina većeg broja biomarkera prikazanih Slikom 5 otkrivena je kao značajno povećana i prije razvoja proteinurije i azotemije (poput KIM-1-a, NGAL-a i L-FABP-a) pa bi oni mogli služiti kao rani markeri za poremećaj funkcije bubrega u babeziozi. S obzirom da se na temelju otkrivenih biomarkera od značaja u urinu može zaključiti da poremećaj funkcije bubrega u pasa s babeziozom postoji i prije povišenja rutinskih dijagnostičkih markera, u svih pasa s dijagnozom invazije s *B. canis* potrebno je pratiti bubrežnu funkciju tijekom i poslije liječenja, i kada ne postoje dokazi za AOB temeljeni na rutinskim laboratorijskim mjerenjima. Uzimajući u obzir kompleksnost bolesti i heterogenost u populaciji, može se predložiti da bi u kliničkoj primjeni od koristi bila analiza panela urinarnih biomarkera prikazanih Slikom 5, kako bi se postigla visoka osjetljivost i specifičnost za dijagnozu poremećaja rada bubrega u pasa invadiranih s *B. canis*, što ponajprije zahtjeva validaciju na većem broju uzoraka. Predloženi

panel biomarkera predstavlja zajednički doprinos rezultata istraživanja uključenih u doktorski rad.

Objedinjeni rezultati ova dva istraživanja upućuju na postojanje oštećenja/poremećaja u funkciji bubrega na razini glomerula, proksimalnih i distalnih kanalića nefrona u pasa s babeziozom, što je u skladu s literaturom (Winiarczyk i sur., 2017). U istraživanjima priloženim ovom radu utvrđeno je da su u urinu pasa s poremećajem funkcije bubrega u babeziozi prisutni proteini sa značajno promijenjenim razinama podrijetlom iz seruma s velikom, srednjom i malom molekulskom masom, kao i proteini bubrežnog podrijetla oslobođeni iz oštećenih distalnih kanalića. Prisutnost serumskih proteina velike molekulske mase u urinu (poput IgG-a) upućuje na poremećaj funkcije glomerularne filtracijske barijere, a serumskih proteina srednje i male molekulske mase (poput ALB-a, RBP-a, VDBP-a, B2M-a) na oštećenje/poremećaj funkcije proksimalnih bubrežnih kanalića u pasa s babeziozom. Potonje može biti posljedica direktnog oštećenja stanica proksimalnih kanalića, ali i zasićenja receptora megalina za reapsorpciju proteina srednje i male molekulske mase zbog kompeticije s drugim prisutnim proteinima u lumenu kanalića. Također, pokazano je da ishemija (smanjen protok krvi) ili proupalni citokini mogu utjecati na smanjenu ekspresiju receptora megalina za reapsorpciju proteina male i srednje molekulske mase u proksimalnim kanalićima (Vinge i sur., 2010). S obzirom da u poremećaju funkcije bubrega u babeziozi važnu ulogu imaju hipoksija i pretjerani upalni odgovor (Zygner i sur., 2014), ovi mehanizmi moguće imaju direktan utjecaj na oštećenje/poremećaj funkcije proksimalnih kanalića.

*2.1.2.1. Proteinski urinarni biomarkeri poremećaja funkcije glomerula*

Povećana koncentracija albumina (ALB) u urinu upućuje na postojanje poremećaja funkcije na razini glomerula budući da se s molekulskom masom od 69 kDa ne bi trebao filtrirati iz krvi. Ipak, manja količina koja prolazi barijeru se reapsorbira u proksimalnim kanalićima, te njegova povećana urinarna koncentracija može upućivati i na poremećaj funkcije kanalića nefrona, čineći ga nespecifičnim markerom za mjesto oštećenja bubrega (De Loor i sur., 2013). U oba naša istraživanja razina urinarnog ALB-a bila je značajno povećana u pasa s babeziozom u odnosu na zdrave, upućujući na poremećaj funkcije u radu glomerula i/ili proksimalnih kanalića, što je u skladu s drugim istraživanjima pasa s bolestima bubrega, primjerice u pasa s nasljednom nefropatijom vezanom uz kromosom X, u pasa genetički predisponiranih za nefropatiju te u pasa s kroničnom bubrežnom bolesti (De Loor i sur., 2013). Ipak, pokazano je da povećane koncentracije urinarnog ALB-a mogu biti prisutne i u bolesti koje nisu bubrežne, smanjujući njegovu specifičnost (Whittemore i sur., 2006).

Specifičniji biomarker za oštećenje glomerula je imunoglobulin G (IgG), protein velike molekulske mase koji se normalno ne filtrira u glomerulima (D'Amico i Bazzi, 2003). U oba istraživanja uključena u ovaj rad nađena je povećana razina urinarnog IgG-a u pasa s babeziozom, što upućuje na oštećenje glomerularnih kapilara, a u skladu je i s drugim istraživanjima pasa invadiranih s *B. rossi* i *B. canis* (Defauw i sur., 2012; Winiarczyk i sur., 2017), kao i pasa s kroničnom bubrežnom bolesti i drugim sistemskim bolestima koje uključuju poremećaj funkcije bubrega (Hokamp i Nabity, 2016).

Jedan od otkrivenih proteina koji vežu masne kiseline (engl. *Fatty Acid-Binding Protein,* FABP) s povećanom razinom u urinu pasa s babeziozom u skupina B i C u odnosu na zdrave je i adipocitni protein koji veže masne kiseline (engl. *Adipocyte Fatty Acid-Binding Protein*, A-FABP), maleni protein koji ima ulogu u prijenosu masnih kiselina u adipocitima. A-FABP je eksprimiran i u makrofazima te epitelnim i endotelnim stanicama različitih tkiva. Porast cirkulirajućeg serumskog A-FABP koji se oslobađa iz adipocita mogao bi služiti kao potencijalni biomarker za metaboličke i vaskularne bolesti. S obzirom da se serumski A-FABP slobodno filtrira u glomerulima, njegov porast bi mogao biti marker za smanjenu brzinu glomerularne filtracije upućujući na oštećenje bubrega (Shi i sur., 2019). A-FABP se nakon filtracije u glomerulima reapsorbira u proksimalnim kanalićima endocitozom ovisnom o megalinu, te bi njegov porast u urinu mogao služiti kao marker poremećaja funkcije/oštećenja proksimalnih kanalića (Shrestha i sur., 2018), ali i glomerula. Naime, pokazano je da se A-FABP ne eksprimira u endotelnim stanicama glomerula u fiziološkim uvjetima, već u oštećenom bubregu gdje je njegova ekspresija povezana s proteinurijom i smanjenim GFR-om, odnosno poremećajem funkcije bubrega u ljudi s bubrežnim oboljenjima (Okazaki i sur., 2014). Dakle, povećana razina urinarnog A-FABP-a utvrđena u proteomskom istraživanju pasa s babeziozom u skupina B i C u odnosu na zdrave može uputiti na postojanje poremećaja funkcije glomerula ili proksimalnih kanalića nefrona.

*2.1.2.2. Proteinski urinarni biomarkeri poremećaja funkcije proksimalnih i distalnih kanalića*

U oba istraživanja uključena u ovaj rad nađena je povećana razina urinarnog retinol-vezujućeg proteina (RBP) u grupa pasa B i C s babeziozom u odnosu na zdrave. Rezultati proteomskog istraživanja za urinarni RBP priloženi su u dopunskim materijalima objavljenog članka (proteini sa značajno promijenjenom razinom, $p < 0.05$ i $1.7 < \log_2 FC < 1.7$). RBP je jetreni protein male molekulske mase (21 kDa) koji ima funkciju prenošenja retinola u krvi, a veže se u kompleks s transtiretinom što sprječava njegovu glomerularnu filtraciju. Slobodni RBP prolazi kroz glomerule, ali se učinkovito reapsorbira u proksimalnim kanalićima nefrona

endocitozom putem receptora megalina i koreceptora kubilina, pa stoga prisutnost RBP-a u urinu upućuje na poremećaj funkcije proksimalnih kanalića (Raila i sur., 2000). RBP je pronađen u urinu u povećanoj koncentraciji u pasa s babeziozom invadiranih s *B. rossi* (Defauw i sur., 2012) i *B. canis* (Winiarczyk i sur., 2017) u odnosu na zdrave, ali i u pasa s piometrom, kamencima u mokraćnom sustavu i kroničnom bubrežnom bolesti (De Loor i sur., 2013). Rezultati oba naša istraživanja u kojima je nađena povećana razina RBP-a u urinu upućuju na postojanje poremećaja funkcije proksimalnih kanalića nefrona u pasa s proteinurijom invadiranih s *B. canis*. Potrebno je napomenuti da je u našem proteomskom istraživanju nađeno da serumski RBP ima smanjenu razinu u pasa s babeziozom u odnosu na zdrave, što je očekivano s obzirom da je on negativni protein akutne faze upale čija se proizvodnja u jetri smanjuje tijekom upale (Rosales i sur., 1996). S obzirom da smanjenje njegove proizvodnje ovisi o jakosti upalnog odgovora (Tóthová i Nagy, 2017), korištenje urinarnog RBP-a kao samostalnog biomarkera za poremećaj rada bubrega u babeziozi je vjerojatno nedostatno.

Još jedan marker oštećenja stanica proksimalnih kanalića je lizozimski enzim N-acetil-glukozaminidaza (NAG), protein velike molekulske mase (150 kDa) koji se uslijed oštećenja cjelovitosti stanica proksimalnih kanalića pojavljuje u urinu (Sato i sur., 2002). Mjerenje njegove aktivnosti u urinu koristi se kao mjera funkcije kanalića nefrona te je njegova povećana aktivnost nađena u urinu ljudi s različitim bubrežnim bolestima (Vaidya i sur., 2008), ali i u pasa s kroničnom bubrežnom bolesti (Hokamp i Nabity, 2016) te početnim stupnjem AOB-a u lišmaniozi (Ruiz i sur., 2023). U našem istraživanju otkrivena je njegova povećana aktivnost korištenjem enzimatskog kolorimetrijskog testa u pasa s babeziozom u odnosu na zdrave, upućujući na postojanje oštećenja stanica proksimalnih kanalića.

U preliminarnom proteomskom istraživanju nađena je povećana razina vitamin D-vezujućeg proteina (engl. *Vitamin D-Binding Protein*, VDBP) u urinu pasa s babeziozom u odnosu na zdrave pse. Isto je nađeno u proteomskom istraživanju s većim brojem uzoraka u svih skupina pasa s babeziozom (A, B i C) u odnosu na zdrave te u pasa s proteinurijom u odnosu na one bez proteinurije u babeziozi (B/A). VDBP je protein koji veže i prenosi vitamin D i njegove metabolite u krvi te se u obliku kompleksa s vitaminom D djelomično filtrira kroz glomerularnu barijeru. Njegove količine u urinu su uobičajeno minimalne budući da se reapsorbira endocitozom preko receptora megalina/kubilina u proksimalnim kanalićima gdje se degradira (De Brito Galvao i sur., 2013). Iz tog razloga njegova povećana koncentracija u urinu upućuje na poremećaj funkcije proksimalnih kanalića, a predloženo je i da može služiti kao marker za tubulointersticijsku fibrozu (Mirković i sur., 2013). U pasa s nasljednom

nefropatijom vezanom uz kromosom X nađeno je povećanje količine VDBP-a u urinu s napretkom bolesti u odnosu na zdrave pse (Vinge i sur., 2010), a u pasa s kroničnom bubrežnom bolesti bez proteinurije i s graničnom proteinurijom, utvrđeno je povećanje koncentracije urinarnog VDBP-a u ranom stupnju bolesti (Chacar i sur., 2017).

Potencijalni urinarni marker za oštećenje proksimalnih kanalića je i beta-2-mikroglobulin (B2M), protein koji se eksprimira na površini stanica s jezgrom kao sastavni dio glavnog sustava tkivne podudarnosti. B2M se slobodno filtrira u glomerulima zbog male molekulske mase, a zatim reapsorbira endocitozom posredstvom receptora megalina/kubilina u epitelnim stanicama proksimalnih kanalića (Schardijn i van Eps, 1987). U preliminarnom proteomskom istraživanju, kao i u objavljenom proteomskom radu nađena je povećana razina B2M-a u urinu pasa s babeziozom svih skupina u odnosu na zdrave što upućuje na poremećaj funkcije proksimalnih kanalića. Urinarni B2M je i otprije detektiran korištenjem spektrometrije masa kao rani biomarker za oštećenje bubrega u pasa s nasljednom progresivnom glomerularnom bolesti (Nabity i sur., 2011) te u pasa otrovanih otrovom zmije *Vipera berus* (Palviainen i sur., 2012).

U našem preliminarnom proteomskom istraživanju razina proteina uromodulina (UMOD) u urinu bila je smanjena u pasa s babeziozom u odnosu na zdrave. Ovo je nadalje potvrđeno u proteomskom istraživanju s većim brojem uzoraka gdje je nađena smanjena razina UMOD-a u svih skupina pasa s babeziozom u odnosu na zdrave, kao i u skupina s babeziozom s proteinurijom (B i C) u odnosu na grupu bez proteinurije. U istraživanju uključenom u ovaj rad gdje je korišten imunotest ELISA nije nađena razlika u urinarnoj koncentraciji UMOD-a u pasa s babeziozom u odnosu na zdrave. Moguće je da je takav rezultat dobiven radi interferencije testa s hemoglobinurijom (prisutnosti hemoglobina u urinu) zbog čega je isključen dio uzoraka iz skupina B i C. UMOD je glikoprotein veličine 100 kDa kojeg se uobičajeno može detektirati u urinu zdravih ljudi i pasa u većoj koncentraciji (Ferlizza i sur., 2020), a ima uloge u vezanju metabolita vitamina A, održavanju ravnoteže vode i elektrolita, obrani protiv infekcija u mokraćnom traktu, prevenciji formiranja bubrežnih kamenaca te urođenoj imunosti bubrega (Rampoldi i sur., 2011). S obzirom da je bubrežnog podrijetla, odnosno proizvode ga isključivo epitelne stanice Henleove petlje (90%) i distalnih kanalića nefrona (10%), njegova smanjena količina u urinu može služiti kao biomarker za oštećenje distalnih dijelova nefrona (Tokonami i sur., 2018). Budući da su stanice koje proizvode UMOD osobito osjetljive na ishemijsku ozljedu radi velike metaboličke aktivnosti, smanjena količina UMOD-a u urinu pasa s babeziozom može uputiti na oštećenje distalnih kanalića radi

hipoksičnih uvjeta u bubrežnom tkivu. Naš nalaz je u skladu s istraživanjem autora Raila i sur. (2014) gdje je korištenjem metode Western blot nađena smanjena količina UMOD-a u urinu pasa s umjerenom i izraženom azotemijom i proteinurijom, ali nije potvrđen istraživanjem autora Winiarczyk i sur. (2017) gdje je pomoću ELISA-e nađena povećana koncentracija urinarnog UMOD-a u pasa s babeziozom. Nedostaci tog istraživanja su uključenost manjeg broja pasa te postojanje hemoglobinurije u većem broju uzoraka urina što je moglo interferirati s testom. U novijim istraživanjima kronične bubrežne bolesti pokazano je da u pasa s proteinurijom postoji smanjena količina uromodulina u urinu (Ferlizza i sur., 2020; Daza i sur, 2024; Marečáková i sur., 2024).

*2.1.2.2.1. Rani proteinski urinarni biomarkeri poremećaja funkcije kanalića*

U urinu pasa s babeziozom u istraživanjima uključenim u ovaj rad otkriveni su proteini koji bi mogli služiti kao rani biomarkeri poremećaja funkcije bubrega. U radu gdje su korištene komercijalne metode, kao rani biomarker se ističe molekula bubrežne ozljede-1 (engl. *Kidney Injury Molecule-1*, KIM-1), a u proteomskom radu urinarni lipokalin udružen s neutrofilnom gelatinazom (engl. *Neutrophil Gelatinase-Associated Lipocalin*, NGAL) te jetreni protein koji veže masne kiseline (engl. *Liver-type Fatty Acid-Binding Protein,* L-FABP). Povećana urinarna koncentracija NGAL-a i L-FABP-a validirana je metodom ELISA u skupina B i C pasa s babeziozom u odnosu na zdrave.

Molekula bubrežne ozljede-1 (KIM-1) je transmembranski glikoprotein čija ekspresija značajno raste nakon bubrežne ozljede u stanicama proksimalnih kanalića nefrona te se stoga KIM-1 smatra specifičnim biomarkerom za njihovo oštećenje (Jin i sur., 2017). U ljudi se koristi kao rani biomarker za AOB gdje je zabilježen porast njegove urinarne koncentracije 2 h nakon ozljede bubrega, a koncentracija je ostala povećana do 48 h nakon ozljede (Yun i Craig, 2011). U istraživanju autora Lippi i sur. (2018) nađena je povećana koncentracija KIM-1 u urinu pasa s AOB-om prije porasta serumskog kreatinina, a isto je nađeno i u nedavnim istraživanjima u urinu pasa s AOB-om prije pojave azotemije u babeziozi (Idress i sur., 2024) i leptospirozi (Dias i sur., 2021; Idress i sur., 2024). Također, razina KIM-1-a u plazmi te omjer razine KIM-1-a i kreatinina u urinu pouzdani su biomarkeri za rano otkrivanje stabilne kronične bolesti bubrega u pasa, čak i prije razvoja azotemije (Yu i Kim, 2026). Iako KIM-1 nije identificiran u urinu pasa u proteomskom istraživanju uključenom u ovaj rad, u istraživanju gdje je korištena ELISA otkrivena je njegova povećana koncentracija u svih skupina pasa s babeziozom u odnosu na zdrave, pa i u grupi A (bez azotemije i proteinurije) čineći ga

potencijalnim ranim biomarkerom koji upućuje na oštećenje proksimalnih kanalića u pasa s poremećajem funkcije bubrega u babeziozi.

Lipokalin udružen s neutrofilnom gelatinazom (NGAL) je protein molekulske mase 25 kDa kojeg izlučuju imunosne stanice poput aktiviranih neutrofila i makrofaga tijekom upale, ali i tubularne stanice u bubrezima nakon ozljede i tijekom regeneracije bubrežnog tkiva (Hsu i sur., 2014). Povišene razine NGAL-a u urinu nađene su u ljudi s AOB-om prije povišenja razine serumskog kreatinina zbog čega se NGAL smatra ranim biomarkerom ozljede bubrega, a do njegovog povećanog lučenja u urin dolazi uslijed povećane sinteze u distalnom dijelu nefrona te smanjene reapsorpcije zbog oštećenja proksimalnih tubula (Romejko i sur., 2023). Iz tog razloga NGAL se smatra biomarkerom za oštećenje i proksimalnih i distalnih dijelova bubrežnih kanalića. U pasa s bolestima bubrega nađene su povećane koncentracije NGAL-a u urinu (Hsu i sur., 2014), što je u skladu i s našim proteomskim istraživanjem gdje je utvrđena povećana razina NGAL-a u urinu svih skupina pasa s babeziozom (A, B i C) u odnosu na zdrave pse, a taj rezultat je validiran pomoću ELISA-e gdje je nađena značajno veća koncentracija urinarnog NGAL-a normaliziranog u odnosu na kreatinin u skupini B i C u odnosu na kontrolnu skupinu. U skladu s time, u istraživanju autora Idress i sur. (2024) korištenjem metode ELISA nađena je značajno povišena koncentracija urinarnog NGAL-a u pasa s AOB-om u babeziozi i prije povišenja serumskog kreatinina.

Rani biomarker poremećaja funkcije bubrega u babeziozi pasa koji je otkriven u urinu i prije razvoja azotemije mogao bi biti jetreni protein koji veže masne kiseline (L-FABP). L-FABP je maleni citosolni protein koji se proizvodi u jetri, crijevima i epitelnim stanicama proksimalnih tubula. Njegova uloga je prijenos masnih kiselina unutar stanice, ali i vezanje produkata lipidne peroksidacije koji nastaju tijekom oksidativnog stresa (Slocum i sur., 2012). L-FABP se smatra potencijalnim ranim urinarnim biomarkerom AOB-a u ljudi koji se javlja tijekom tubulointersticijske ozljede kao odgovor na hipoksiju ili oksidativni stres (Kamijo i sur., 2004). Naš nalaz povećane razine L-FABP-a u urinu pasa s babeziozom u odnosu na zdrave pse, što je potvrđeno metodom ELISA u skupini B i C u odnosu na kontrolnu skupinu, u skladu je s radom autora Takashima i sur. (2021) koji su utvrdili značajno povećanu koncentraciju L-FABP-a u urinu pasa s bolestima bubrega u odnosu na zdrave.

Osim L-FABP-a, proteomskom metodom je u pasa s babeziozom u odnosu na zdrave uočena povećana urinarna razina i nekoliko drugih proteina iz obitelji proteina koji vežu masne kiseline (engl. *Fatty Acid-Binding Protein,* FABP). FABP-ovi su maleni citosolni proteini koji vežu i prenose dugolančane masne kiseline, ali i produkte lipidne peroksidacije koji nastaju

tijekom oksidativnog oštećenja tkiva, postižući zaštitni antioksidativni učinak (Slocum i sur., 2012). Njihova povećana razina u urinu pasa s babeziozom skupina A, B i/ili C u odnosu na zdrave upućuje na hipoksiju kao uzrok oštećenja bubrega, što je u skladu i s bioinformatičkom analizom gdje su utvrđeni značajni pojmovi ″stanični odgovor na reaktivne kisikove oblike″ te ″uklanjanje superoksidnih radikala″. Ovaj nalaz pridonosi i pretpostavci da je hipoksija u tkivu bubrega glavni uzrok ozljede bubrega u kontekstu babezioze (Zygner i Gójska-Zygner, 2014).

Povećana razina urinarnog srčanog proteina koji veže masne kiseline (engl. *Heart-type fatty acid-binding protein*, H-FABP) otkrivena je u preliminarnom proteomskom istraživanju u pasa s babeziozom u odnosu na zdrave, kao i u svih skupina pasa s babeziozom u proteomskom radu. H-FABP je maleni citosolni protein koji ima ulogu u metabolizmu masnih kiselina u tkivima s velikim energetskim zahtjevima pa se ponajviše eksprimira u srcu, mišićima, mozgu, bubrezima i žljezdanim tkivima (Rezar i sur., 2020). Njegova ekspresija je otkrivena i u distalnim kanalićima nefrona (Kimura i sur., 1999). U zdravih ljudi H-FABP ima vrlo niske serumske koncentracije, no uslijed ozljede srca oslobađa se iz citoplazme miocita u krvotok te je stoga prepoznat kao novi osjetljivi biomarker za srčani infarkt (Rezar i sur., 2020). Njegova uloga u bubrežnim oboljenjima je slabije istražena, no pokazano je da je osjetljiv marker za toksičnu ozljedu distalnih kanalića nakon primjene lijeka gentamicina (Pelsers, 2008) te za subklinički AOB u kontekstu operacije aneurizme aorte u ljudi (Kokot i sur., 2014). Povećana razina urinarnog H-FABP-a mogla bi služiti kao rani biomarker oštećenja distalnih kanalića u pasa s poremećajem funkcije bubrega u babeziozi.

### *2.1.3. Mehanizmi uključeni u poremećaj funkcije bubrega u babeziozi pasa*

Bioinformatička analiza proteina sa značajno promijenjenim razinama u oba uzorka (serumu i urinu) otkrivenih u pasa invadiranih s *B. canis* u odnosu na zdrave upućuje na biološke puteve uključene u tijek bolesti i patološke procese u bubregu. Od značaja su u kontekstu poremećaja rada bubrega metabolizam hemoglobina, aktivacija koagulacijskog sustava, detoksikacija reaktivnih kisikovih vrsta, aktivacija odgovora akutne faze upale, aktivacija komplementa i interakcije u izvanstaničnom matriksu. U skladu s time je metabolomsko istraživanje provedeno u urinu pasa s promijenjenom funkcijom bubrega u babeziozi gdje je nađeno da važnu ulogu imaju upalni odgovor domaćina, oksidativni stres, metabolizam masnih kiselina i energetski metabolizam (Kuleš i sur., 2021). Također, u istraživanju proteoma seruma pasa invadiranih vrstom *Babesia rossi* identificirani su biološki putevi aktivacije hemostaze, urođenog imunosnog sustava, upale te promjena u metabolizmu lipida (Kuleš i sur., 2023).

Jedan od značajnih bioloških puteva detektiran u našem radu jest metabolizam hemoglobina. Kako bi se istražili mehanizmi poremećaja funkcije bubrega u babeziozi pasa, u radu autora Lobetti i sur. (1996) ispitan je eksperimentalno izazvan učinak hemoglobinemije, hipoksije uslijed anemije i kombinacije ova dva čimbenika. Izazivanje hemoglobinemije nije rezultiralo značajnom bubrežnom bolesti, hipoksija izazvana anemijom je rezultirala tek blagom bubrežnom bolesti, a oba čimbenika u kombinaciji nisu pojačala nefropatiju, što znači da je hipoksija značajniji faktor od hemoglobinemije koji pridonosi poremećaju funkcije bubrega u pasa. U uzorcima urina pasa s babeziozom nerijetko je prisutan hemoglobin u većoj koncentraciji (hemoglobulinurija), a isto je detektirano i u proteomskom istraživanju u skupina pasa B i C s babeziozom u odnosu na zdrave. U stanju teške hemolize višak hemoglobina koji se ne veže za haptoglobin u krvi prolazi kroz glomerularnu barijeru, reapsorbira se u proksimalnim kanalićima posredstvom receptora megalina/kubilina te razgrađuje unutar stanica, no u većoj količini hemoglobin ispoljava toksičnost za epitelne stanice proksimalnih kanalića. Hemoglobin također pridonosi oštećenju bubrega radi vazokonstrikcijskog djelovanja te opstrukcije distalnih kanalića stvaranjem precipitata hemoglobina u lumenu kanalića (Gburek i sur., 2002). S obzirom na nađenu povećanu razinu hemoglobina u urinu pasa s proteinurijom u babeziozi, ne mogu se zanemariti njegovi učinci koji bi mogli pridonijeti oštećenju i proksimalnih i distalnih kanalića, iako se prema literaturi ne smatra da je glavni uzrok poremećaja funkcije bubrega u babeziozi toksični učinak hemoglobina, već hipoksija i upalni odgovor (Zygner i sur., 2014).

Naime, osim hemoglobinemije u pasa invadiranih s *B. canis* postoje dodatni hemodinamski i upalni faktori izazvani prisutnošću parazita koji utječu na nastanak poremećaja funkcije bubrega. U istraživanju autora Dubova i sur. (2023) predloženo je da poremećaj funkcije bubrega u babeziozi može biti opisan kao napredujuća akutna ozljeda bubrega koja nastupa kao rezultat upale bubrežnih glomerula radi poremećaja u mikrocirkulaciji glomerula, što je uzrokovano padom volumena krvi u cirkulaciji i stanja pojačanog zgrušavanja. Naime, paraziti vrste *Babesia* izazivanjem intenzivne hemolize i upale uzrokuju promjene u krvnim kapilarama i pokreću mehanizme zgrušavanja krvi, gdje u kompliciranim slučajevima dolazi do razvoja stanja šoka s niskim krvnim tlakom i ponekad diseminirane intravaskularne koagulacije (DIK). U DIK-u dolazi do nekontroliranog aktiviranja sustava zgrušavanja u krvi, što kao posljedicu ima krvarenje zbog potrošnje faktora koagulacije (Kuleš i sur., 2010). Ovo je u skladu s našim istraživanjem gdje je kao značajan biološki put otkrivena aktivacija koagulacijskog sustava. Razvijanje stanja šoka s niskim krvnim tlakom u pasa s babeziozom

ima kao posljedicu hipoksiju u tkivu bubrega, koje je zbog velike metaboličke aktivnosti osobito na nju osjetljivo. Hipoksija vodi povećanom oslobađanju reaktivnih kisikovih vrsta koji djeluju kao signalne molekule kako bi se aktivirala prilagodba stanica na stres, primjerice stabilizacijom hipoksijom induciranog čimbenika 1-alfa (Guzy i Schumacker, 2006). Ovo je u skladu s našim radom gdje je detoksikacija reaktivnih kisikovih vrsta otkrivena kao put od značaja.

Sistemski upalni odgovor domaćina, osim što ima ulogu u obrani organizma od parazita, može pretjeranom aktivacijom voditi oštećenju tkiva i organa, a smatra se da tijekom babezioze pasa pridonosi nastanku komplikacija, pa tako i razvoju AOB-a (Jacobson i Clark, 1994). Nekontrolirani upalni odgovor povezan je s pretjeranom proizvodnjom proupalnih citokina poput monocitnog kemotaktičnog proteina-1 i KC-like (engl. *Keratinocyte Chemotactic-like*) čije bi povećane količine na prvi dan dijagnoze babezioze u pasa mogle služiti kao prognostički faktor za razvoj komplikacija (Galán i sur., 2018). U istraživanju uključenom u ovaj rad otkrivena je analizom razlikovnih proteina u urinu aktivacija odgovora akutne faze upale, dok je analizom zajedničkih razlikovnih proteina u serumu i urinu nađena aktivacija kaskade komplementa. Sukladno tome, u istraživanju autora Winiarczyk i sur. (2019) je analizom proteoma urina pasa s babeziozom u ranoj fazi AOB-a pomoću dvodimenzionalne elektroforeze i spektrometrije masa otkrivena uključenost imunosnog i upalnog odgovora posredovanog djelovanjem kemokina i citokina u oštećenju bubrega. Autori tog rada su pretpostavili važnu ulogu epitelno-mezenhimalne tranzicije koja u početnoj fazi upale potiče strukturnu regeneraciju u tkivu bubrega nakon stresa, no dugoročno vodi do patološke fibroze i poremećaja rada bubrega. U epitelno-mezenhimalnoj tranziciji dolazi do promjene stanica epitela renalnih tubula u miofibroblaste koji u odgovoru na ozljedu tkiva izlučuju komponente izvanstaničnog matriksa (poput kolagena tipa I i III) te tako sudjeluju u cijeljenju rane, no trajnom aktivacijom vode do fibrozne degeneracije organa (Bochaton-Piallat i sur., 2016). Smatra se da povećano odlaganje izvanstaničnog matriksa u bubrezima tijekom fibroze vodi do smanjenog lučenja kolagenskih fragmenata u urinu (Metzger i sur., 2010). Ovo je u skladu s našim nalazom smanjene razine više tipova kolagena (tipa I, II, III, VI, XII, XVIII) u urinu pasa s poremećajem rada bubrega u babeziozi u odnosu na zdrave, kao i u pasa s babeziozom s proteinurijom u odnosu na skupinu bez proteinurije (B/A). Smanjene razine kolagenskih fragmenata nađene su u urinu ljudi s AOB-om, kao i u urinu ljudi i pasa s kroničnom bolesti bubrega (Metzger i sur., 2010; Pelander i sur., 2019). Uzimajući u obzir i promijenjenu razinu nekoliko drugih komponenti izvanstaničnog matriksa u urinu pasa s babeziozom u odnosu na zdrave u našem

radu, poput fibulina, fibronektina, vitronektina te proteoglikana specifičnog za bazalnu membranu, može se pretpostaviti da dolazi do remodeliranja tkiva bubrega u pasa s poremećajem rada bubrega u babeziozi. Fibroza u bubrežnom tkivu nije uobičajeno obilježje u babeziozi pasa, već u kroničnoj bubrežnoj bolesti, ali je moguće da se razvije uslijed dugotrajne upale pri čemu se kolagen i fibrozno tkivo nakuplja te tako narušava funkciju bubrega (Wynn i Ramalingam, 2012).

Prema literaturi je poremećaj rada bubrega u pasa s babeziozom najvjerojatnije rezultat aktivacije upalnih posrednika kao odgovora na prisustvo parazita te hipoksije radi anemije i sistemskog niskog tlaka uz moguće sinergističke učinke sa slobodnim hemoglobinom (Jacobson i sur., 2000; Zygner i Gójska-Zygner, 2014), što je u skladu s našim istraživanjem. Dakle, osim otkrića potencijalnih proteinskih biomarkera u serumu i urinu pasa, rezultati našeg rada doprinose pronalasku patofizioloških mehanizama koji su povezani s poremećajem rada bubrega u babeziozi pasa, kao što su upala, aktivacija hemostaze, metabolizam reaktivnih kisikovih vrsta, metabolizam hemoglobina i interakcije u izvanstaničnom matriksu. Najznačajniji potencijalni biomarkeri za poremećaj funkcije bubrega otkriveni u serumu i urinu pasa s babeziozom te mehanizmi u koje su uključeni prikazani su u obliku Tablice 2 (Prilog 6).

Iako su se proteomska metoda istraživanja i korištenje komercijalnih testova pokazali kao odlični alati za otkriće relevantnih biomarkera i patoloških procesa vezanih uz poremećaj funkcije bubrega u babeziozi, istraživanja uključena u ovaj rad imaju nekih ograničenja. To je ponajprije manji broj uzoraka koji je korišten te bi se u budućnosti trebalo provesti ispitivanje s većim brojem uzoraka seruma i urina radi validacije jednog ili više (panela) biomarkera. Također, u proteomskom istraživanju mogla bi se provesti deplecija proteina s velikom zastupljenošću u serumu i urinu kako bi se otkrili značajni proteini prisutni u manjoj količini.

### *2.2. Poremećaj funkcije srca u pasa*

U znanstvenim člancima koji su sastavni dio ovog rada potvrđena je hipoteza da postoje razlike između proteoma seruma pasa s idiopatskom dilatativnom kardiomiopatijom (iDKM-om) i zdravih pasa (Prilog 4), odnosno proteoma seruma pasa s kroničnom bolesti srčanih zalistaka (KBSZ-om) i zdravih pasa (Prilog 5), koje odražavaju promjene uslijed poremećaja funkcije srca. U istraživanjima uključenim u ovaj rad identificirane su promijenjene razine serumskih proteina pasa s iDKM-om (razreda II ili IIIa prema sustavu ISACHC) u odnosu na zdrave pse, kao i promjene u proteomu seruma pasa s KBSZ-om (stupnja C prema sustavu ACVIM) u usporedbi sa zdravim psima, te su razlikovni proteini analizirani bioinformatički kako bi se otkrili mehanizmi uključeni u patofiziologiju istraživanih bolesti. U istraživanjima je korištena kvantitativna proteomska analiza LC-MS/MS pomoću obilježavanja izobarnim TMT privjescima, a sličan pristup je rabljen u proteomskim istraživanjima srčanih oboljenja u ljudi, poput istraživanja autora Cao i sur. (2020) korištenjem izobarnih privjesaka TMT i LC-MS/MS-a gdje su identificirani proteini s promijenjenom razinom u tkivu lijevog atrija za dijagnostiku perzistentne fibrilacije atrija.

#### *2.2.1. Proteinski serumski biomarkeri*

U istraživanjima pasa s iDKM-om i KBSZ-om objavljenim u priloženim znanstvenim radovima izmjerene su povišene koncentracije serumskog srčanog troponina I te N-terminalnog proBNP-a; poznatih biomarkera za bolesti srca u ljudi i pasa, što je u skladu s literaturom (Spratt i sur., 2005; Ljungvall i sur., 2010; Wess i sur., 2011; Wolf i sur., 2013). S obzirom na nespecifičnost ovih biomarkera (kako je opisano u potpoglavlju 1.2.2.), na temelju istraživanja uključenih u ovaj rad mogu se predložiti novi proteini kao potencijalni biomarkeri za iDKM i KBSZ u pasa koji bi mogli biti od dijagnostičkog ili prognostičkog značaja.

U proteomskom istraživanju pasa s iDKM-om koje je sastavni dio ovog rada nađene su značajno promijenjene razine 12 proteina u serumu oboljelih pasa u odnosu na zdrave, a neki od njih su potencijalni kandidati za biomarkere u dijagnostici ili prognozi ove bolesti.

Jedan od potencijalnih biomarkera za iDKM u pasa u priloženom radu predstavljaju povećane razine serumskog proteina povezanog s mikrofibrilima 4 (engl. *Microfibril-associated protein 4*, MFAP4), što je potvrđeno i metodom Western blot. MFAP4 je glikoprotein eksprimiran u izvanstaničnom matriksu u elastičnim tkivima, poput srca, pluća, krvnih žila i kože koji tijekom različitih bolesti u interakciji s integrinima vodi do aktivacije stanica, upale i fibroze te je predložen kao serumski biomarker za težinu fibroze jetre (Kanaan

i sur., 2022). U istraživanjima plazme na velikom broju uzoraka MFAP4 je prepoznat kao jedan od potencijalnih biomarkera za zatajenje srca u ljudi (Sækmose i sur., 2015; Shah i sur., 2024), kao i u proteomskom istraživanju zatajenja srca u pasa u induciranom modelu bolesti (Yang i sur., 2015). Gen za MFAP4 je prepoznat kao jedan od novih biomarkera za zatajenje srca u radu autora Jin i sur. (2025), a u nedavnom istraživanju zatajenja srca koje je inducirano DKM-om gen za MFAP4 je također zabilježen kao jedan od ključnih gena s povećanom ekspresijom u srčanim fibroblastima koji bi mogao pridonijeti fibrozi u miokardu i razvoju zatajenja srca (Yu i sur., 2026). S obzirom da u iDKM-u dolazi do remodeliranja izvanstaničnog matriksa i razvoja fibroze u srčanom tkivu (Gilbert i sur., 1997; Louzao-Martinez i sur., 2016), MFAP4 bi mogao služiti kao serumski biomarker za remodeliranje u srčanom mišiću koje vodi zatajenju srca u pasa s iDKM-om. Tomu u prilog ide i njegova povećana razina u serumu potvrđena metodom Western blot u pasa sa uznapredovalim kongestivnim zatajenjem srca (ISACHC IIIa) u odnosu na zdrave pse u našem radu. Uloga serumskog MFAP4-a kao biomarkera za razvoj zatajenja srca u iDKM-u pasa bi trebala biti dodatno ispitana u istraživanju s većim brojem jedinki.

Na aktivaciju upalnih procesa u pasa s iDKM-om upućuju nađene povišene razine proteina odgovora akutne faze upale u istraživanju uključenom u ovaj rad. Naime, u pasa s iDKM-om nađene su povećane serumske razine dvaju proteina akutne faze upale koji pripadaju obitelji inter-alfa inhibitora, što je i potvrđeno metodom Western blot. To su teški lanci 3 i 4 inter-alfa-tripsin inhibitora (engl. *Inter-alpha-trypsin inhibitor heavy chain 3 i 4*, ITIH3 i ITIH4) koji imaju prepoznate uloge u različitim patofiziološkim procesima poput upale i karcinogeneze (Sun i sur., 2021). Pokazano je da je ITIH3 pozitivni protein akutne faze upale u ljudi (Salier i sur., 1996), a ITIH4 u svinja, krava i pasa koji djeluje protuupalno potiskivanjem fagocitoze u polimorfonuklearnim imunosnim stanicama (Soler i sur., 2016). U ljudi je ITIH3 otkriven u proteomskom istraživanju plazme kao mogući novi biomarker za srčani udar (Chen i sur., 2022) te kao biomarker za peripartalnu kardiomiopatiju u trudnica i rodilja (Kodogo i sur., 2023). U pasa je pokazano da serumske razine ITIH4-a rastu nakon operacije i kao odgovor na infekciju čineći ga proteinom akutne faze upale s umjerenim porastom (Soler i sur., 2021). Također, ITIH3 i ITIH4 stabiliziraju izvanstanični matriks kovalentnim vezanjem za hijaluronan tijekom upale i remodeliranja matriksa u fiziološkim i patofiziološkim procesima (Zhang i sur., 2024). Stoga povećane razine ITIH3-a i ITIH4-a u serumu pasa s iDKM-om upućuju na aktivaciju odgovora akutne faze upale i/ili na remodeliranje u izvanstaničnom matriksu srčanog mišića.

U istraživanju pasa s KBSZ-om uključenom u ovaj doktorski rad otkriveno je 15 proteina sa smanjenom razinom u serumu oboljelih pasa u odnosu na zdrave, a neki od njih mogli bi biti od koristi u dijagnostici ili prognozi ove bolesti.

Tako su u proteomskom istraživanju pasa s KBSZ-om nađene smanjene razine još jednog proteina iz obitelji inter-alfa inhibitora; teškog lanca 1 inter-alfa-tripsin inhibitora (engl. *Inter-alpha-trypsin inhibitor heavy chain 1*, ITIH1). ITIH1 je otkriven kao pozitivni i negativni protein akutne faze upale u različitim stanjima s prepoznatom ulogom u upali i karcinogenezi (Sun i sur., 2021). U našem istraživanju pasa s KBSZ-om, kao i u proteomskom istraživanju autora Rešetar Maslov i sur. (2023), nađene su smanjene razine serumskog ITIH1-a kao moguće posljedice vezanja cirkulirajućih molekula ITIH1-a za hijaluronan u oboljelim zaliscima. Naime, u ljudi i pasa s KBSZ-om nađeno je da količina hijaluronana kao glavnog glikozaminoglikana raste u svim slojevima oboljelih zalistaka što vodi njihovom zadebljanju i gubitku funkcije (Aupperle i Disatian, 2012).

Nadalje, u istraživanju pasa s KBSZ-om je otkriven i dvjema neovisnim metodama validiran protein adiponektin čije bi niske serumske koncentracije mogle poslužiti kao marker za težinu bolesti. Naime, i u drugom proteomskom istraživanju autora Saril i sur. (2022) je utvrđena smanjena količina serumskog adiponektina u pasa sa stupnjem D KBSZ-a u odnosu na zdrave, iako postoji i suprotan nalaz u radu autora Kim i sur. (2016). Adiponektin je najzastupljeniji adipocitokin kojeg luči masno tkivo, a sudjeluje u kardiovaskularnim, metaboličkim, imunosnim i upalnim procesima (Radin i sur., 2009). Visoke razine adiponektina u ljudi su povezane s niskim rizikom od kardiovaskularnih bolesti i usporavanjem njihovog napredovanja (Hui i sur., 2012), stoga bi njegove niske koncentracije nađene u pasa sa stupnjem C i D KBSZ-a mogle biti rizični faktor za loš ishod. Međutim, s obzirom na nekonzistentne nalaze (Kim i sur., 2016), a moguće zbog korištenja različitih metoda te bioloških različitosti pasa uključenih u istraživanja, potrebna su daljnja ispitivanja upotrebe adiponektina kao biomarkera za KBSZ u pasa.

Nalazi značajno smanjenih razina proteina tetranektina i gelsolina u serumu pasa s KBSZ-om u odnosu na zdrave pse u našem radu u skladu su s druga dva provedena proteomska istraživanja (Saril i sur., 2022; Rešetar Maslov i sur., 2023). Tetranektin i gelsolin su predloženi kao novi biomarkeri za kardiovaskularne bolesti u ljudi u radu autora Yin i sur. (2014) na temelju proteomskih istraživanja, dok je u drugom istraživanju nađeno da bi tetranektin mogao biti proteinski biomarker za predviđanje ishoda u kardiovaskularnim bolestima (Ho i sur., 2018). Tetranektin sudjeluje u regulaciji hemostaze vezivanjem na plazminogen pospješujući

njegovu aktivaciju i tako potiče razgradnju ugrušaka (Mogues i sur., 2004). Smanjena razina serumskog tetranektina je potencijalno povezana s njegovom povećanom potrošnjom u oštećenom srčanom tkivu gdje pridonosi razgradnji fibrina u ugruščima. Psi s KBSZ-om koji razviju kongestivno zatajenje srca nalaze se u prokoagulantnom stanju (Prihirunkit i sur., 2014), što je u skladu s našim nalazom smanjene razine serumskog tetranektina u oboljelih pasa sa stupnjem C KBSZ-a. Naš nalaz smanjene razine serumskog tetranektina u pasa s KBSZ-om sa stupnjem C, u skladu je s istraživanjem pasa s KBSZ-om autora Rešetar Maslov i sur. (2023), kao i u pasa s KBSZ-om stupnja D (Saril i sur., 2022). Također, u ljudi je utvrđeno da su niske razine tetranektina povezane sa zatajenjem srca više nego natrijuretski peptidi B-tipa te je predloženo da je tetranektin potencijalni biomarker za zatajenje srca povezan s procesima fibroze u srčanom mišiću (McDonald i sur., 2020). Tetranektin predstavlja obećavajući biomarker čije su snižene razine u krvi povezane s razvojem bolesti koronarnih arterija i zatajenja srca, a njegovo korištenje u kombinaciji s biomarkerom NT-proBNP-om unaprjeđuje dijagnostičku točnost (Vulciu i sur., 2025). Snižene razine serumskog tetranektina nađene su i u ljudi s iDKM-om (Saha i sur., 2026). Uzimajući u obzir navedeno, uloga tetranektina u KBSZ-u pasa bi trebala biti dodatno ispitana. Nalaz smanjene razine serumskog gelsolina u pasa s KBSZ-om potvrđen je i u drugom proteomskom istraživanju (Rešetar Maslov i sur., 2023). Gelsolin u plazmi ima ulogu u uklanjanju aktinskih filamenata koji se otpuštaju u krv tijekom oštećenja tkiva prilikom zatajenja srca, a njegove smanjene razine predložene su kao biomarker za reumatski endokarditis (Argun i sur., 2015).

### *2.2.2. Mehanizmi uključeni u poremećaj funkcije srca u pasa*

Otkriće bioloških puteva posredstvom proteomske i bioinformatičke analize u istraživanjima uključenim u ovaj rad pridonosi pronalasku patofizioloških mehanizama uključenih u razvoj iDKM-a i KBSZ-a u pasa. Rezultati analize proteina od značaja objavljeni u radovima o bolestima srca pasa u velikoj su mjeri međusobno usklađeni, a pokazuju i konzistentnost s nalazima istraživanja u ljudi. Tako su u istraživanju pasa s iDKM-om uključenom u ovaj rad otkrivene značajne uloge aktivacije komplementa, zgrušavanja krvi, oblikovanja elastičnih vlakana, homeostaze plazmatskih lipoproteinskih čestica te metabolizma i prijenosa retinoida, a u pasa s KBSZ-om uloge cijeljenja rane, aktivacije plazminogena, organizacije izvanstaničnih struktura, regulacije razine plazmatskih lipoproteinskih čestica, homeostaze lipida i metabolizma retinoida.

U skladu s tim, u sveobuhvatnoj meta-analizi koja je uključila proteomska istraživanja kardiovaskularnih bolesti u ljudi otkriveni su biološki putevi od značaja poput upalnog

odgovora, cijeljenja rane, zgrušavanja krvi, proteolize, organizacije izvanstaničnog matriksa, metabolizma kolesterola i lipoproteinskih čestica (Mokou i sur., 2017). U radu autora Feig i sur. (2019) proteomska analiza seruma ljudi s DKM-om uputila je na uključenost lipidnog metabolizma, upale i koagulacije kao najznačajnije promijenjenih puteva u oboljelih ljudi. Nadalje, u proteomskom radu autora Klimentova i sur. (2024) nađeno je u ljudi s DKM-om da su plazmatski proteini od značaja uključeni u puteve upalnog odgovora, odgovora proteina akutne faze, aktivacije komplementa i trombocita, zgrušavanja krvi i metabolizma lipida. U novijem istraživanju autora Saha i sur. (2026) provedena je kombinirana analiza proteina i lipida u plazmi ljudi s iDKM-om pri čemu su otkrivene promjene u metabolizmu membranskih lipida, energetskom metabolizmu i oksidativnom stresu, aktivaciji imunosnog sustava te remodeliranju izvanstaničnog matriksa. Također, u nedavnom istraživanju koje je uključilo analizu proteoma seruma i histološku analizu oboljelih zalistaka u ljudi s KBSZ-om različitog primarnog uzroka, otkrivena je važna uloga upale i remodeliranja izvanstaničnog matriksa (Garcia-Peña i sur., 2026).

U istraživanjima uključenim u ovaj rad nađena je važna uloga imunosnih i upalnih procesa u patofiziologiji iDKM-a i KBSZ-a u pasa. Ovdje treba napomenuti da u bioinformatičkoj analizi proteina sa značajno promijenjenom razinom u serumu pasa s KBSZ-om nije direktno otkrivena uključenost upalnih procesa kao u pasa s iDKM-om. Ipak, određeni proteini od značaja poput ITIH1-a, adiponektina i gelsolina imaju prepoznate uloge u upalnim procesima što upućuje na upalnu komponentu u KBSZ-u u pasa. U pasa s KBSZ-om se u literaturi mogu pronaći dokazi o uključenosti upalnih procesa kao što je nalaz značajnog porasta razine serumskih citokina ovisno o stupnju napretka bolesti, kao i porast broja regulatornih T-limfocita koje održavaju imunosnu toleranciju na vlastito tkivo (Piantedosi i sur., 2022). Također, poznato je da je mikrookoliš u promijenjenim zaliscima pasa i ljudi s KBSZ-om u upalnom stanju (Grzeczka i sur., 2025), pa je primjerice u endotelu oboljelih zalistaka pasa nađena povećana ekspresija nekoliko gena za upalne citokine što ih čini direktnim izvorom posrednika upale (Aupperle i Disatian, 2012). U skladu s istraživanjem uključenim u ovaj rad, i druga proteomska istraživanja pružaju dokaz o uključenosti imunosnih i upalnih procesa u patologiji KBSZ-a u pasa (Saril i sur., 2022; Rešetar Maslov i sur., 2023). U istraživanju seruma pasa s iDKM-om uključenim u ovaj rad nađene su povišene razine proteina uključenih u imunosni odgovor (komponente komplementa C4-A i C9) te odgovora akutne faze upale (ITIH3, ITIH4), što je u skladu s nedavno provedenim istraživanjem proteoma plazme ljudi s DKM-om, gdje su utvrđene povećane količine proteina uključenih u imunosni odgovor i upalu,

poglavito sustava komplementa (Klimentova i sur., 2024). Dok je uloga imunosnih procesa u patogenezi DKM-a u ljudi poznata, poput odlaganja imunoglobulina G te komponenti komplementa na oštećenom srčanom tkivu te povećane količine proteina upalnog odgovora u biopsijama miokarda u pacijenata s DKM-om (Klimentova i sur., 2024), dokazi za uključenost imunosnog odgovora u pasa s ovom bolesti je dosada slabo dokumentirana. U nekim starijim radovima nađena su serumska autoimuna i anti-mitohondrijska protutijela u dijelu pasa s DKM-om (Day, 1996; Buse i sur., 2008). Nadalje, Rubio i sur. (2020) našli su povećane razine odabranih pozitivnih proteina odgovora akutne faze upale (poput CRP-a) i proupalnih citokina te smanjene razine antioksidativnih enzima u serumu pasa sa uznapredovanim zatajenjem srca oboljelih od KBSZ-a i DKM-a, upućujući na ulogu upale i oksidativnog stresa u razvoju zatajenja srca. Ovo je u skladu s nalazom povećane razine CRP-a izmjerene biokemijskom metodom u oboljelih pasa sa srčanim zatajenjem u oba naša rada.

U oba istraživanja bolesti srca pasa nađeni su pomoću bioinformatičke analize procesi povezani s remodeliranjem izvanstaničnog matriksa; u pasa s iDKM-om oblikovanje elastičnih vlakana, a u pasa s KBSZ-om organizacija izvanstaničnih struktura. Otprije je u pasa s iDKM-om otkriveno difuzno remodeliranje srčanog mišića s fokalnim oštećenjima karakteriziranim fibrozom, nastankom novih krvnih žila, infiltracijom makrofaga i propadanjem kardiomiocita, odnosno mjestima gubitka kardiomiocita i posljedično manje kontraktilnosti (Gasparini i sur., 2020). Prema autorima ovog istraživanja, navedeni nalazi upućuju da je inicijalni patološki proces u iDKM-u pasa oštećenje i gubitak pojedinačnih kardiomiocita s ulogom upalnih procesa posredovanih makrofazima što vodi nastanku oštećenja te remodeliranju miokarda, te naposlijetku širenju srčanih komora i promijenjenoj funkciji srca. Nakon oštećenja kardiomiocita slijedi upalna reakcija posredstvom različitih imunosnih stanica što je regulirano citokinima, adhezijskim molekulama i enzimima iz izvanstaničnog matriksa. Protuupalni citokini sudjeluju u fazi popravka oštećenja tkiva gdje dolazi do povećanog odlaganja izvanstaničnog matriksa i nastanka tkiva ožiljka, odnosno fibroze. U iDKM-u pasa potvrđena je povećana ekspresija proupalnih citokina i enzima koji remodeliraju izvanstanični matriks u miokardu (Fonfara i sur., 2013). U pasa s KBSZ-om su već dugo prepoznate promjene u izvanstaničnom matriksu zalistaka koje vode njihovom zadebljanju i poremećaju funkcije. Promjene uključuju nakupljanje komponenti izvanstaničnog matriksa – glikozaminoglikana i proteoglikana, promjene ekspresije komponenti bazalne membrane i kolagena, promjene u raspodjeli i razinama enzima koji razgrađuju izvanstanični matriks (matriks metaloproteinaza i njihovih inhibitora), što vodi smanjenoj razgradnji i nakupljanju komponenti izvanstaničnog

matriksa u zahvaćenim zaliscima te naposlijetku promjeni njihove strukture i gubitku funkcije (Aupperle i sur., 2009; Fox, 2012). U pasa pasmine *Cavalier King Charles* španijela s kongestivnim zatajenjem srca uzrokovanim KBSZ-om analiza transkriptoma je pokazala uključenost organizacije izvanstaničnog matriksa, oštećenja endotela i gena odgovornih za kontraktilnost kardiomiocita (Reimann i sur., 2024).

U patofiziologiji obje bolesti srca pasa istraživanih ovim radom prepoznata je važna uloga hemostaze na temelju bioinformatičke analize te je nađeno da se psi s KBSZ-om nalaze u prokoagulantnom stanju, što je u skladu s literaturom. Tarnow i sur. (2007) našli su promijenjene koncentracije i aktivnosti biomarkera hemostaze u pasa sa zatajenjem srca oboljelih od DKM-a ili KBSZ-a koji upućuju na prokoagulantno stanje. U suglasju s tim je naš nalaz snižene serumske razine antitrombina III, alfa-2-antiplazmina, tetranektina te kininogena-1 u pasa s KBSZ-om. Antitrombin III je prirodni antikoagulans koji inhibira proces zgrušavanja krvi vezanjem na trombin (Hsu i Moosavi, 2023), a njegova smanjena aktivnost nađena je u pasa sa zatajenjem srca oboljelih od KBSZ-a (Prihirunkit i sur., 2014). U pasa sa zatajenjem srca dolazi do smanjenog srčanog minutnog volumena krvi te nepravilnosti u krvnom protoku što rezultira povećanom viskoznosti krvi i potiče zgrušavanje krvi (Prihirunkit i sur., 2014). I u pasa s iDKM-om u priloženom radu nađena je uključenost aktivacije koagulacijskog sustava pomoću bioinformatičke analize, vjerojatno kao posljedice promjena u protoku krvi zbog proširenja srčanih komora i smanjenog srčanog minutnog volumena krvi (Tarnow i sur., 2007).

U oba istraživanja pasa s poremećajem funkcije srca obuhvaćenih ovim radom nađena je uključenost procesa vezanih za homeostazu lipida i dinamiku lipoproteinskih čestica u krvi. Konzistentan je nalaz u oba rada smanjena razina različitih serumskih apolipoproteina, odnosno proteina koji vežu lipide i tako tvore lipoproteine koji prenose kolesterol i trigliceride kroz krvotok. Ovaj nalaz je u skladu s proteomskim istraživanjem plazme u ljudi s DKM-om gdje je nađena smanjena razina različitih apolipoproteina (ApoC1, ApoA1, ApoA2 i ApoA4) (Klimentova i sur., 2024). Dok lipoproteini imaju važnu fiziološku ulogu u prijenosu lipida, promjene u njihovom metabolizmu su povezane s kardiovaskularnim bolestima i zatajenjem srca. Apolipoproteini su uključeni u različite patofiziološke mehanizme vezane uz aterosklerozu, napredovanje zatajenja srca i remodeliranje lijevog ventrikula poput upale, oksidativnog stresa, fibroze i apoptoze (Lemesle i sur., 2020). U proteomskim istraživanjima uključenim u ovaj rad otkrivene su i nezavisnom metodom validirane smanjene razine apolipoproteina B-100 (ApoB-100) i apolipoproteina D (ApoD) u pasa s KBSZ-om, te apolipoproteina A4 (ApoA4) u pasa s iDKM-om. U ljudi sa zatajenjem srca nađeno je da je

razina ApoB-100 u serumu značajno manja u bolesnika koji su preminuli od kardiovaskularne bolesti u odnosu na preživjele (Lemesle i sur., 2020), dok je smanjena razina ApoA4 bila povezana s rizikom od iznenadne smrti (Kronenberg, 2017). ApoB-100 je ključan strukturni protein u svim značajnim lipoproteinima koji sudjeluju u aterosklerozi te ima ulogu u regulaciji metabolizma lipida (Behbodikhah i sur., 2021). ApoA4 je glikoprotein koji cirkulira u krvi u slobodnom obliku ili vezan za hilomikrone te je dokazana njegova zaštitna uloga u kardiovaskularnim bolestima u ljudi zbog uključenosti u crijevnu apsorpciju lipida i homeostazu glukoze te posjedovanja antioksidativnih i protuupalnih svojstava (Li i sur., 2022). Stoga, smanjena serumska razina ApoB-100 u pasa s KBSZ-om te ApoA4 u pasa s iDKM-om bi mogla služiti u procjeni rizika razvoja i napredovanja ovih bolesti.

U istraživanjima obje bolesti srca pasa koja su priložena ovom radu, nađena je uključenost metabolizma retinoida koji se odnosi na niz biokemijskih reakcija kojima organizam apsorbira, skladišti, pretvara i eliminira retinoide (derivate vitamina A). U istraživanju autora Zhang i sur. (2025) usporedbom proteoma zdravog srca i srca s razvojem DKM-a otkriveno je da je najznačajniji mehanizam izražen u lijevom ventrikulu srca s DKM-om put sinteze retinoične kiseline te se tretman s trans-retinoičnom kiselinom pokazao učinkovit za srčani udar na mišjem modelu, onemogućujući povećanje srčanog mišića i vraćajući normalnu srčanu funkciju. Također, u nedavnom istraživanju autora Bilgiç i sur. (2025) detektirana je smanjena razina retinola u plazmi pasa s DKM-om te je preporučena suplementacija vitaminom A uz standardni protokol za liječenje DKM-a u pasa. Uzimajući navedeno u obzir, učinak retinoične kiseline u terapiji srčanih oboljenja u pasa i ljudi treba biti dodatno ispitana.

Zajednički doprinos proteomskih istraživanja dvije najčešće bolesti srca u pasa koja su uključena u ovaj rad jest prepoznavanje sličnosti u patofiziološkim mehanizmima, kao i sličnosti s procesima uključenim u ove bolesti u ljudi na temelju pregleda literature. Navedeno čini rezultate ovih istraživanja potencijalno primjenjivim u ljudskoj medicini. Najznačajniji potencijalni biomarkeri za poremećaj funkcije srca otkriveni u serumu pasa s iDKM-om i KBSZ-om te mehanizmi u koje su uključeni prikazani su u obliku Tablice 3 (Prilog 7).

Ograničenja provedenih proteomskih istraživanja ponajviše se odnose na manji broj analiziranih uzoraka te korištenje istih uzoraka za analizu proteoma i validaciju rezultata neovisnom metodom. Rezultati istraživanja bolesti srca u pasa koji su uključeni u ovaj doktorski rad mogu se stoga smatrati preliminarnim te im je potrebna daljnja potvrda korištenjem većeg broja uzoraka.

# 3. ZAKLJUČAK

Na temelju rezultata istraživanja uključenih u ovaj doktorski rad, mogu se donijeti slijedeći objedinjeni zaključci:

1. LC-MS/MS proteomskom analizom utvrđeno je da postoje značajne razlike između proteoma seruma i urina pasa s poremećajem funkcije bubrega invadiranih s protozoonom *Babesia canis* i proteoma seruma i urina zdravih pasa. Bioinformatičkom analizom proteina sa značajno različitom razinom utvrđeno je da je poremećaj funkcije bubrega u babeziozi pasa povezan s metabolizmom hemoglobina, aktivacijom koagulacijskog sustava, detoksikacijom reaktivnih kisikovih vrsta, aktivacijom odgovora akutne faze upale, aktivacijom komplementa i interakcijama u izvanstaničnom matriksu.
2. U istraživanju pasa s poremećajem funkcije bubrega u babeziozi, LC-MS/MS proteomskom analizom pomoću obilježavanja s izobarnim TMT privjescima otkriveno je 150 reprezentativnih proteina u serumu pasa, od čega je zabilježena značajno različita razina 58 proteina između pasa s poremećajem funkcije bubrega u babeziozi i zdravih pasa. Pritom značajno povećane razine serumskog LRG-1-a i AGP-a imaju potencijal relevantnog biomarkera za poremećaj funkcije bubrega u babeziozi pasa.
3. U istraživanju pasa s poremećajem funkcije bubrega u babeziozi, LC-MS/MS proteomskom analizom otkriveno je 450 reprezentativnih proteina u urinu pasa, od čega je zabilježena značajno različita razina 259 proteina između pasa s poremećajem funkcije bubrega u babeziozi i zdravih pasa. Pritom bi značajno povećane razine NGAL-a i L-FABP-a u urinu mogle služiti kao osjetljivi rani biomarkeri i prije povišenja serumskog kreatinina u pasa s poremećajem funkcije bubrega u babeziozi.
4. Na temelju biomarkera od značaja otkrivenih u priloženim istraživanjima može se zaključiti da poremećaj funkcije bubrega u pasa s babeziozom postoji i prije povišenja rutinskih dijagnostičkih markera te je stoga u svih pasa s dijagnozom invazije s *B. canis* potrebno pratiti bubrežnu funkciju tijekom i nakon liječenja. Analizom panela urinarnih biomarkera otkrivenih u istraživanjima uključenim u ovaj rad može se postići dijagnostička specifičnost i precizno praćenje oboljelih pasa.
5. Na temelju objedinjenih rezultata dvaju objavljenih istraživanja pasa s poremećajem funkcije bubrega u babeziozi utvrđeno je da se u oboljelih pasa javlja poremećaj funkcije nefrona koji zahvaća glomerularnu filtracijsku barijeru, proksimalne i distalne kanaliće. U pasa s poremećajem funkcije bubrega u babeziozi povišene razine IgG-a i ALB-a u urinu

ukazuju na oštećenje glomerula, povišene razine L-FABP-a, VDBP-a, B2M-a, RBP-a, KIM-1-a i NAG-a potvrđuju poremećaj funkcije i/ili direktno oštećenje proksimalnih kanalića, dok smanjena razina UMOD-a uz povišenu razinu NGAL-a u urinu ukazuje na oštećenje distalnih dijelova bubrežnih kanalića.

6. LC-MS/MS proteomskom analizom pomoću obilježavanja s izobarnim TMT privjescima utvrđeno je da postoje razlike u proteomu seruma između pasa s idiopatskom dilatativnom kardiomiopatijom **(**iDKM-om) i zdravih pasa, koje odražavaju sistemske promjene uslijed poremećaja funkcije srca. Analizom je otkriveno 134 reprezentativnih proteina u serumu pasa, od čega je zabilježena značajno različita razina 12 proteina između pasa s iDKM-om i zdravih pasa. Pritom, povećane razine serumskog MFAP4-a predstavljaju potencijalno osjetljiv biomarker za zatajenje srca u pasa s iDKM-om.
7. U istraživanju pasa s kroničnom bolešću srčanih zalistaka (KBSZ-om), LC-MS/MS proteomskom analizom pomoću obilježavanja s izobarnim TMT privjescima utvrđeno je da postoje razlike u proteomu seruma između pasa s KBSZ-om i zdravih pasa, koje odražavaju promjene uslijed poremećaja funkcije srca. Otkriveno je 290 reprezentativnih proteina u serumu pasa, od čega je zabilježena značajno različita razina 15 proteina između pasa s KBSZ-om i zdravih pasa. Pritom smanjene serumske razine adiponektina i ApoB-100 predstavljaju potencijalne biomarkere za KBSZ u pasa.
8. Bioinformatičkom analizom proteina sa značajno različitom razinom u priloženim istraživanjima otkriveni su ključni biološki putevi uključeni u patofiziologiju iDKM-a i KBSZ-a u pasa, pri čemu su nalazi u velikoj mjeri usklađeni međusobno te konzistentni s rezultatima istraživanja u ljudi. U obje bolesti potvrđena je značajna uključenost remodeliranja izvanstaničnog matriksa, aktivacije koagulacijskog sustava, metabolizma retinoida i dinamike lipoproteinskih čestica.

## 4. POPIS LITERATURE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## 5. PRILOZI

PRILOG 1

Bilić, P., Horvatić, A., Kuleš, J., Gelemanović, A., Beer Ljubić, B., Mũnoz-Prieto, A., Gotić, J., Žubčić, D., Barić Rafaj, R., Mrljak, V. (2023) Serum and urine profiling by high-throughput TMT-based proteomics for the investigation of renal dysfunction in canine babesiosis. *Journal of Proteomics* 270, 104735.

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# Serum and urine profiling by high-throughput TMT-based proteomics for the investigation of renal dysfunction in canine babesiosis

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ABSTRACT

Canine babesiosis is a tick-borne disease caused by *Babesia canis*, with acute kidney injury as one of the common complications. In the study 8 healthy control dogs and 22 dogs with naturally occurring babesiosis were enrolled, with the aim to analyse differences in serum and urinary proteomes between healthy dogs and dogs with different degree of renal dysfunction in babesiosis using a label-based high-throughput quantitative proteomic approach. In serum, 58 proteins were found differentially abundant between healthy controls and groups of dogs with different degrees of renal dysfunction in babesiosis, while in urine there were 259 differentially abundant proteins. In addition, altered biological pathways were detected in the diseased dogs using bioinformatics tools and validation of several candidate biomarkers was performed.

*Significance:* The main aim of this comprehensive study was to perform analyses of serum and urinary proteomes of dogs with renal dysfunction in babesiosis compared to healthy dogs using, for the first time, a high-throughput proteomic method and functional enrichment analyses. Serum and urine samples of the same dogs were investigated in order to gain a more complete picture of pathologic changes taking place in renal dysfunction in babesiosis. We highlighted two putative biomarkers validated herein which could be of importance for early diagnosis of renal dysfunction in canine babesiosis, as they are easily accessible from urine and their concentration rises before the appearance of azotaemia: urinary neutrophil gelatinase-associated lipocalin (NGAL) and urinary liver-type fatty acid-binding protein (L-FABP).

## 1. Introduction

Canine babesiosis is a tick-borne disease caused by the intraerythrocytic protozoan parasites of *Babesia* genus species. The most common cause of the disease in Europe is the species *Babesia canis* (*B. canis*) with prevalence from 2.3% up to 44.8% in different countries, based on molecular studies [1]. The main hallmark of the disease is anaemia due to haemolysis of red blood cells happening upon the release of the parasites into circulation. Clinically, the disease can have an uncomplicated or complicated form, latter being marked by development of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) [2].

Acute kidney injury (AKI), presenting as anuria or oliguria despite adequate rehydration therapy, is one of the recognized complications in canine babesiosis whose incidence varies in literature due to different diagnostic criteria. One of the most common parameters used in the diagnosis of kidney function impairment is azotaemia, which is an elevation of blood urea and creatinine levels due to reduced glomerular filtration in kidneys. Azotaemia was reported to range from 0 to 36% in relevant studies of dogs infected with various *Babesia* species [3]. Minimal renal damage is more common than severe acute kidney injury in dogs infected with *Babesia*, making ŕenal involvement´more proper term for renal pathologic changes in this context [4].

The exact mechanisms leading to the development of kidney dysfunction in canine babesiosis are still unknown, but several factors were proposed. Haemoglobinuria occurring upon glomerular filtration

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of excess haemoglobin from lysed erythrocytes into urine was attributed to damaged kidneys in canine babesiosis, but a study by Lobetti et al. showed it did not induce significant nephropathy in an experimental model [5]. Renal dysfunction could more probably be a result of hypoxia due to anaemia and/or hypotension developing in canine babesiosis [6,7]. Additionally, renal damage was speculated to be caused by inflammatory processes taking place during the course of the disease [8].

Kidney function is accurately evaluated with glomerular filtration rate (GFR), but methods for GFR measurement are expensive and time-consuming hindering their use in routine clinical setting. Level of serum creatinine, a molecule freely filtered by glomeruli without renal secretion and extra renal metabolism, is commonly used as an indirect measure of GFR. However, serum creatinine is an insensitive marker of renal function as it does not increase above reference interval until 75% of nephrons are non-functional [9]. Additionally, other factors can influence its concentration; such as dog age, breed, dehydration, drugs and body mass [10]. Furthermore, elevations in serum bilirubin and haemoglobin due to haemolysis present in canine babesiosis can negatively interfere with the measurement of serum creatinine when certain methods are used, resulting in undetected cases of renal dysfunction in dogs infected with *Babesia* [11]. Persistent proteinuria, which is defined as presence of elevated amounts of proteins in urine, is also used as a marker of renal damage by indicating renal loss of plasma proteins that should normally not be filtered by glomeruli or should be reabsorbed by kidney tubular cells. It is expressed as the urine protein to creatinine ratio (UPC), measured in a single random urine sample, which has a good correlation to the 24-hour urine protein quantification [12]. Proteinuria is a common finding in dogs infected with *B. canis*, whose severity increases with the severity of the disease [4].

Since it is recognized that renal dysfunction in canine babesiosis can be a serious complication, resulting in a 5-fold increased risk of death compared to other complications [13], early diagnosis is of notable interest. Moreover, in both human and veterinary medicine there is a need to identify new sensitive and precise biomarkers of renal dysfunction which would ideally indicate not only early stage of disease, but also severity, progression and location of kidney injury. The tests for biomarkers are preferred to be non-invasive, rapid and low cost. Taking into account that urine is easily accessible in large quantities, the urinary proteome is an especially attractive source of biomarkers in kidney pathologies. Moreover, urinary biomarkers could be used to precisely locate kidney injury with high or intermediate molecular weight proteins (MW $>$ 69 kDa) found in elevated amounts in urine pointing out to glomerular dysfunction and low MW proteins to tubular dysfunction [14].

New high-throughput proteomic technologies offer advantageous promises to discover a number of new candidate biomarkers for renal dysfunction in canine babesiosis, which could eventually find their way into routine practices. Also, changed proteome profiles in serum and urine of diseased dogs in comparison to healthy dogs can provide an insight into molecular mechanisms leading to renal damage in canine babesiosis. New proteomic technologies are underutilised in the research of canine renal diseases, with few studies found in the literature [15,16]. To the authors´knowledge, there is only one proteomic study of urinary proteome of dogs with kidney injury during babesiosis, where two-dimensional electrophoresis followed by protein identification using MALDI-TOF (matrix-assisted laser desorption ionization coupled to time of flight) mass spectrometry was used [17].

Therefore, the aim of the current study was to analyse differences in serum and urinary proteomes between healthy dogs and dogs with renal dysfunction in babesiosis using label-based high-throughput quantitative proteomic approach. In addition, changed biological pathways were detected in the diseased dogs using bioinformatics tools and validation of several candidate biomarkers was performed.

## 2. Materials and methods

### 2.1. Study design

Study design is shown in the Fig. 1. Since serum and urine proteome differences between healthy controls and studied groups of dogs with babesiosis are result of systemic and renal pathologic processes, it can be challenging to identify certain differential proteins as kidney function impairment biomarkers. Therefore, in order to study kidney dysfunction in babesiosis, it could be more instructive to address serum and urine proteome differences between groups of dogs with babesiosis with different degrees of renal damage (A, B and C). As stated by the International Renal Interest Society, there are 3 substages of proteinuria in dogs: non-proteinuric with UPC $<$ 0.2, borderline proteinuria with UPC 0.2 to 0.5 and proteinuric with UPC $>$ 0.5.

In the study, 8 healthy control dogs (group H) and 22 dogs with naturally occurring babesiosis were enrolled. Dogs with babesiosis were divided into 3 groups: group A consisted of 6 non-azotaemic dogs (serum creatinine $<$140 μmol/L) with normal UPC (UPC $<$ 0.5), group B of 10 non-azotaemic dogs with proteinuria (UPC $>$ 0.5) and group C of 6 azotaemic dogs (serum creatinine $>$140 μmol/L) and significant proteinuria (UPC $>$ 2). Serum and urine samples were collected from all dogs and analysed separately in serum and urine proteomic experiments. Serum and urine protein samples were individually labelled using isobaric Tandem Mass Tag (TMT) labels and then grouped into sets. In both serum and urine proteomic experiments, samples were grouped into 6 sixplex experimental sets so that each set contained samples of healthy dogs, dogs of groups A, B and C and an internal standard sample (IS). IS was made as a pool of equal protein amounts from all 30 samples and was used for normalization to allow analysis of data across all 6 sixplex sets of an experiment. Data acquired by LC-MS/MS analysis of sets was processed statistically to identify differentially abundant proteins, which were further analysed by bioinformatics tools to find biological pathways modulated in renal dysfunction in dogs with babesiosis caused by *B. canis*. Concentrations of some of the differential proteins were measured by canine-specific ELISAs in serum and urine of dogs included in the proteomic experiment in order to validate proteomics results. Recommendations concerning study design, sample collection, processing workflows, MS data acquisition, data processing, and bioinformatic analysis were followed in order to minimize systematic preanalytical variation that can result in significant proteome change [18].

### 2.2. Animals

The study was approved by the Committee on the Ethics of the University of Zagreb, Faculty of Veterinary Medicine (Permit Number: 640–01/14–305/16, 251–61-01/139–14-28). All dogs were admitted during a 3-year period (year 2015–2017) to the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia. Informed owner consents were acquired and all procedures performed in accordance with EU Directive 2010/63/EU for animal experiments.

Diseased dogs were diagnosed with babesiosis based on detection of the intra-erythrocytic parasite in thin blood smears stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Infection with *B. canis* subspecies was confirmed with PCR, as described by Beck et al. [19]. Serum and urine samples of diseased dogs were collected prior to treatment with one dose (6 mg/kg) of imidocarb dipropionate (Imizol® 12%, Schering-Plough, Kenilworth, USA), which was given subcutaneously on the admission day. Diseased dogs were of different breeds and sex, among them 9 females (2 spayed) and 13 males, aged between 1 and 13 years (median age of 3 years).

In the healthy control group there were dogs of various breeds, of which 4 females (3 spayed) and 4 males (3 castrated), aged between 1 and 13 years (median age of 3.5 years). Control dogs were considered healthy based on clinical examination, as well as haematological, serum

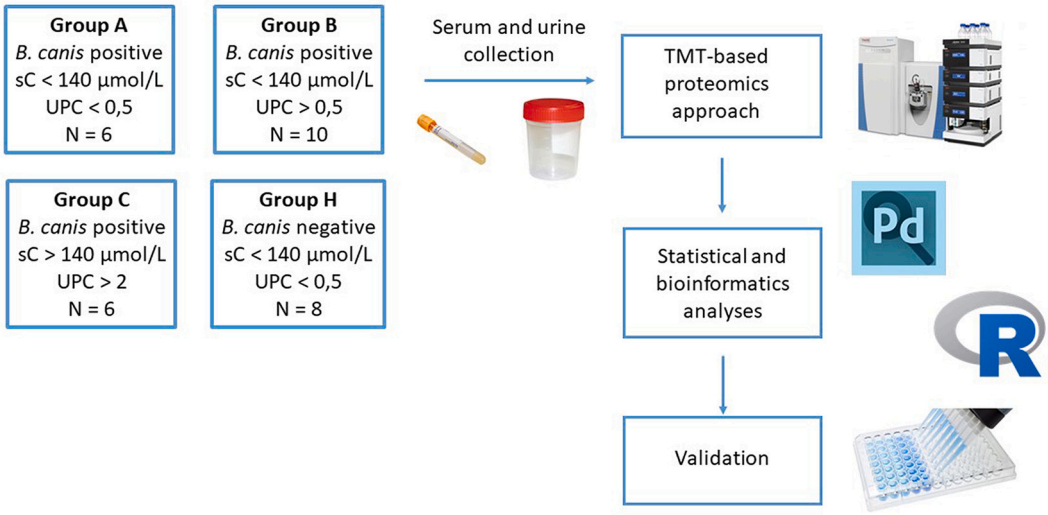


**Fig. 1.** Study design. In the study dogs were divided into 4 groups. Serum and urine were analysed using Tandem Mass Tag (TMT) – based proteomics approach. Acquired data was processed by statistical and bioinformatics tools, and for validation of proteomics results canine specific ELISAs were used. sC - serum creatinine concentration. UPC - urinary protein to creatinine ratio.

biochemical and urine analyses, which gave results within normal reference ranges.

### 2.3. Serum and urine samples preparation

Blood samples were collected from all 30 dogs and serum obtained by centrifugation at 3500 ×*g* for 10 min at room temperature. Serum aliquots were stored at −80 °C until analysed. Samples were thawed just once, immediately before proteomic analysis. Once thawed, protein concentration in serum was determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) following manufacturer's guidelines.

Mid-stream urine samples were collected by free catch into sterile tubes from healthy and diseased dogs. Urine samples were analysed by semiquantitative dipstick test (Multistix 10SG, Siemens Healthcare, Zagreb, Croatia) on an automated analyser (CLINITEK Status+Analyser, Siemens Healthcare, Zagreb, Croatia) and then centrifuged at 500 ×*g* at 4 °C for 10 min. Acquired sediment was analysed microscopically at high power field (400×) and supernatant was used for urine protein and creatinine concentration determination using commercial kits on an automated chemistry analyser (Olympus AU 640, Hamburg, Germany). Remaining urine supernatants were stored at −80 °C and thawed just once, when analysed. In order to purify and concentrate proteins, thawed urine samples were individually processed by ultrafiltration using Amicon® Ultra centrifugal filter devices (Merck, Darmstadt, Germany) with a 10-kDa molecular weight cut-off. Individual urine samples were added to the device, centrifuged at 5500 ×*g* for 10 min at 4 °C and washed twice with 1 mL of ultrapure water using same conditions. Then, 1 mL of 100 mM triethylammonium bicarbonate buffer (TEAB) (Sigma Aldrich, St. Louis, USA), compatible with further proteomic analysis, was added and samples recentrifuged at 5500 ×*g* for 10 min at 4 °C. Finally, the ultrafiltrate was collected by a reverse spin at 1000 ×*g* for 3 min at 4 °C. Protein concentration in ultrafiltrate urine samples was determined by colorimetric method using a commercial Urinary/CSF Protein reagent (Beckman Coulter, CA, USA) according to manufacturer's instructions on an automated chemistry analyser (Olympus AU 640, Hamburg, Germany).

### 2.4. TMT labelling

TMT reagents (Thermo Scientific, Rockford, USA) were used in order to multiplex identification and quantitation of proteins in samples by tandem mass spectrometry (MS/MS). Serum and urine protein samples were labelled in the same way using TMT reagents according to manufacturer's instructions. In brief, 35 micrograms of each protein sample and IS samples were reduced with 200 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, USA), alkylated with 375 mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) and precipitated with ice-cold acetone (VWR, Pennsylvania, USA) overnight at −20 °C. Then, samples were centrifuged at 8000 ×*g* for 10 min at 4 °C and acetone was removed. Pellets were resuspended with 50 µL of 100 mM TEAB buffer and subjected to trypsin digestion (Promega, Madison, USA) overnight at 37 °C (1 µg of trypsin per 35 µg of protein). Peptide samples were then labelled randomly with TMT-127, TMT-128, TMT-129, TMT-130 and TMT-131 reagents to avoid labelling preference, while IS samples were all labelled with TMT-126 reagent. TMT label reagents were dissolved in anhydrous acetonitrile LC-MS grade (Thermo Scientific, Rockford, USA), added to each sample (18 µL of the reagent to 35 µg of protein) and reaction incubated for 1 h at room temperature. Reaction was quenched by adding 5% hydroxylamine (Thermo Scientific, Rockford, USA) and incubation at room temperature for 15 min. Finally, samples were mixed at equal amounts into sixplex sets for serum and urine proteomic experiments, subsequently (described above). Each mixed sample set (7 µg of protein) was vacuum-dried for 15 min and stored at −80 °C until LC-MS/MS analysis.

### 2.5. LC-MS/MS analysis

TMT-labelled peptides were separated by applying on-line Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) with a trap (C18 PepMap100, 5 µm, 100A, 300 µm × 5 mm) and the analytical (PepMap™ RSLC C18, 50 cm × 75 µm) column during a 120 min linear gradient of 5–45% mobile phase B (0.1% FA in 80% ACN) at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1% FA. The eluate was electrosprayed into the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) through a nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) containing a 10 µm-inner diameter stainless steel emitter (Thermo Scientific, Rockford, USA). Q Exactive Plus was operated in positive ion mode using data dependent acquisition (DDA) Top8 method as described before [20]. The MS setting was acquired with source voltage of +2.00 kV, sheath gas of 0 (arbitrary units), auxiliary gas of 0 (arbitrary units), and capillary temperature of 275 °C. S-lens RF level was 60.0 (arbitrary units). The resolution was set to 70,000 for full MS, 17,500 for MS/MS and a full

scan of the *m/z* range of 350–1800. The AGC targets for full MS and MS/MS were $1 \times 10^6$ and $2 \times 10^5$, respectively. Isolation window was 2.0 *m/z* and dynamic exclusion 30.0 s. HCD fragmentation was performed at step collision energy (29% and 35% normalized collision energy), and precursor ions with unassigned charge state and charge states of +1 and more than +7 were excluded from fragmentation.

### 2.6. MS/MS data analysis

Thermo raw files were analysed using Proteome Discoverer (version 2.0., Thermo Fisher Scientific) and for protein identification and relative quantification. Database search against *Canis lupus familiaris* NCBInr FASTA files (downloaded 13/10/2016, 41,787 entries) was performed using SEQUEST algorithm according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) as fixed peptide modification, oxidation (M), deamidation (N,Q) and TMT sixplex (peptide N-terminus, K) as dynamic modifications. The false discovery rate (FDR) at peptide level was calculated using the Percolator algorithm and the acceptance limit was set at 1% FDR. Proteins with at least two unique peptides and high (1% FDR) and medium (5% FDR) confidence were reported for identification. Protein relative quantification was accomplished by comparing the intensities of the reporter ions at low mass region of MS/MS spectra. Internal standard was used to compare relative quantification results among the TMT experimental sets. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD017263 [21].

### 2.7. Validation of proteomics results

Validation of proteomics results was performed using commercially available canine-specific ELISAs according to manufacturer's instructions. Assays were performed using the samples of the same patients as in proteomic analysis. Selection of biomarker candidates for immuno-assay based validation was based on biological significance and availability of canine-specific assays. Serum alpha 1 acid glycoprotein (AGP) concentration was measured by canine AGP ELISA assay (Shanghai BlueGene Biotech CO, Huissen, the Netherlands), urinary concentration of neutrophil gelatinase-associated lipocalin (NGAL) by canine NGAL ELISA assay (Bioporto diagnostics, Hellerup, Denmark) and urinary concentration of liver-type fatty acid-binding protein (L-FABP) by canine L-FABP ELISA assay (Shanghai BlueGene Biotech CO, Huissen, the Netherlands).

### 2.8. Statistics and bioinformatics analysis

Statistical analyses of proteomics results were performed using R v3.2.2 [22]. First, sample outliers were detected per each group for each of the identified protein using the Dixon's test from R package *outliers* v0.14 [23]. If any sample outlier was significant ($P < 0.05$) it was removed from further analysis. As majority of the analysed proteins did not follow normal distribution, tested by Shapiro-Wilk test, to test the difference in protein abundance between groups Kruskal-Wallis test was performed. Due to multiple comparisons problem, *P*-values were adjusted using the false discovery rate (FDR) q-value from R package *qvalue* v2.2.2 [24]. If any protein showed to be significant (FDR < 0.05), Conover *post-hoc* test was performed for pairwise multiple comparisons using the R package *PMCMR* v4.3 [25]. Only pairwise multiple comparisons with significant adjusted *P*-value (FDR < 0.05) after Conover *post-hoc* tests were considered for further analysis. Protein abundance fold changes between two groups were calculated as mean (Group2)/mean (Group1) and expressed on $\log_2$ scale.

Principal component analysis (PCA) and volcano plots were designed using R package *ggplot2* v3.1.1 [26], Venn diagrams were designed using web tool InteractiVenn (http://www.interactivenn.net/) [27], and heatmap was designed with R package *pheatmap* v1.0.12.

Proteins GI accession numbers were converted into official gene symbol either by DAVID conversion tool (https://david.ncifcrf.gov/conversion.jsp), UniProtKB ID mapping (https://www.uniprot.org/uploadlists/) or from Mascot search engine implemented into Proteome Discoverer. Functional enrichment analyses were performed using R packages *clusterProfiler* v3.16.1 [28] and *ReactomePA* v1.32.0 [29] with Gene Ontology (GO) and Reactome Pathways annotations. Redundant enriched GO terms were removed using *simplify* function with similarity threshold of 0.3 to retain only representative terms. Significantly enriched terms were those with FDR adjusted *P*-value <0.05.

Differences in concentration of potential serum and urine biomarkers measured by ELISAs between healthy and diseased groups were assessed by Kruskal-Wallis test with *post-hoc* Dunn test using GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA). Urinary concentration of potential biomarkers was expressed as a ratio to urine creatinine concentration in order to adjust the marker concentration for variations in urine volume and concentration [30]. For all statistical comparisons, $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Identification and quantification of serum and urine proteins

TMT label-based approach was used in the serum proteomic experiment in order to analyse 6 sixplex sets of 30 serum samples and 6 IS samples, used in each set for normalization. Analysis of data acquired from all 6 sets identified 240 canine serum proteins with high and medium confidence, which were grouped into 150 groups by Proteome Discoverer software. Top-ranking proteins of those groups (master proteins) identified by minimum two unique peptides were used in statistical analysis, which included 150 proteins. There were 58 proteins with significantly different abundances between groups H, A, B and C. The most significant proteins (FDR adjusted *P*-value <0.05, $\log_2FC > 0.5$ or $\log_2FC < -0.5$ for any of the comparisons) are listed in Table 1, while those with $P < 0.05$ and $-0.5 < \log_2FC < 0.5$ are provided in Supplementary Table S1. Distribution of significantly differential serum proteins between groups is shown with Venn diagrams (Fig. 2, A and B).

In the urine proteomic experiment, the same strategy was used as with serum to analyse 6 sixplex sets of 30 urine samples plus IS samples. There were 971 canine urine proteins identified by at least two unique peptides, of which 450 master proteins were used in statistical analysis. The analysis yielded 259 proteins with significantly differential abundances between groups H, A, B and C, of which the most significant are shown in Table 2 (*P*-value <0.05; $\log_2FC \geq 1.7$ or $\log_2FC \leq -1.7$ for any of the comparisons) and the remaining ($P < 0.05$ and $-1.7 < \log_2FC < 1.7$) are listed in the Supplementary Table S2. As we detected higher number of proteins in urine, higher threshold for significantly abundant proteins in urine than in serum samples was selected to be able to focus to the most altered proteins. Distribution of significantly differential proteins in urine between groups is shown with Venn diagrams (Fig. 2, C and D).

In order to examine whether biological replicates of the groups form clusters that differ based on identified protein datasets, PCA plots based on relative abundance values were designed and given in Fig. 3. Of note is that replicate samples formed separated clusters between groups in both serum and urine experiments, with a more clear separation of the groups for acquired urine datasets.

There were 32 statistically significant differential proteins overlapping between serum and urine proteomic experiments, presented in Fig. 4 and listed in Supplementary Table S3. These proteins predominantly followed two patterns, increased abundance in both serum and urine from diseased dogs (with larger fold changes in urine) and increased abundance in urine with decreased abundance in serum. Only three proteins (apolipoprotein H, fibronectin and IGH) showed lower

**Table 1**
Canine serum proteins with significantly differential abundances between groups H, A, B and C identified using TMT approach.

| *GI Accession** | *Description* | *Coverage (%)* | *Unique peptides (N)* | *$log_2$ (FC)* | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | A/H | B/H | C/H | B/A | C/A | C/B |
| 3915605 | apolipoprotein E | 67.54 | 2 | 0.59 | 0.58 | 0.77 | NS | NS | NS |
| 545496287 | complement component C6 | 20.61 | 17 | 0.48 | 0.58 | 0.73 | NS | 0.25 | NS |
| 545496317 | complement component C9 | 27.82 | 12 | 0.58 | 0.65 | 0.79 | NS | NS | NS |
| 223556019 | carbonic anhydrase 2 | 27.31 | 6 | 0.54 | 1.10 | 1.83 | 0.56 | 1.29 | 0.73 |
| 163954 | glycoprotein 80 | 33.26 | 14 | 0.33 | 0.40 | 0.70 | NS | 0.37 | 0.30 |
| 936976329 | C-reactive protein | 38.57 | 6 | 1.20 | 1.32 | 1.31 | NS | NS | NS |
| 545524897 | fibrinogen gamma chain | 32.13 | 13 | 0.93 | 0.97 | 1.61 | NS | 0.68 | 0.64 |
| 227343816 | Chain A, Crystal Structure Of Dog Hemoglobin | 70.92 | 8 | NS | 0.76 | 2.25 | NS | 1.62 | 1.49 |
| 227343817 | Chain B, Crystal Structure Of Dog Hemoglobin | 86.99 | 12 | 1.01 | 1.45 | 2.83 | NS | 1.82 | 1.38 |
| 73957095 | haptoglobin-like | 67.72 | 23 | NS | NS | 1.13 | 0.85 | 1.48 | NS |
| 73988725 | hemopexin | 63.32 | 19 | NS | NS | −0.60 | NS | −0.93 | −0.50 |
| 545553762 | histidine-rich glycoprotein | 22 | 12 | −0.67 | −0.62 | −0.83 | NS | NS | NS |
| 598107 | IgA heavy chain constant region partial | 66.47 | 15 | NS | 0.62 | 1.46 | NS | 1.08 | 0.84 |
| 928186325 | inter-alpha-trypsin inhibitor heavy chain H4 | 46.58 | 38 | 0.61 | 0.87 | 1.51 | 0.26 | 0.90 | 0.64 |
| 345789637 | lipopolysaccharide-binding protein | 36.79 | 13 | 1.58 | 1.76 | 2.04 | NS | 0.46 | 0.28 |
| 345777714 | alpha-1-acid glycoprotein 1 | 49.76 | 11 | 1.10 | 0.92 | 1.30 | NS | NS | 0.38 |
| 345779666 | immunoglobulin J chain | 35.85 | 5 | NS | 0.21 | 0.62 | NS | NS | NS |
| 928186331 | inter-alpha-trypsin inhibitor heavy chain H3 | 18.58 | 13 | 0.57 | 0.54 | 0.60 | NS | NS | NS |
| 545535128 | leucine-rich alpha-2-glycoprotein | 41.81 | 13 | 0.57 | 0.76 | 0.77 | 0.19 | 0.20 | NS |
| 928139154 | sulfhydryl oxidase 1, partial | 34.03 | 17 | 0.46 | 0.52 | 0.72 | NS | 0.26 | 0.20 |
| 928175781 | retinol-binding protein 4 | 39.30 | 6 | −1.18 | −0.86 | −0.43 | NS | 0.75 | 0.44 |
| 545535768 | serum amyloid A1 | 44.19 | 8 | 1.64 | 2.03 | 2.63 | NS | 0.99 | NS |
| 73964432 | alpha-1-antichymotrypsin | 52.49 | 18 | 0.37 | 0.53 | 1.07 | 0.16 | 0.71 | 0.54 |
| 359322074 | peroxiredoxin-2 | 12.63 | 2 | NS | NS | 1.61 | NS | 1.42 | 1.37 |

Group A – dogs with babesiosis with serum creatinine <140 μmol/L and with normal UPC (UPC < 0.5). Group B – non-azotaemic dogs with babesiosis with proteinuria (UPC > 0.5). Group C - azotaemic dogs with babesiosis (serum creatinine >140 μmol/L) and significant proteinuria (UPC > 2). Group H – healthy dogs.
* Accession number from NCBI protein database for *Canis lupus familiaris*, NS- nonsignificant.

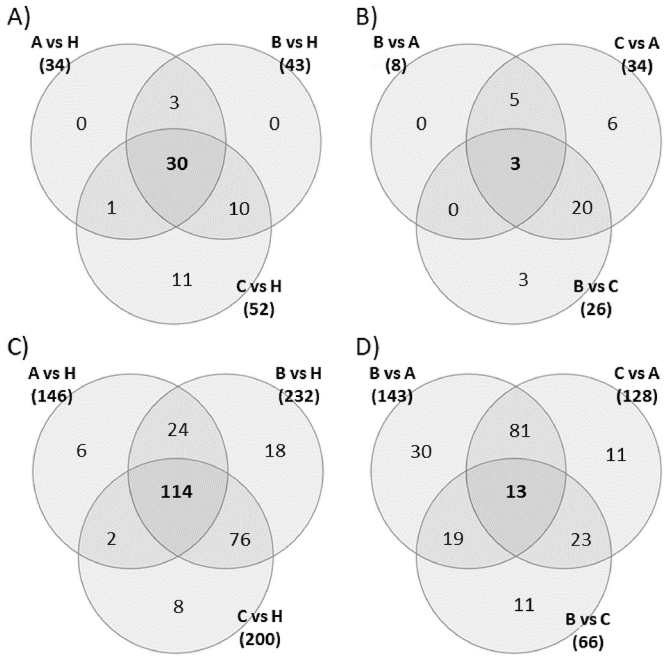


**Fig. 2.** Venn diagrams showing distribution of significantly differential proteins: A) in serum in comparison with healthy group, B) in serum, comparison between groups with babesiosis, C) in urine in comparison with healthy group, D) in serum, comparison between groups with babesiosis. H – control group ($N$ = 8). A - non-azotemic dogs with babesiosis with normal urine protein to creatinine ratio (UPC < 0.5) (N = 6). B - non-azotemic dogs with babesiosis and UPC > 0.5 (N = 10). C - azotemic dogs with babesiosis and UPC > 2 ($N$ = 6).

abundance in both serum and urine.

Graphical representation of the results is depicted in the form of volcano plots showing upregulated and downregulated proteins between the groups H, A, B and C and are showed in Supplementary Fig. S1A and B. There were larger magnitude fold-changes found for proteins with significantly differential abundances between the groups in urine, than in serum.

### 3.2. Functional enrichment analyses of differential proteins

Proteins with significantly differential abundances between groups in serum and urine were functionally annotated using Gene Ontology (GO) database (Supplementary Figs. S2). After removal of redundant enriched GO terms and selecting only those with minimum of 10 proteins, in total 16 Biological Process terms were enriched. Serum proteins with higher abundance were enriched only for protein activation cascade. Urine proteins with higher abundance were enriched for acute inflammatory response, cellular oxidant detoxification, negative regulation of endopeptidase activity and platelet degranulation, while urine proteins with lower abundance were enriched for extracellular matrix, cell-substrate adhesion and regulation of peptidase activity. Interestingly, neutrophil mediated immunity was enriched with urine proteins with decreased abundance in comparisons between babesiosis group A and B *versus* healthy, while it was associated with urine proteins with higher abundance in comparison between babesiosis group C *versus* healthy (Supplementary Fig. S2).

Reactome pathway analysis of 32 statistically significant differential proteins overlapping between serum and urine showed alterations in 32 different pathways, including platelet degranulation, response to elevated platelet cytosolic Ca2+, platelet activation, signaling and aggregation, post-translational protein phosphorylation, binding and uptake of ligands by scavenger receptors, scavenging of heme from plasma, neutrophil degranulation, complement cascade (Fig. 5).

### 3.3. Validation of proteomics results

Concentration of serum AGP measured by ELISA assay was significantly higher in group B (median, interquartile range: 3.9 mg/mL, 3.0–4.1 mg/mL) and group C (4.3 mg/mL, 3.3–4.9 mg/mL) of dogs with babesiosis compared to healthy controls (0.7 mg/mL, 0.5–1.3 mg/mL) (Fig. 6A). Urinary NGAL to creatinine ratio (NGAL/C) and urinary L-

**Table 2**
Canine urine proteins with significantly differential abundances between groups H, A, B and C identified using TMT approach.

| *GI Accession** | *Description* | *Coverage (%)* | *Unique peptides (N)* | *$log_2$ (FC)* | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | A/H | B/H | C/H | B/A | C/A | C/B |
| 345801505 | alpha-hemoglobin-stabilizing protein | 40.22 | 3 | NS | 1.90 | 3.90 | 1.25 | 3.26 | NS |
| 6687188 | Albumin | 93.26 | 2 | 1.78 | 2.50 | 1.24 | NS | NS | NS |
| 928133662 | apolipoprotein A-I | 65.41 | 17 | NS | 1.86 | 2.31 | 1.10 | 1.56 | NS |
| 40950172 | beta amyloid precursor protein | 5.06 | 4 | NS | −1.48 | −1.79 | −0.86 | −1.17 | NS |
| 548923914 | beta-2-microglobulin | 58.4 | 4 | 0.72 | 2.47 | 1.34 | 1.75 | NS | −1.13 |
| 73953824 | complement component C7 | 3.13 | 2 | −1.08 | −2.11 | −1.61 | −1.03 | NS | NS |
| 223556021 | carbonic anhydrase 1 | 61.69 | 11 | 0.49 | 1.84 | 1.21 | 1.36 | 0.72 | NS |
| 223556019 | carbonic anhydrase 2 | 18.08 | 4 | NS | 0.76 | 2.35 | NS | 1.92 | NS |
| 545506465 | charged multivesicular body protein 1b | 10.55 | 3 | −0.92 | −1.74 | −1.00 | −0.81 | NS | 0.73 |
| 50978940 | collagen alpha-2(I) chain precursor | 3.07 | 3 | NS | −1.31 | −1.82 | −0.99 | −1.50 | NS |
| 928173058 | collagen alpha-1(II) chain | 3.29 | 3 | NS | −3.52 | −2.79 | −2.44 | −1.71 | NS |
| 74004777 | collagen alpha-1(III) chain | 1.84 | 3 | NS | −2.21 | −1.95 | −1.64 | −1.38 | NS |
| 345790427 | cathepsin B | 45.13 | 14 | NS | 1.20 | 1.78 | 1.13 | 1.71 | NS |
| 545486348 | cathepsin L2 | 12.91 | 4 | NS | NS | 1.81 | NS | 2.03 | 1.47 |
| 359320080 | dihydrolipoyllysine-residue succinyltransferase component | 11.21 | 4 | −1.03 | −1.73 | −1.41 | −0.70 | −0.38 | NS |
| 545551941 | dentin matrix acidic phosphoprotein 1 | 12.02 | 5 | NS | −2.16 | −1.54 | −1.60 | −0.98 | NS |
| 928183085 | junctional adhesion molecule A | 55.90 | 11 | −0.74 | −1.74 | −1.69 | −0.99 | −0.95 | NS |
| 559098436 | fatty acid-binding protein, liver | 59.06 | 6 | 0.65 | 1.71 | 0.78 | 1.06 | NS | −0.93 |
| 57109396 | fatty acid-binding protein, intestinal | 41.67 | 6 | NS | 1.75 | NS | NS | NS | NS |
| 545491467 | fatty acid-binding protein, heart | 56.39 | 7 | 1.38 | 1.27 | 2.34 | NS | 0.96 | NS |
| 73999292 | fatty acid-binding protein, adipocyte | 37.12 | 4 | NS | 1.19 | 1.88 | 1.18 | 1.87 | NS |
| 345798728 | membrane protein FAM174A | 17.89 | 2 | −1.50 | −2.36 | −1.93 | −0.87 | NS | NS |
| 73978329 | fibrinogen alpha chain | 26.57 | 17 | NS | 0.53 | 2.05 | NS | 1.75 | NS |
| 545521763 | vitamin D-binding protein | 72.99 | 29 | 1.14 | 2.13 | 0.92 | 0.99 | NS | −1.21 |
| 928144044 | Granulins | 22.12 | 7 | −0.84 | −1.69 | −1.81 | −0.85 | −0.97 | NS |
| 227343816 | Chain A, Crystal Structure Of Dog Hemoglobin | 91.49 | 3 | NS | 2.38 | 2.70 | NS | NS | NS |
| 227343817 | Chain B, Crystal Structure Of Dog Hemoglobin | 93.84 | 9 | NS | 2.47 | 2.47 | NS | NS | NS |
| 928179879 | protein HEG homolog 1 | 6.48 | 6 | −1.07 | −2.10 | −1.87 | −1.10 | −0.80 | NS |
| 359321488 | insulin-like growth factor-binding protein 3 | 6.19 | 2 | NS | −1.76 | NS | −1.16 | NS | 1.59 |
| 17066528 | immunoglobulin gamma heavy chain C | 43.46 | 11 | 0.81 | 1.41 | 1.71 | NS | NS | NS |
| 345789637 | lipopolysaccharide-binding protein | 29.94 | 11 | 0.72 | 1.72 | 2.63 | 1.00 | 1.91 | 0.91 |
| 57092313 | neutrophil gelatinase-associated lipocalin | 11.11 | 2 | 1.31 | 1.49 | 1.71 | NS | NS | NS |
| 345777714 | alpha-1-acid glycoprotein 1 | 66.83 | 15 | 2.61 | 2.39 | 1.64 | NS | NS | −0.75 |
| 928157352 | regenerating islet-derived protein 3-gamma-like | 25.91 | 5 | NS | 1.12 | 2.75 | NS | NS | NS |
| 399567842 | hemoglobin subunit alpha-like | 88.73 | 2 | NS | 3.16 | 2.94 | 2.22 | NS | NS |
| 928143149 | CMRF35-like molecule 1 | 20.88 | 3 | 2.40 | NS | NS | −2.05 | −2.43 | −0.38 |
| 928167632 | Serotransferrin | 79.15 | 52 | 0.70 | 1.82 | 0.97 | 1.12 | NS | −0.84 |
| 73957095 | haptoglobin-like | 67.72 | 22 | NS | NS | 1.89 | NS | 1.05 | 0.99 |
| 73966900 | regakine-1-like | 31.91 | 3 | NS | 0.89 | 2.40 | 0.88 | 2.39 | 1.51 |
| 345806460 | lymphocyte antigen 6D | 52.34 | 4 | −1.58 | −2.43 | −2.46 | −0.84 | −0.88 | NS |
| 665505916 | lysozyme C | 65.54 | 9 | NS | 0.92 | 1.87 | 0.99 | 1.95 | 0.95 |
| 928132033 | multimerin-2 | 4.00 | 3 | −1.08 | −1.76 | −1.17 | −0.68 | NS | 0.59 |
| 345805210 | tumor necrosis factor receptor superfamily member 16 | 6.99 | 3 | −1.01 | −1.73 | −1.78 | −0.72 | −0.76 | NS |
| 345779081 | protein NOV | 33.99 | 8 | −1.05 | −1.99 | −1.88 | −0.94 | −0.83 | NS |
| 359322074 | peroxiredoxin-2 | 41.41 | 7 | NS | 1.29 | 1.91 | 0.70 | 1.31 | NS |
| 356582340 | ubiquitin-40S ribosomal protein S27a | 37.82 | 7 | NS | 0.57 | 1.73 | 0.33 | 1.49 | NS |
| 545531229 | Uteroglobin | 41.76 | 4 | −1.99 | −2.78 | −1.94 | −0.79 | NS | NS |
| 73964432 | alpha-1-antichymotrypsin | 26.84 | 9 | 0.87 | 2.11 | 1.44 | 1.25 | 0.57 | NS |
| 545501607 | Leukosialin | 4.07 | 2 | NS | −1.73 | −1.61 | −1.14 | −1.02 | NS |
| 30144609 | trefoil factor 1 | 56.79 | 5 | −1.44 | −2.27 | −2.14 | −0.83 | −0.70 | NS |
| 925114886 | uromodulin | 51.63 | 27 | −1.00 | −1.88 | −1.58 | −0.88 | −0.58 | 0.30 |

Group A – dogs with babesiosis with serum creatinine <140 μmol/L and with normal UPC (UPC < 0.5). Group B – non-azotaemic dogs with babesiosis with proteinuria (UPC > 0.5). Group C - azotaemic dogs with babesiosis (serum creatinine >140 μmol/L) and significant proteinuria (UPC > 2). Group H – healthy dogs.
* Accession number from NCBI protein database for *Canis lupus familiaris*, NS- nonsignificant.

FABP to creatinine ratio (L-FABP/C) were also significantly higher in group B (NGAL/C: 92.4 ng/mg, 73.4–111.5 ng/mg; L-FABP/C: 3.1 μg/g, 1.6–4.2 μg/g) and group C (NGAL/C: 287.7 ng/mg, 185.2–578.3 ng/mg; L-FABP/C: 12.1 μg/g, 5.0–15.6 μg/g) compared to healthy control dogs (NGAL/C: 0.2 ng/mg, 0.1–0.5 ng/mg; L-FABP/C: 0.6 μg/g, 0.4–0.8 μg/g (Fig. 6B and C, respectively).

## 4. Discussion

The main aim of the current study was to perform analyses of serum and urinary proteomes of dogs with renal dysfunction in babesiosis compared to healthy dogs using, for the first time, a high-throughput proteomic method and functional enrichment analyses. To the authors' knowledge, this is the first comprehensive study where both serum and urine proteome samples of the same dogs with renal dysfunction in babesiosis were investigated in order to gain a more complete picture of pathologic changes taking place.

Using TMT label-based quantitative proteomics method, we found 58 serum and 259 urine differentially abundant proteins between healthy controls and dogs with different degrees of renal dysfunction in babesiosis (Fig. S1).

### 4.1. Serum proteome changes during the development of renal dysfunction in babesiosis

Several components of the innate host immune response were found to be upregulated in serum of dogs with babesiosis compared to healthy dogs, such as complement component C6, complement component C9, as well as acute phase proteins: lipopolysaccharide binding protein (LBP), C-reactive protein, fibrinogen, inter-alpha-trypsin inhibitor

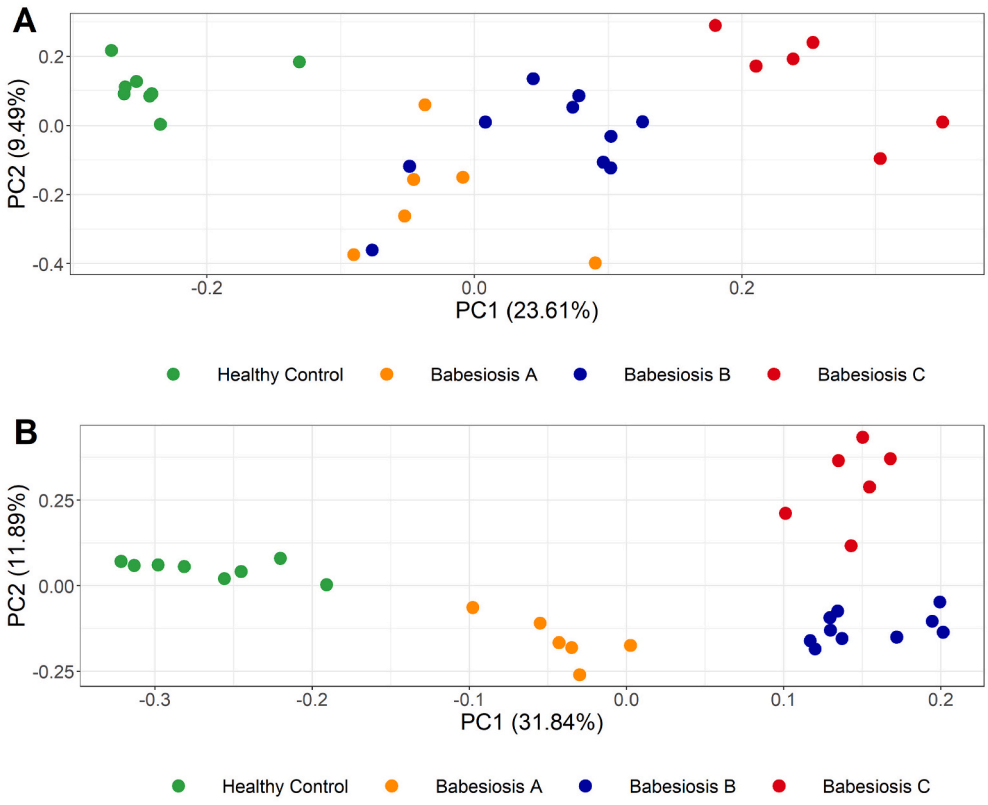


**Fig. 3.** PCA score plot showing distribution of groups in A) serum and B) urine.
Healthy control – control group ($N = 8$). Babesiosis A - non-azotemic dogs with babesiosis with normal urine protein to creatinine ratio (UPC $< 0.5$) ($N = 6$). Babesiosis B - non-azotemic dogs with babesiosis and UPC $> 0.5$ ($N = 10$). Babesiosis C - azotemic dogs with babesiosis and UPC $> 2$ (N = 6).

heavy chain H4 (ITIH4), inter-alpha-trypsin inhibitor heavy chain H3, alpha-1-acid glycoprotein 1, leucine-rich alpha-2-glycoprotein (LRG1) and serum amyloid A1 (Table 1). Bioinformatic analysis of serum proteome differences between studied groups of dogs revealed involvement of acute inflammatory response, acute-phase response, regulation of wound healing and negative regulation of blood coagulation. This is in line with previous reports of serum proteome changes in dogs naturally infected with *B. canis*, which found involvement of acute phase response, complement activation, coagulation cascade and oxidative stress in babesiosis [31,32].

We report herein for the first time the up-regulation of lipopolysaccharide binding protein (LBP) in serum and urine of dogs with babesiosis. LBP is an acute phase protein secreted by the liver usually in response to the Gram-negative bacterial infection, but it was also found increased in serum in human malaria [33]. LBP acts detoxifying toward bacterial lipopolysaccharide endotoxin and triggers activation of innate immune response [34]. Based on our results it may also have a protective role in babesiosis.

Obtained differences in serum proteome are predominantly indicative of disease pathogenesis and host immune response taking place during the course of babesiosis. Nevertheless, several serum proteins can be proposed to be of relevance in context of renal dysfunction in babesiosis, such as leucine-rich alpha-2-glycoprotein, retinol-binding protein 4 and alpha-1-acid glycoprotein 1.

Leucine-rich alpha-2-glycoprotein is an acute-phase protein whose plasma levels are reported to be increased in several pathologies, including kidney diseases [35]. LRG1 was found up-regulated in plasma of human patients during renal function decline in chronic kidney disease [36] and plasma LRG1 concentration was inversely correlated with estimated glomerular filtration rate in patients with type 2 diabetes [37]. In our study urinary LRG1 was increased in both serum and urine of dogs with babesiosis compared to healthy and it was previously proposed to be a biomarker of renal tubular injury in different renal diseases [38].

Plasma retinol-binding protein 4 (RBP4), specific transport protein of retinol, is a negative acute phase protein whose synthesis in liver is reduced during inflammation [39]. This is in line with our results, where serum RBP4 is reduced in groups A, B and C of dogs with babesiosis compared to healthy controls. Due to its small size of 21 kDa, plasma RBP4 is filtered at glomeruli and then reabsorbed by receptor-mediated endocytosis in proximal renal tubular cells. When those cells are damaged, there is an appearance of RBP4 in urine. Urinary RBP4 is a sensitive biomarker of proximal renal tubular damage in various kidney diseases [40], however, due to its decrease depending on the strength of acute inflammatory response [41], we can speculate it is not a suitable biomarker candidate of renal dysfunction in context of babesiosis.

Alpha-1-acid glycoprotein 1 (AGP) is described as moderate acute phase protein (APP) in dogs, commonly reported in malignancies, inflammatory diseases and severe infections [42]. Its role was also supported in this study, both by proteomics and immunoassay. In healthy people urinary AGP secretion is low, however a higher level was detected in patients with renal damage [43–45]. The renal filtration of AGP is more permeable than albumin due to its smaller size, indicating it could be a more sensitive marker of glomerular permeability [46]. Similar to other studies related to inflammatory diseases [47], increase of urinary AGP was more considerable than that of serum AGP in babesiosis.

### *4.2. Urine proteome changes during the development of renal dysfunction in babesiosis*

Several proteins recognized from the literature as biomarkers of renal dysfunction in humans and dogs were significantly changed in the urine of dogs with babesiosis compared to controls and in between

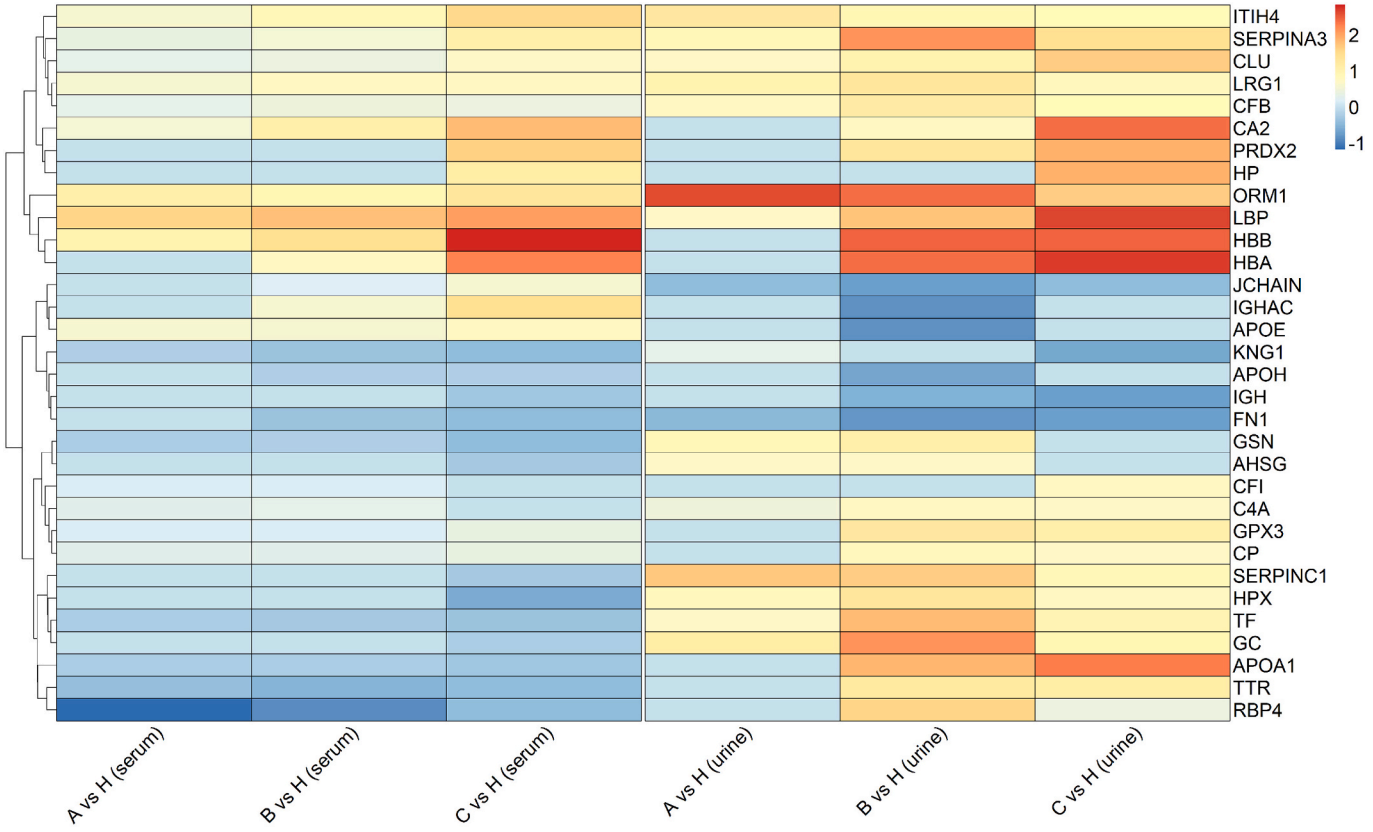


**Fig. 4.** Heat map showing changes in abundance for 32 statistically significant differential proteins overlapping between serum and urine proteomic experiments. H – control group (N = 8). A - non-azotemic dogs with babesiosis with normal urine protein to creatinine ratio (UPC < 0.5) (N = 6). B - non-azotemic dogs with babesiosis and UPC > 0.5 (N = 10). C - azotemic dogs with babesiosis and UPC > 2 (N = 6). Full description of proteins names can be found in Supplementary table 3.

groups A, B and C. These are albumin, immunoglobulin gamma (IgG), NGAL, uromodulin, beta-2-microglobulin and vitamin D-binding protein. Albumin as an intermediate molecular weight (MW) protein with negative charge is almost excluded from filtration by the glomeruli and any that is filtered is reabsorbed by proximal tubular cells. It was shown to be increased in early AKI in humans, as well as in dogs with chronic kidney disease (CKD), X-linked hereditary nephropathy and those experimentally infected with *Dirofilaria immitis* [14]. Elevated urinary albumin levels detected in dogs with babesiosis compared to healthy controls in our study can therefore point out to both glomerular and proximal tubular damage, as previously assessed in dogs with babesiosis and renal dysfunction [48]. Furthermore, we also found increased urinary IgG in dogs with babesiosis, similarly detected previously in dogs with CKD and those with renal dysfunction in several systemic diseases, as well as in babesiosis [48,49]. IgG is a high MW plasma protein which should not be filtered by the glomeruli, and its elevation in urine indicates presence of glomerular damage. NGAL is a 25 kDa protein synthesized in neutrophils and various organs, including the kidneys, where it is released from renal tubules upon injury. Urinary NGAL level was shown to be a good indicator of renal damage in humans with AKI and CKD, as well as in dogs with kidney diseases [50]. Herein its concentration normalized against creatinine was increased in dogs with babesiosis compared to controls, which was further validated by ELISA with urinary NGAL/C ratio elevated in groups B and C compared to controls, making it a promising biomarker in this context (Fig. 6B). Uromodulin (UMOD) is a protein normally found in urine of healthy humans and dogs as it is exclusively produced in the epithelial cells of the distal part of tubules. In canine urine UMOD may have a special role in binding of vitamin A metabolites and its decreased concentration points out to damage of distal tubular cells, as shown in moderately to severely azotaemic and proteinuric dogs with naturally occurring renal disease [51]. Accordingly, our finding of reduced urinary UMOD in dogs with babesiosis compared to controls and in groups B and C compared to A suggests distal tubular damage is also present in dogs with renal dysfunction in babesiosis. Beta-2-microglobulin (B2M) is a small 11.8 kDa protein present in cell membranes as part of major histocompatibility complex class I and is almost completely filtered by the glomeruli and reabsorbed by the proximal tubular cells under physiological conditions. Increased urinary B2M levels were detected in humans with proximal tubular injury due to viral infection, ischemia and toxicity [52]. In dogs with X-linked hereditary nephropathy urinary B2M level was an independent predictor of GFR [16] and urinary vitamin D-binding protein level increased compared to healthy dogs [14]. Increased urinary levels of both B2M and vitamin D-binding protein were found herein in dogs with renal dysfunction in babesiosis, indicating presence of proximal tubular damage.

Several fatty acid binding proteins (FABPs) had increased abundances in urine of dogs with babesiosis compared to healthy dogs and in between groups A, B and C. FABPs are small cytosolic proteins which bind and transport long-chain fatty acids, but also lipid peroxidation products which emerge upon oxidative damage during tissue injury, thereby exerting a protective antioxidative role [53]. Liver-type fatty acid-binding protein (L-FABP) is produced in the liver, intestines and epithelial cells of renal proximal tubules and can be found in urine during hypoxic acute and chronic tubulointerstitial injury in humans [14]. Although well known as promising urinary biomarker of early AKI in humans [54], L-FABP is reported herein for the first time as a potential biomarker of canine renal injury. Moreover, increased urinary L-FABP/C ratio in groups B and C compared to healthy control, measured by ELISA, confirms proteomics results and suggests potential use of this marker in diagnosis of renal dysfunction in babesiosis (Fig. 6C). Increased level of L-FABP in urine of dogs with renal dysfunction indicates hypoxia-

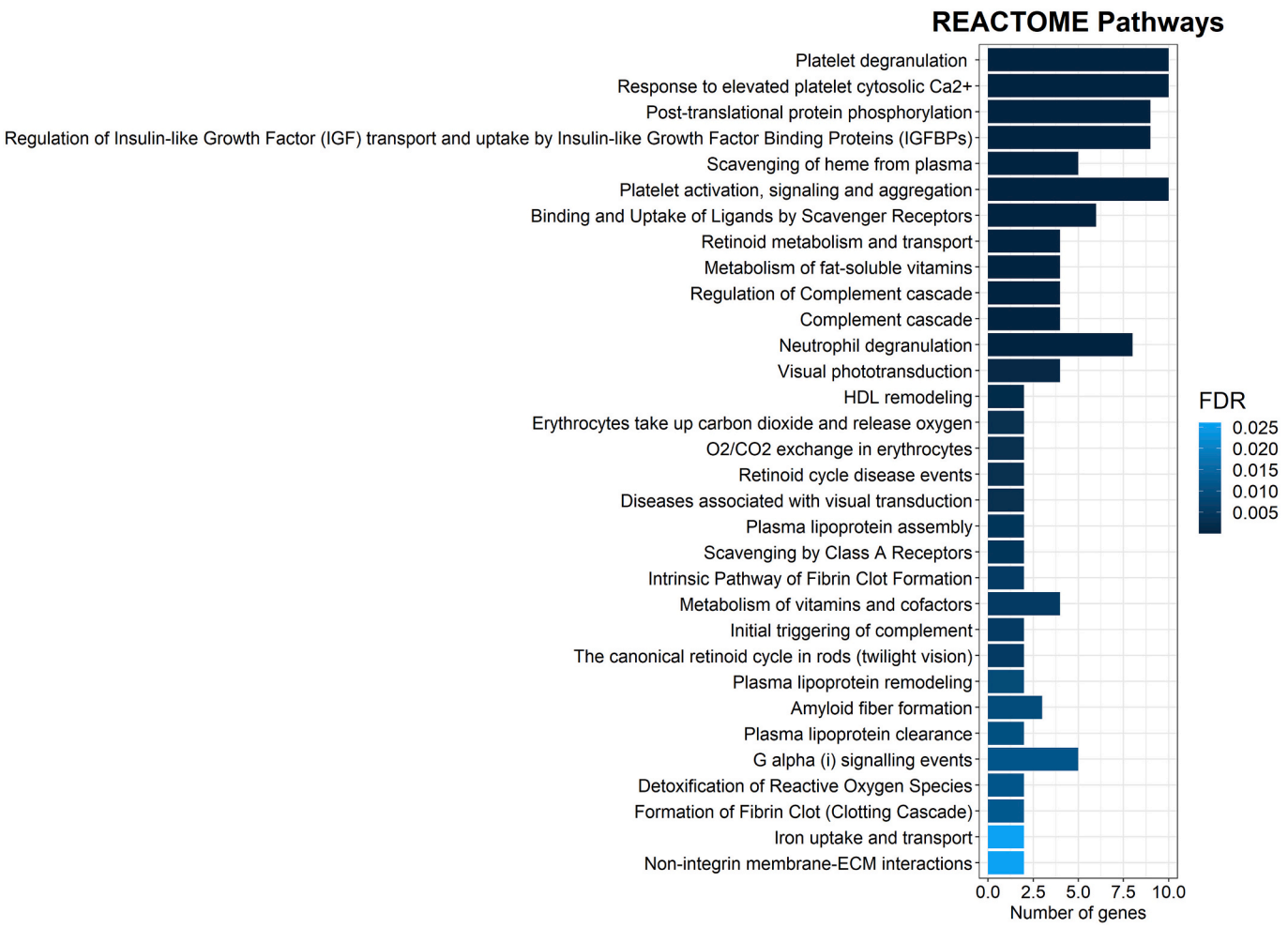


**Fig. 5.** Reactome pathway analysis of 32 statistically significant differential proteins overlapping between serum and urine.

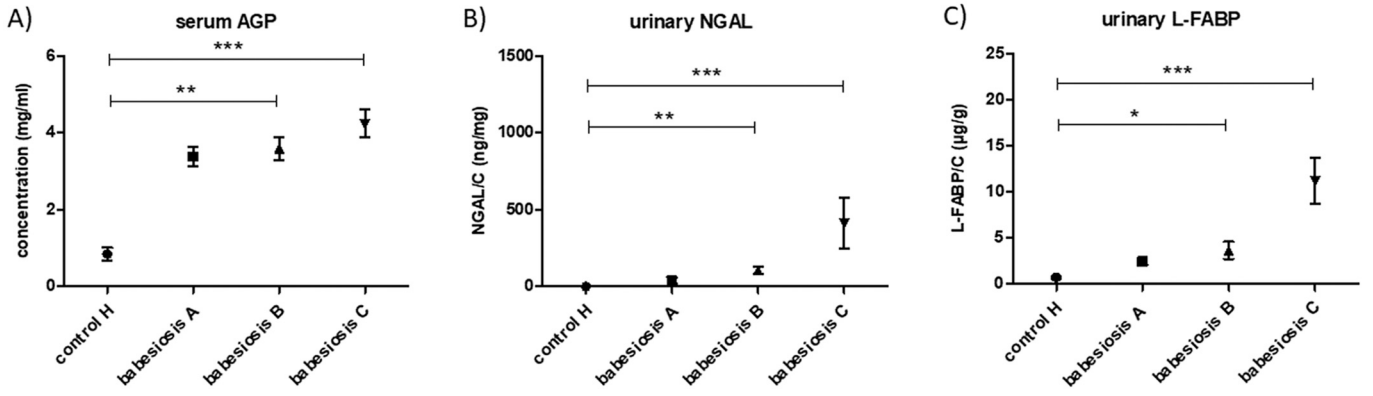


**Fig. 6.** Concentrations of A) serum alpha 1 acid glycoprotein (AGP), B) urinary neutrophil gelatinase-associated lipocalin (NGAL) to creatinine ratio (NGAL/C) and C) urinary liver-type fatty acid-binding protein (L-FABP) to creatinine ratio (L-FABP/C) between all four groups assessed by ELISA and presented as median with interquartile range and corresponding *P* value. Babesiosis A - non-azotemic dogs with babesiosis with normal urine protein to creatinine ratio (UPC < 0.5) (N = 6). Babesiosis B - non-azotemic dogs with babesiosis and UPC > 0.5 (N = 10). Babesiosis C - azotemic dogs with babesiosis and UPC > 2 (N = 6). Control H – healthy dogs (N = 8).

induced kidney injury in babesiosis, also detected by enrichment analysis as significant pathway terms ″cellular response to reactive oxygen species″ and ″removal of superoxide radicals″. Another FABP, heart-type fatty acid-binding protein (H-FABP) was significantly increased in urine of dogs with babesiosis. H-FABP is mainly produced in the heart and has recently emerged as a serum biomarker of myocardial injury [55]. It is also expressed in kidney distal tubular cells and its role in kidney pathologies is less known than that of L-FABP, with some studies showing relation to membranous nephropathy [56] and subclinical AKI [57]. We also detected increased urinary levels of adipocyte-type FABP (A-FABP) in diseased dogs, which is expressed in adipocytes, macrophages and endothelial capillary cells of glomeruli and was shown to be

potential novel biomarker of glomerular damage in humans [58]. Increased urinary FABPs detected in dogs with renal dysfunction in babesiosis may suggest the presence of renal damage of both proximal (L-FABP) and distal (H-FABP) tubular cells, as well as glomeruli (A-FABP).

An interesting finding are the decreased urinary levels of several collagens (type I, II, III, VI, XII, XVIII) in dogs with babesiosis with renal dysfunction compared to controls, as well as dogs with babesiosis with proteinuria compared to the non-proteinuric group. This is in agreement with decreased levels of collagen fragments found in urine of human patients with both AKI and CKD [59], as well as dogs with CKD in a recent study [60]. It is hypothesized that increased extracellular matrix (ECM) deposition in kidneys happening upon fibrosis leads to decreased urinary excretion of collagen fragments [59]. Changed urinary abundances of matrix metalloproteinase-9 and its tissue inhibitor of matrix metalloproteinase-1, as well as of several other ECM components such as fibulins, fibronectin, vitronectin and basement membrane-specific heparan sulfate proteoglycan, further support the notion of ECM remodelling taking place in kidneys of dogs with renal dysfunction in babesiosis. This is also in line with a study by Winiarczyk et al. [17] where urinary proteome of dogs with subclinical kidney injury during babesiosis was compared to that of healthy dogs, using gel-based proteomic approach. Authors hypothesized that epithelial-mesenchymal transition, which is a process of transition of polarized epithelial cells into ECM secreting mesenchymal cells that can in long term lead to fibrous degeneration and organ disorder, may have an important role in pathological changes in renal tissue during babesiosis.

In addition to proteins previously described to be promising biomarkers of renal dysfunction, we can propose several candidate proteins which could be of further research interest, such as trefoil factor 1, uteroglobin, cathepsins B and L2 and granulins. Decreased urinary trefoil factor 3 level was reported to be a novel biomarker for early detection of drug-induced acute renal tubular injury in rats [61]. Uteroglobulin is a potent endogenous immunomodulatory and anti-inflammatory protein, and study on uteroglobulin knock-out mices showed its role in the development of IgA glomerulopathy [62]. The urinary excretion of the lysosomal hydrolase cathepsin B was increased in different models of renal disease in rats, indicating tubular damage [63].

### 4.3. Differential proteins overlapping between serum and urine during the development of renal dysfunction in babesiosis

Reactome pathway analysis of significant differential proteins in both serum and urine compared to healthy pinpointed several pathways reflective of both systemic and renal pathologic processes taking place during the disease (Fig. 5). Nevertheless, pathways such as detoxification of reactive oxygen species, non-integrin membrane-ECM interactions and complement cascade could be highlighted in the context of renal dysfunction. Of 32 statistically significant differential proteins overlapping between serum and urine, the most highlighted pattern of protein abundance is higher abundance in both serum and urine samples of dogs with babesiosis compared to healthy controls (Fig. 4). Among these proteins, there are acute phase proteins (LRG1, AGP, ceruloplasmin, ITIH4, LBP), complement components (complement C4-A, complement factor B, complement factor I), antioxidant enzymes (glutathione peroxidase, peroxiredoxin-2) and haemoglobin chains. Proteins that followed second pattern showed lower abundance in serum and higher in urine of dogs with babesiosis compared to healthy ones. Most of them belong to vitamin transport proteins (RBP4, vitamin D binding protein, gelsolin, transthyretin), haem metabolism (transferrin, hemopexin) and apolipoproteins (Apo A1, Apo E). As abundance changes of these proteins are reflective of systemic changes during babesiosis, differences between groups with different degree of renal dysfunction indicate presence of renal involvement at the same time.

### 4.4. Limitations of the study

There are some limitations of our study, including small sample size, using same samples for initial proteomic experiments and validation, as well as a challenge to distinguish relevant proteome changes reflective of renal pathologic processes from those occurring systemically during the course of the disease. Therefore, confirmation of obtained results using larger sample size is needed in the future. Furthermore, no depletion of high abundant proteins were performed in this study, leading to possible bias against low abundant proteins. Depletion and enrichment methods can help access low abundance proteins, however, their use also comes with disadvantages including increased sample handling, lower reproducibility/throughput, carry-over concerns, and co-depletion of proteins bound to high abundant proteins [18]. Nevertheless, omics technologies approach used in this study proved to be helpful for discovery of potential biomarkers and pathologic mechanisms of renal dysfunction in canine babesiosis.

## 5. Conclusions

We can highlight two putative biomarkers validated herein which could be of importance for early diagnosis of renal dysfunction in canine babesiosis, as they are easily accessible from urine and their concentration rises before appearance of azotaemia (groups A and B). These are urinary L-FABP, acting as potential marker of hypoxic acute and chronic tubulointerstitial injury and urinary NGAL, potential marker of renal tubular injury. Significant finding of our study is also evidence of renal injury location at level of glomeruli, as well as proximal and distal tubules based on significantly changed abundances of >15 relevant urinary proteins between studied groups. This study contributes to the revelation of still unknown mechanisms leading to the development of kidney dysfunction in canine babesiosis. Our results support the notion that renal dysfunction could be a result of systemic hypoxia. In addition, we can speculate that renal dysfunction is, at least in part, related to inflammatory processes triggering fibrous kidney degeneration taking into account involvement of immune response activation and ECM remodelling pathways in kidneys of dogs with renal dysfunction in babesiosis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2022.104735.

## Declaration of Competing Interest

None to declare.

## Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017263

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PRILOG 2

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Original article

# Glomerular and tubular kidney damage markers in canine babesiosis caused by *Babesia canis*

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ABSTRACT

Canine babesiosis is a tick-borne disease caused by the haemoprotozoan parasites of the genus *Babesia*. The aim of this study was to assess renal dysfunction in dogs with babesiosis caused by *B. canis*, using serum and urinary markers for both glomerular and tubular dysfunction. Assays previously not validated for use in canine samples were validated and the potential interference of haemoglobin, lipids and bilirubin with these analyses was additionally considered.

In this study 42 dogs naturally infected with *B. canis* and 14 healthy dogs were included. Dogs with babesiosis were divided into 3 groups: group A consisted of 9 non-azotemic dogs with normal urine protein to creatinine ratio (UPC < 0.5), group B of 27 non-azotemic dogs with UPC > 0.5 and group C of 6 azotemic dogs with UPC > 2. The concentrations of urinary immunoglobin G (IgG), retinol binding protein (RBP), uromodulin, kidney injury molecule – 1 (KIM-1), and serum symmetric dimethylarginine were measured by ELISA assays, while urinary albumin and N-acetyl-b-D-glucosaminidase (NAG) were evaluated by an immunoturbidimetric and enzymatic colorimetric assay, respectively. Urinary markers were normalized to urine creatinine concentration.

All tested markers, with exception of uromodulin, showed significant differences between dogs with babesiosis and healthy dogs, and also showed strong or very strong positive correlation with UPC. Increases of urinary albumin and IgG suggested glomerular damage, and increases of KIM-1, RBP and NAG proximal tubular damage in dogs with babesiosis. They demonstrated clear advantages compared to conventional parameters by showing earlier changes in detecting renal damage.

## 1. Introduction

Canine babesiosis is a tick-borne disease that is caused by the haemoprotozoan parasites of the genus *Babesia*. Acute kidney injury (AKI) is considered one of the most prevalent complication of canine babesiosis with a prevalence varying from 2.2% to 36% (Jacobson and Clark, 1994; Máthé et al., 2006; Matijatko et al., 2012). This complication leads to a decrease of glomerular filtration rate and in consequence causing azotaemia and uraemia in some dogs. Renal damage is identified more often than overt acute kidney injury, making "renal involvement" a more suitable description for this complication (Lobetti and Jacobson, 2001). It is interesting to note that the exceptionally high incidence of renal dysfunction is confirmed in *Babesia canis* infection. The research conducted in Croatia confirmed that AKI was the most common complication (Matijatko et al., 2009), and similar findings were reported in Poland and Hungary (Adaszek et al., 2009; Máthé et al., 2006). Furthermore, high mortality rate of about 50% was reported in dogs with this complication (Máthé et al., 2006).

Different mechanisms of renal damage in babesiosis have been proposed (Jacobson and Clark, 1994; Koster et al., 2015; Lobetti et al.,

*Abbreviations:* TNF-α, tumour necrosis factor alpha; SDMA, symmetric dimethylarginine; KIM-1, kidney injury molecule – 1; NAG, N-acetyl-b-D-glucosaminidase; uriALB, urinary albumin; uromod/C, uromodulin-to-creatinine ratio; KIM-1/C, urinary kidney injury molecule-1-to-creatinine ratio; IgG/C, urinary immunoglobulin G-to-creatinine ratio; RBP/C, urinary retinol-binding protein-to-creatinine ratio; NAG/C, *N*-acetyl-b-D-glucosaminidase-to-creatinine ratio; uriALB/C, urinary albumin-to-creatinine ratio; AUC, area under curve; SE, standard error; CI, confidence intervals; AKI, acute kidney injury; GFR, glomerular filtration rate; IRIS, International Renal Interest Society; HMW, high molecular weight; MMW, middle molecular weight; LMW, low molecular weight; XLHN, X-linked hereditary nephropathy; CKD, chronic kidney disease

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1996). Renal hypoxia caused by anaemia and systemic hypotension seems to be the primary cause of renal damage (Ayoob et al., 2010). Renal dysfunction could also be explained by the cytokine release due to a systemic inflammatory response (Galán et al., 2018; Jacobson and Clark, 1994). Increase in serum concentration of proinflammatory cytokine tumour necrosis factor alpha (TNF-α) was found in canine babesiosis, which was related to hypotension and renal failure. This could be due to the induction of vasodilatation and hypotension by TNF-α, which possibly leads to renal ischaemia and hypoxia, finally causing renal failure (Zygner et al., 2014). Haemoglobinaemia is considered by many clinicians to be the main cause of damage to the kidneys, although experimental studies carried out on healthy dogs administered haemoglobin intravenously was unable to demonstrate that nephropathy occurred in these individuals (Lobetti et al., 1996). It has also been suggested that methaemoglobinuria, reported in canine babesiosis, may be another cause of renal damage, as methaemoglobin has been shown to be nephrotoxic in dogs (Koster et al., 2015; Lobetti and Reyers, 1996).

Current conventional diagnostic tests of kidney damage in blood (serum creatinine and urea) and urine (urine protein creatinine ratio and urine specific gravity) are widely used for diagnosis and monitoring of kidney disease. However, they all have important limitations, such as serum creatinine concentration will only be increased when already 75% of functional renal mass is lost (Braun et al., 2003), and also might be inaccurate in some cases such as in patients with low muscle mass or with fluid overload (Hokamp and Nabity, 2016). It is also important to recognize that changes in serum creatinine and blood urea nitrogen concentrations primarily reflect functional changes in filtration capacity and are not true “injury markers”. Measurement of glomerular filtration rate (GFR) provides the most accurate assessment of renal function and is the most sensitive method for early detection of kidney dysfunction. However, many clearance tests are costly and time-consuming and not suitable for widespread use as a screening test. Recently, Cystatin C has been proposed as more sensitive marker than creatinine for reduced GFR, but concentrations of Cystatin C in dogs with babesiosis were within reference range (de Scally et al., 2006; Ghys et al., 2014). While urine specific gravity is widely used as an estimate of urine osmolality, it was showed to be unreliable in evaluating renal azotaemia in dogs with babesiosis, misdiagnosing 71% of dogs as having prerenal azotaemia (Defauw et al., 2017), and was not measured in our study.

The urine protein to creatinine ratio (UPC) performed on a single random urine sample, has a close correlation to the 24 h urine protein quantification (Harley and Langston, 2012). UPC has become the gold standard test for proteinuria and there are different guidelines for cut-off values. According to the International Renal Interest Society (IRIS), significant proteinuria is manifested with a UPC > 0.5 for dogs, with borderline proteinuria 0.2 to 0.5. In azotemic dogs, a UPC > 1 is associated with increased risk of uremic crisis and death. Prospective monitoring sufficient to accomplish timely detection of any worsening trends is recommended for non-azotemic dogs with persistent microalbuminuria and non-azotemic dogs with persistent renal proteinuria and UPC values > 0.5 (Lees et al., 2005).

Therefore, early diagnosis of decreased kidney function remains a challenge in veterinary medicine, and there is a need for novel and validated markers for early and site-specific detection of kidney dysfunction. In general, the presence of high molecular weight (HMW; > 80 kDa) proteins and middle molecular weight (MMW; approximately 60–80 kDa) proteins in the urine are indicative of glomerular damage, while low-molecular weight (LMW; < 60 kDa) proteins and enzymes in the urine suggest tubular damage due to decreased reabsorption of proteins, direct tubular damage, or both. Additional markers of glomerular filtration rate and glomerular and tubular damage are desirable, particularly for earlier detection of kidney disease when therapy is most effective.

In babesiosis, serum urea concentration should not be used in the diagnosis of renal failure, as it can be elevated due to extra-renal causes such as haemolysis and rhabdomyolysis, both of which occur in babesiosis (de Scally et al., 2004). Furthermore, serum haemoglobin and bilirubin can interfere with the chemical analysis of serum creatinine, leading to an underestimation of its concentration in several methods of creatinine measurement. Although some improvements are implemented in creatinine measurement regarding interferences, e.g. bichromatic measurements and enzymatic methods (Greenberg et al., 2012), new sensitive biomarkers are necessary for the early diagnosis and localisation of renal dysfunction in canine babesiosis.

Novel serum and urine biomarkers are being evaluated in human and veterinary patients in an attempt to improve the ability to accurately diagnose both acute and chronic kidney diseases including the potential for early disease detection. Biomarkers that have demonstrated their potential for early detection and localisation of transitory kidney damage in human studies are gaining interest in companion animals studies. Among others, these urinary markers include intermediate weight proteins such as urinary albumin (uriALB), low molecular weight proteins such as retinol-binding protein (RBP), HMW proteins such as urinary immunoglobulin G (IgG), tubular proteins such as uromodulin and kidney injury molecule - 1 (KIM-1), and urinary enzymes such as N-acetyl-b-D-glucosaminidase (NAG), as well as serum marker symmetric dimethylarginine (SDMA) (Cobrin et al., 2013; Hokamp and Nabity, 2016; Loor et al., 2013). Immunoassays have become the method of choice for measurement of diagnostic markers in different biofluids, such as serum and urine, which are relatively easy to collect. Before a method can be applied in clinical settings or research environments, validation of method is essential. Validation of RBP and IgG ELISA assays, which we used, has already been performed (Maddens et al., 2010b), so we performed analytical validation of other employed assays.

The aim of this study was to assess renal dysfunction in dogs with babesiosis caused by *B. canis*, using serum and urinary markers for both glomerular and tubular dysfunction. Assays previously not validated for use in canine samples were validated and the potential interference of haemoglobin, lipids and bilirubin with these analyses was additionally considered.

## 2. Materials and methods

### 2.1. Study design

In the study 42 dogs naturally infected with *Babesia canis* and 14 healthy dogs were included. Dogs with babesiosis were divided into 3 groups: group A consisted of 9 non-azotemic dogs (serum creatinine < 140 μmol/L) with normal urine protein to creatinine ratio (UPC < 0.5), group B of 27 non-azotemic dogs with UPC > 0.5 and group C of 6 azotemic dogs (serum creatinine > 140 μmol/L) with UPC > 2 (Fig.1).

### 2.2. Animals

This study was approved by the Committee on the Ethics of the University of Zagreb, Faculty of Veterinary Medicine (Permit Number: 640-01/14-305/16, 251-61-01/139-14-28) and conducted from February till June 2017. Blood and urine samples were collected prior to treatment from 42 dogs of various breeds and sex with naturally occurring babesiosis caused by *B. canis*, who were admitted to the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia. There were 15 females (8 spayed) and 27 males (2 neutered), with median age of 3 years (min-max: 6 months – 13 years). The diagnosis of babesiosis was confirmed by demonstration of the parasites within the infected erythrocytes in thin blood smears stained with May-Grünwald-Giemsa stain (Merck, Darmstadt, Germany). Subspecies were confirmed using PCR, as described previously (Beck et al., 2009). One dose (6 mg/kg) of imidocarb dipropionate (Imizol®

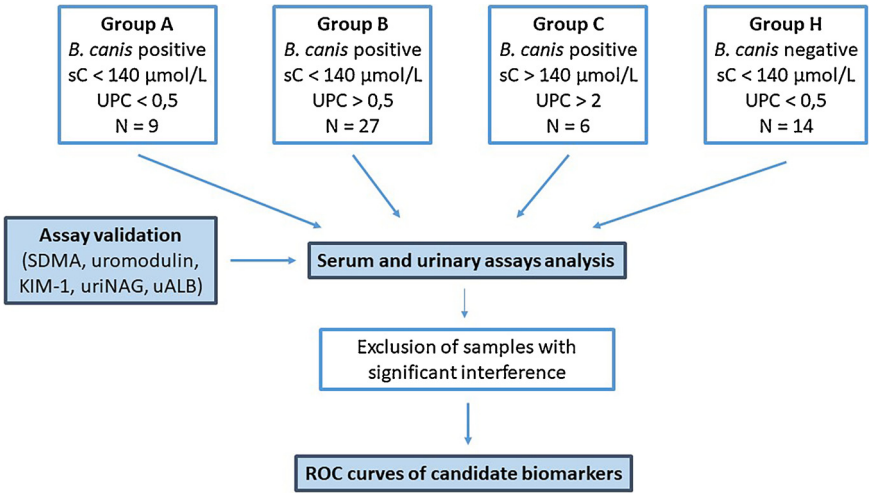


**Fig. 1.** Schematic overview of study design.

12%, Schering-Plough, Kenilworth, NJ, USA) was administered to all the dogs subcutaneously on the day of admission. As needed, other therapy was given to support renal function with intravenous fluids, diuretic therapy, intravenous antibiotics, oxygen supplementation via intranasal tubes or oxygen cage.

The control group consisted of 14 healthy dogs, 6 females (5 spayed) and 8 males (4 neutered), with median age of 4 years (1–13 years). At the time of enrollment, none of the healthy dogs had histories of previous illness. Routine haematologic and biochemical analyses with urinalysis were performed, and all of the obtained results were within reference ranges. PCR was performed as for infected dogs, to rule out sublinical infection.

### 2.3. Urine and serum sample preparation

Blood samples were collected by jugular venipuncture, using the Vacutainer blood collection system (Becton, Dickinson and Co., Rutherford, NJ). Serum was separated by centrifugation at 1000*g* at 4 °C for 15 min, within 1 h of collection, and aliquots were stored at −80 °C until analysis. Voided midstream urine samples were collected into sterile tubes from healthy dogs and dogs with babesiosis. After urinalysis, the remaining urine was centrifuged for 10 min at 500*g* at 4 °C, and supernatant was aliquoted and stored at −80 °C until analysis.

### 2.4. Methods

Urine samples were evaluated by routine urinalysis including semi-quantitative dipstick test (Multistix 10SG, Siemens) on an automated analyser (CLINITEK Status + Analyzer, Bayer) and the microscopic sediment analysis at high (400x) power field. Urine protein and creatinine concentrations were determined using commercial kits on an automated chemistry analyser (Olympus AU 640, Hamburg, Germany). The assessment of lipemia/turbidity, icterus and haemolysis in serum and urine samples was performed by photometric test (LIH, Beckman Coulter, Brea, USA) on an automated chemistry analyser (Olympus AU 640, Hamburg, Germany).

Frozen serum samples were thawed and analysed with competitive enzyme-linked immunosorbent assay (ELISA) for determination of endogenous symmetric dimethylarginine (SDMA) in serum (DLD Diagnostika GmbH, Hamburg, Germany), according to manufacturer's instructions.

The concentrations of IgG and RBP were measured in urine by ELISA assays (ICL, Portland, USA) previously validated for use in canine urine (Maddens et al., 2010b). Canine-specific ELISAs were used for determination of urinary uromodulin (BioVendor – Laboratorni medicina a.s., Brno, Czech Republic) and KIM-1 concentrations (ICL, Portland, USA). Urinary NAG was evaluated by an enzymatic colorimetric assay (Diazyme Laboratories, USA) and uriALB with a human immunoturbidimetric assay (Microalbumin OSR6167 Olympus system reagent) on an automated chemistry analyser (Olympus AU 640, Hamburg, Germany), which were previously validated for use in canine urine samples (Gentilini et al., 2005; Nabity et al., 2012). All assays were performed according to manufacturer's instructions, and preliminary tests were used to determine appropriate sample dilutions.

### 2.5. Analytical validation

For analytical performance of the assays, assay precision, accuracy and limit of detection were calculated as previously described in similar validation studies (Kjelgaard-Hansen and Jacobsen, 2011; Martínez-Subiela and Cerón, 2005; Tvarijonaviciute et al., 2012). All samples used for repetitive analysis were frozen in aliquots and only vials needed for each assay run were used, to avoid possible changes caused by repetitive thawing and freezing. For intraassay precision, two pools of samples with different concentrations of analytes that corresponded to low and high ranges of the assays were prepared from serum and urine samples obtained from healthy dogs and dogs with babesiosis. For inter-assay precision, one pool was divided into aliquots and stored in plastic vials at −20 °C until analysis. Intra-assay coefficient of variation (CV) was calculated, after analysis of the high and low pools, six times in a single assay run. Inter-assay CV was determined by analysing the same samples in five separate runs carried out on different days. The accuracy of the assays was evaluated indirectly by recovery studies. As certified species-specific reference material was not available, to perform a spiking recovery test, two samples with different amount of analytes were mixed in different ratios. Test recovery, expressed as a percentage, was calculated for each dilution for comparison of expected versus measured concentration. Limit of detection was calculated on the basis of data from 11 replicate determinations of the zero standard, as mean value plus three standard deviations.

To investigate the effects of haemoglobin, lipid and bilirubin interference, canine sample pools were mixed with different concentrations of haemoglobin, lipid or bilirubin solution, as described previously (Martínez-Subiela and Cerón, 2005). To determine if interference was present, cut-off limits for acceptable deviation from the original results were set to 10% from original results (Dimeski, 2008; Ryder and Glick, 1993).

To investigate the effect of haemoglobin, a fresh haemolysate was prepared by sonication (Qsonica, Newtown, USA) of full blood from a healthy dog (5 cycles for 10 s at maximum amplitude) followed by

centrifugation for 10 min at 16 000 × *g*. The haemoglobin concentration in the haemolysate was determined by using a veterinary animal blood counter (Horiba Vet ABC; Montpellier, France) and then serially diluted with sample buffer, so 10 µl of each dilution was added to the 90 µl of canine samples. The final haemoglobin concentrations were 8, 4, 2, 0.5 and 0 *g*/L (10 µl of sample diluent buffer were added to the samples to give 0 g/L concentration). These haemoglobin concentrations would correspond to slight haemolysis (0.5 *g*/L), moderate haemolysis (2 *g*/L) and marked haemolysis (4 and 8 *g*/L) (Martínez-Subiela and Cerón, 2005).

To investigate the effects of lipids, a commercial fat emulsion (SMOFlipid 20%, Fresenius Kabi, Graz, Austria) with a triglyceride concentration of 50 *g*/L was serially diluted with sample diluent buffer; 10 µl of each dilution was added to 90 µl of canine sample. Sample homogeneity was then achieved by vortexing. The final triglyceride concentrations were 5, 2.5, 1.25, 0.625 and 0 *g*/L. These triglyceride concentrations would correspond to slight lipaemia/lipiduria (0.7 mmol/L), moderate lipaemia/lipiduria (1.41 and 2.83 mmol/L) and marked lipaemia/lipiduria (5.65 mmol/L) (Martínez-Subiela and Cerón, 2005).

To study the effects of bilirubin interference, 6 mg of bilirubin (Sigma Chemical, St Louis, MO, USA) were suspended in 1 ml of the dimethyl sulfoxide. This stock solution was serially diluted with sample buffer and 10 µl of each dilution was added to the samples of 90 µl of canine plasma and urine. The final bilirubin concentrations were 0.15, 0.075, 0.037, 0.009 and 0 *g*/L. These bilirubin concentrations would correspond to slight bilirubinaemia/bilirubinuria (15.3 µmol/L), moderate bilirubinaemia/bilirubinuria (63.27 µmol/L) and marked bilirubinaemia/bilirubinuria (128.25 and 256.5 µmol/L) (Martínez-Subiela and Cerón, 2005).

### 2.6. Statistical analysis

Statistical analysis was performed using the statistical software, GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). All urinary biomarkers were expressed as a ratio to urine creatinine concentration. Because the total amount of creatinine excreted daily in urine changes little, dividing by the urine creatinine concentration effectively adjusts the marker concentration for variations in urine volume and concentration. This method allows the measurement of the urinary marker in spot urine samples without the necessity of 24-hour urine collection (Gaspari et al., 2006; Pressler, 2015).

Differences between healthy and diseased dogs were assessed by Mann-Whitney test or Kruskal-Wallis test with *post-hoc* Dunn test, depending on number of groups compared. Non-parametric statistics was chosen due to the small sample size. Correlation between investigated markers and conventional parameters (UPC, serum creatinine, urea and albumin) were calculated by non-parametric Spearman's rank correlation coefficient. In order to test the discriminatory power of selected markers, ROC curves were constructed. Differences with P-value $< 0.05$ were considered statistically significant for all performed tests.

## 3. Results

### 3.1. Assays validation

Mean, standard deviation and intra- and interassay CVs are presented in the Table 1. CVs of the pools were below 10% for all assays, except for SDMA which had intra-assay CV of the low pool 10.18%.

The recovery between observed and expected concentrations ranged from 90 to 117%, with a mean of 109% for SDMA; 93–97%, with a mean of 95% for uromodulin; 94–108% with a mean of 101% for KIM-1; 94–97% with a mean of 95% for NAG; and 102–116% with a mean of 108% for uriALB. The detection limit for SDMA assay was 005 µmol/L (mean 0.2 µmol/L, SD 0.01 µmol/L), for uromodulin 0.06 µg/mL (mean 0.03 µg/mL, SD 0.01 µg/mL) and for KIM-1 0.01 ng/mL (mean 0.005 ng/mL, SD 0.002 ng/mL).

**Table 1**
Intra- and interassay coefficients of variation (CV) of symmetric dimethylarginine (SDMA), uromodulin, kidney injury molecule - 1 (KIM-1), *N*-acetyl-b-D-glucosaminidase (NAG) and urinary albumin (uriALB) assays in canine serum and urine pools.

| Assay | Comparison | Pool | Mean | SD | CV (%) |
|---|---|---|---|---|---|
| SDMA (µmol/L) | Intra | High | 1.486 | 0.09 | 6.51 |
| | | Low | 1.039 | 0.11 | 10.18 |
| | Inter | | 0.856 | 0.08 | 9.39 |
| Uromodulin (µg/mL) | Intra | High | 84.128 | 4.05 | 4.81 |
| | | Low | 36.578 | 1.25 | 3.40 |
| | Inter | | 83.486 | 2.10 | 2.52 |
| KIM-1 (ng/mL) | Intra | High | 62.57 | 5.53 | 8.83 |
| | | Low | 12.03 | 1.2 | 9.98 |
| | Inter | | 13.81 | 1.16 | 8.39 |
| NAG (U/L) | Intra | High | 177.7 | 2.47 | 1.39 |
| | | Low | 100.5 | 1.617 | 1.61 |
| | Inter | | 177.0 | 2.51 | 1.42 |
| uriALB (mg/L) | Intra | High | 417.8 | 5.093 | 1.22 |
| | | Low | 2.428 | 0.165 | 6.80 |
| | Inter | | 425.6 | 4.919 | 1.16 |

Incidence of haemolysis, lipemia/lipiduria and bilirubinaemia/bilirubinuria was assessed both for serum and urine (Table 2). In healthy dogs haemolysis, lipaemia/lipiduria and bilirubinaemia/bilirubinuria were not evidenced. Interferograms showing effects of haemoglobin, lipids and bilirubin on SDMA, uromodulin, KIM-1, NAG and uriALB determination are presented in Fig. 2. The addition of haemoglobin significantly interfered with all performed assays: SDMA and NAG concentration determination was affected in the presence of > 3*g*/L of haemoglobin; KIM-1 in the presence of > 5*g*/L of haemoglobin, while uromodulin and uriALB assay showed interference in the presence of > 1*g*/L of haemoglobin. For addition of lipids, interference was shown for SDMA assay (cut-off > 1.41 mmol/L), KIM-1 (cut-off > 5.65 mmol/L), NAG (cut-off > 0.71 mmol/L) and uriALB (cut-off > 0.71 mmol/L). The addition of bilirubin significantly interfered with SDMA assay (cut-off > 15 µmol/L), uromodulin (cut-off > 2.55 µmol/L) and NAG (cut-off > 62.9 µmol/L), while no interference was found for KIM-1 and uriALB assay. As different degrees of interferences affected measured concentrations or activities, those samples were excluded from further calculations.

### 3.2. Assessment of serum and urinary markers

All tested markers, with exception of uromodulin, showed significant differences between dogs with babesiosis and healthy dogs (Table 3). Groups A, B and C were combined together for uromodulin, NAG and uriALB, due to the assays limitations and exclusion of samples with significant interference.

SDMA serum concentration was significantly lower in dogs from group A comparing to healthy dogs ($p < 0.001$). Urinary KIM-1/C ratio was significantly higher in all groups of dogs with babesiosis (A: $p < 0.01$ ; B: $p < 0.001$; C: $p < 0.01$) comparing to healthy dogs.

Urinary IgG/C and RBP/C ratios had similar patterns, as they were significantly higher in dogs from group B compared to group A ($p < 0.05$, for both) and healthy dogs ($p < 0.001$). IgG/C and RBP/C ratios from group C were significantly higher comparing to healthy dogs ($p < 0.001$) and group A ($p < 0.05$).

Both NAG/C and uriALB/C ratios were significantly higher in dogs with babesiosis compared to healthy dogs ($p < 0.001$; $p < 0.05$, respectively).

### 3.3. Correlations

Correlations between investigated markers and conventional parameters (UPC, serum creatinine, urea and albumin) are showed in the

**Table 2**
Incidence of different degrees of haemolysis, lipaemia/lipiduria and bilirubinaemia/bilirubinuria in serum/urine of non-azotemic dogs with babesiosis with normal urine protein to creatinine ratio (UPC < 0.5) (group A), non-azotemic dogs with babesiosis and UPC > 0.5 (group B), azotemic dogs with babesiosis and UPC > 2 (group C).

| | Serum | | | Urine | | |
|---|---|---|---|---|---|---|
| | haemolysis | lipaemia | bilirubinaemia | haemolysis | lipiduria | bilirubinuria |
| **Group A** (N = 9) | 2 slight<br>2 moderate | – | – | 2 slight | – | – |
| **Group B** (N = 27) | 6 moderate<br>13 marked | 2 slight<br>3 marked | 3 marked | 7 slight<br>5 moderate<br>10 marked | 4 slight<br>1 moderate<br>9 marked | 1 slight<br>1 moderate<br>10 marked |
| **Group C** (N = 6) | 1 moderate<br>5 marked | 1 slight<br>1 moderate<br>3 marked | 1 slight<br>1 moderate<br>3 marked | 2 slight<br>1 moderate<br>1 marked | 1 slight<br>1 marked | 1 slight<br>1 marked |

Haemolysis: slight (< 1g/L), moderate (1–3g/L), marked (> 3g/L). Lipaemia/lipiduria: slight (< 1.1 mmol/L), moderate (1.1–3.4 mmol/L), marked (> 3.4 mmol/L). Bilirubinaemia/bilirubinuria: slight (< 50 μmol/L), moderate (50–85 μmol/L), marked (> 85 μmol/L).

Table 4. All urinary markers, with exception of uromodulin, had strong or very strong positive correlations with UPC. Also, they were positively correlated with each other, showing a strong or very strong association.

### 3.4. ROC curves

ROC curves are shown in Fig. 3. Sensitivity and specificity with corresponding 95% confidence intervals (CI), area under curve (AUC) and p values, and cut-off are presented in the Table 5. All 3 biomarkers yielded high AUC values (above 0.95), as well as sensitivity and specificity indicating their good performance as potential markers of kidney dysfunction in canine babesiosis.

## 4. Discussion

A common task in clinical laboratories is to validate new tests, assuring that test results reflect the status of the animals, minimising the amount of the error. Validation studies must ensure that analytical methods can detect the corresponding analyte and provide repeatedly accurate results. All assays evaluated in the present study showed adequate precision with intra- and inter-assay CVs lower than 15%, the limit of the objective analytic performance standard for precision (FDA, 2001). Moreover, CVs were below 10% for all assays, except for SDMA which had intra-assay CV of the low pool 10.18%. Assays for NAG and uriALB gave results with the best precision, possibly because they are automated. Automated assays are usually more robust and have the additional advantage of more rapid turnaround time. All assays showed acceptable range for recovery, which is 80–120% (Andreasson et al., 2015).

Compared to kits manufacturers' data, CVs were similar or even better in case of uromodulin (reported inter-assay CV 5–9%) and NAG (reported intra-assay CV 0.99–4.23%). Data about interferences are more scarce, usually limited to note to avoid haemolytic or lipemic samples (uromodulin, SDMA), or excessive haemolysis (KIM-1); while only for NAG manufacturer reported that interference occurred in presence of triglycerides (> 11.3 mmol/L) and bilirubin (> 85.5 μmol/L). Our interference assessment showed lower concentration of triglycerides (> 0.71 mmol/L) to interfere with NAG measurement, while for bilirubin similar value was established. Samples from dogs with babesiosis, including serum and urine, can be challenging to analyse due to the high frequency of haemolysis and haemoglobinuria. Presence of hyperlipidaemia and hyperbilirubinaemia is also not uncommon in this haemolytic disease (Koster et al., 2015; Solano-Gallego et al., 2016). For this reason, assay validation of interference of haemoglobin, triglycerides and bilirubin was necessary.

All tested markers, with exception of uromodulin, showed significant differences between dogs with babesiosis and healthy dogs, showing their potential to detect and localise transitory kidney damage in babesiosis. Acute kidney injury is considered to be one of the most prevalent complications of canine babesiosis although there are very few previously published studies on markers of renal damage in babesiosis. Babesiosis caused by *B. rossi* and babesiosis caused by *B. canis* are considered to be different diseases, with different clinical manifestations (Schetters et al., 2007). The possible explanation for this difference could be the high incidence of hypotension and consequential development of septic shock in *B. canis* infection unlike in the South African form of babesiosis caused by *B. rossi* (Jacobson and Clark, 1994; Matijatko et al., 2009).

Concentrations of the three urinary markers (IgG, CRP, and RBP) were significantly higher in dogs with naturally occurring uncomplicated canine babesiosis caused by *B. rossi*, compared to healthy dogs, indicating the presence of both glomerular and tubular dysfunction (Defauw et al., 2012). Another study found higher concentrations of IgG, RBP and uromodulin in the urine of 10 dogs with babesiosis caused by *B. canis* compared to control group (Winiarczyk et al., 2017). In canine babesiosis caused by *B. vogeli* three urinary markers (IgG, CRP, and RBP) were not significantly different between dogs with babesiosis and healthy dogs, possibly due to less severe clinical presentation and low mortality rate compared to *B. rossi* and *B. canis*, but a limitation of this study was that it included only 6 infected dogs (Sungpradit et al., 2016).

Our study added new perspectives on this topic by accessing a wide panel of serum and urinary biomarkers, validated and tested in dogs with babesiosis caused by *B. canis* that were divided into subgroups based on conventional parameters of kidney damage in dogs.

Albumin (MW 69 kDa), the most abundant protein found in plasma, was one of the first candidate urinary biomarkers extensively studied in dogs. Microalbuminuria was shown to be a good indicator of early glomerular dysfunction in dogs with X-linked hereditary nephropathy (XLHN), in dogs experimentally infected with Dirofilaria immitis and in Soft-coated Wheaten Terriers genetically at risk for protein-losing enteropathy and nephropathy (Loor et al., 2013). Persistent microalbuminuria or proteinuria with inactive urine sediment strongly suggests the presence of canine chronic kidney disease (CKD). However, some studies also observed microalbuminuria in non-renal diseases, questioning the specificity of this condition analyte for diagnosis of renal diseases in dogs (Whittemore et al., 2006). In addition, the presence of uriALB is not site-specific, because glomerular damage allows increased leakage of albumin and tubular damage decreases the ability of the nephron to reabsorb even small amounts of albumin in the glomerular filtrate (Loor et al., 2013). In this study, all dogs with babesiosis were presented with markedly higher uriAlb/C ratio compared to controls ($p < 0.001$), probably due to both glomerular and tubular damage. The ratio uriAlb/C also showed a very strong correlation with UPC ($R = 0.902$, $p < 0.001$), as well as with other urinary markers, RBP/C ($R = 0.831$, $p < 0.001$), IgG/C ($R = 0.897$, $p < 0.001$) and

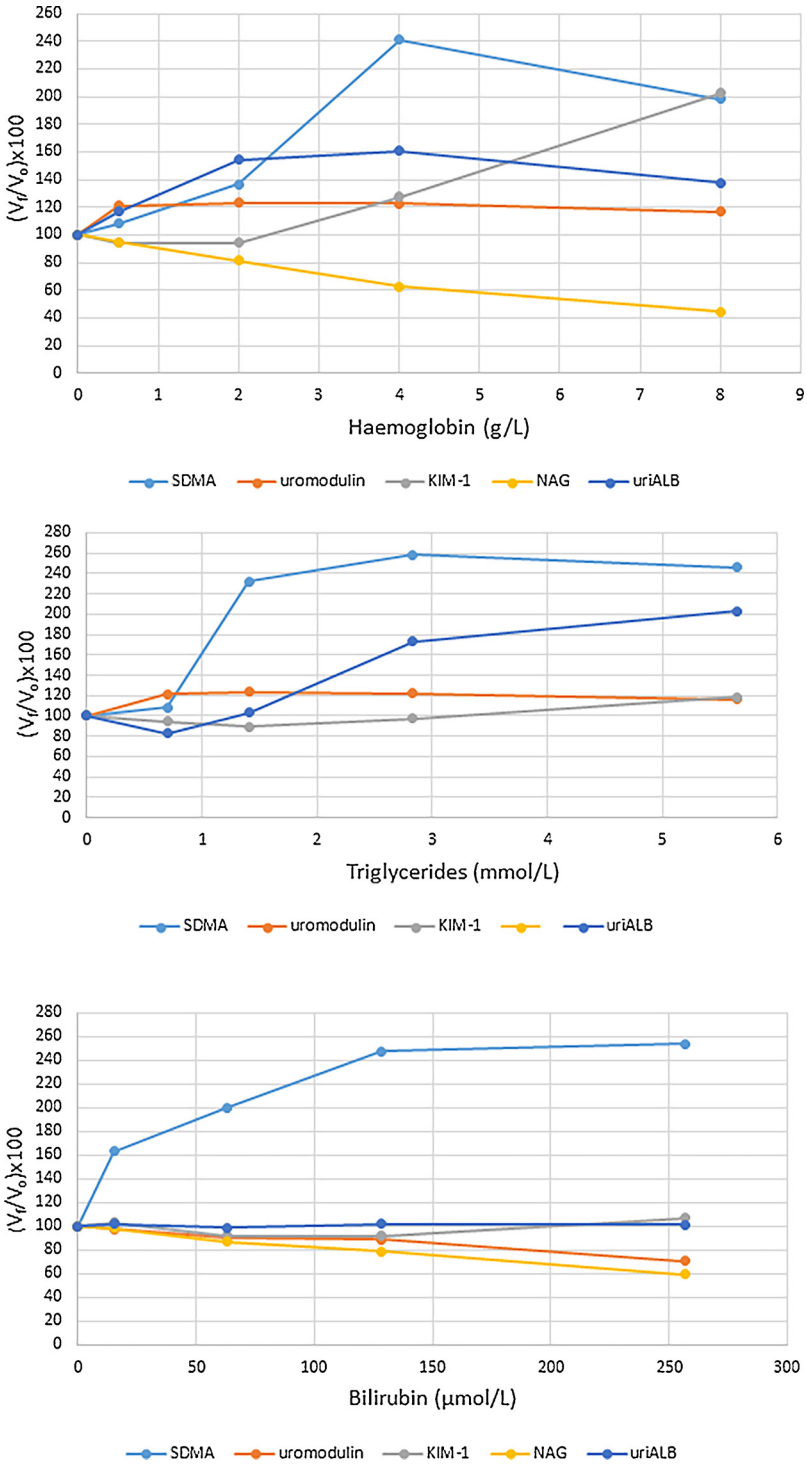


**Fig. 2.** Interferograms corresponding to the effect of haemoglobin, triglycerides and bilirubin concentrations on symmetric dimethylarginine (SDMA), uromodulin, kidney injury molecule - 1 (KIM-1), N-acetyl-b-D-glucosaminidase (NAG) and urinary albumin (uriALB) determination in canine serum/urine pool. X-axes show increasing concentrations of haemoglobin, triglycerides or bilirubin, whilst Y-axes show percentage of change in analyte $(V_f/V_0) \times 100$. $V_f$ - final value; $V_0$ - original value. Data for effect of triglycerides on NAG determination were not presented due to very high interference determined.

KIM-1/C ($R = 0.748$, $p < 0.001$).

Glomerular damage consequently leads to the urinary presence of HMW proteins (D'Amico and Bazzi, 2003). Theoretically, IgG is a better and more selective marker of the severity of damage to the glomerular capillary wall than the overall degree of proteinuria, because increasing amounts of this HMW protein cross the glomerular barrier with an increasing severity of glomerular lesions (D'Amico and Bazzi, 2003). Immunoglobulin G was found to be increased in the urine of dogs with leishmaniasis, pyometra, leptospirosis, Cushing's syndrome, and XLHN (Loor et al., 2013; Maddens et al., 2010a, 2011). The increased level of IgG/C in dogs with babesiosis confirms the findings of other studies in dogs with *B. canis* and *B. rossi* (Defauw et al., 2012; Winiarczyk et al., 2017). Of note is the finding that there is no significant difference between healthy dogs and dogs from group A, suggesting intact glomerular function in that group. Apart being associated with RBP/C ($R = 0.767$, $p < 0.001$), KIM-1/C ($R = 0.632$, $p < 0.001$) and

**Table 3**
Comparison of serum and urinary markers in non-azotemic dogs with babesiosis with normal urine protein to creatinine ratio (UPC < 0.5) (group A), non-azotemic dogs with babesiosis and UPC > 0.5 (group B), azotemic dogs with babesiosis and UPC > 2 (group C) and healthy dogs (group H). Data are presented as median (Q1-Q3). P value for Mann-Whitney or Kruskal-Wallis test is given.

| Parameter | Group A | Group B | Group C | Group H | P - value |
|---|---|---|---|---|---|
| **SDMA (μmol/L)** | 0.51 (0.40-0.79) (N = 8) | 0.85 (0.55-1.03) (N = 12) | – | 1.14 (1.05-1.21) (N = 8) | **0.001** |
| **uromod/C (mg/g)** | 34.76 (28.67-61.55) (N = 16) | | | 30.56 (20.32-51.20) (N = 14) | 0.493 |
| **KIM-1/C (mg/g)** | 0.027 (0.024-0.036) (N = 9) | 0.034 (0.025-0.048) (N = 17) | 0.042 (0.030-0.075) (N = 6) | 0.011 (0.009-0.014) (N = 14) | < **0.001** |
| **IgG/C (mg/g)** | 6.93 (3.69-11.34) (N = 8) | 55.08 (28.22-105.9) (N = 27) | 118.6 (24.83-852.0) (N = 6) | 0.36 (0.19-0.58) (N = 14) | < **0.001** |
| **RBP/C (mg/g)** | 0.015 (0.011-0.069) (N = 9) | 0.028 (0.024-0.036) (N = 27) | 0.399 (0.096-0.608) (N = 6) | 0.009 (0.004-0.014) (N = 14) | < **0.001** |
| **NAG/C (UI/g)** | 12.39 (5.76-39.54) (N = 15) | | | 1.37 (0.51-5.14) (N = 6) | **0.014** |
| **uriALB/C (g/g)** | 172.8 (55.10-854.10) (N = 16) | | | 3.58 (2.24-8.36) (N = 14) | < **0.001** |

Groups A, B and C were combined together for uromodulin, NAG and uriALB, due to the assays limitations and exclusion of samples with significant interference. SDMA – symmetric dimethylarginine, uromod/C – uromodulin-to-creatinine ratio, KIM-1/C - urinary kidney injury molecule-1-to-creatinine ratio, IgG/C - urinary immunoglobulin G-to-creatinine ratio, RBP/C - urinary retinol-binding protein-to-creatinine ratio, NAG/C - N-acetyl-b-D-glucosaminidase-to-creatinine ratio, uriALB/C – urinary albumin-to-creatinine ratio. Significant p values (< 0.05) are bolded.

uriALB/C as previously stated, IgG/C also has strong correlation with another urinary marker NAG/C (R = 0.656, p = 0.001) and UPC (R = 0.847, p < 0.001) and moderate correlation with serum urea concentration (R = 0.467, p < 0.001).

The LMW protein – RBP (MW 21 kDa) was measured as a marker of proximal tubular dysfunction. The RBP-retinol complex is bound to transthyretin in plasma, and this binding prevents its glomerular filtration, while uncomplexed RBP is freely filtered by the glomeruli and efficiently reabsorbed by the proximal tubular cells. Increased levels of RBP are expected in dogs with proximal tubule disorders, as previously reported in dogs with pyometra, urolithiasis, CKD, and Cushing's syndrome (Forterre et al., 2004; Loor et al., 2013; Maddens et al., 2010a, 2011; Raila et al., 2000).

Our finding of increased level of RBP/C in urine also confirmed previous reports of this marker in babesiosis (Defauw et al., 2012; Winiarczyk et al., 2017). This marker also had a strong correlation with other urinary markers, IgG/C, KIM-1/C (R = 0.673, p < 0.001), uriALB/C, NAG/C (R = 0.668, p = 0.001), but also with UPC (R = 0.861, p < 0.001) in this study. Increase of RBP in urine could be a result of the saturation of the tubular reabsorption mechanisms with HMW proteins or their competition for receptor-binding sites, but also a result of direct tubular damage. Various studies have shown that exposure of tubular epithelial cells to ischaemia or cytokines have effects on decreasing the expression of receptors and specific transporters responsible for RBP (and other LMW proteins) reabsorption (Vinge et al., 2010). It has already been documented that tissue hypoxia present in babesiosis has a nephrotoxic effect causing kidney damage (Ayoob et al., 2010), and the excessive production of pro-inflammatory cytokines are important mediators of pathogenesis in babesiosis leading to SIRS and/or MODS development (Galán et al., 2018). Therefore, the combination of hypoxia and inflammatory cytokine production may have a direct effect to tubular damaging effect in babesiosis.

N-acetyl-b-D-glucosaminidase (NAG), a high molecular weight (150 kDa) lysosomal enzyme present in the proximal tubular cells, appears in urine after disruption of cell integrity. In healthy humans and animals, NAG activity is low. Urinary NAG activity is a widely used measure of tubular function, and elevations have been detected in humans with kidney disease secondary to various conditions (Vaidya et al., 2008). Increased NAG activity has also been used to detect the onset of AKI associated with pyometra and leishmaniasis (Maddens et al., 2010a; Palacio et al., 1997). In concert with RBP, significantly higher urinary levels of this marker confirmed proximal tubular damage in babesiosis. Urinary NAG/C also had a strong correlation with other urinary markers, RBP/C, IgG/C, KIM-1/C (R = 0.600, p = 0.004), as well as with UPC (R = 0.658, p = 0.001), which evidented its utility as marker of transitory kidney damage.

**Table 4**
Correlation between serum and urinary markers and routine parameters for kidney dysfunction. Spearman's rank correlation coefficients are shown (*p < 0.05).

| | SDMA (μmol/L) | RBP/C (mg/g) | IgG/C (mg/g) | KIM-1/C (mg/g) | uromod/C (mg/g) | uriALB/C (g/g) | NAG/C (UI/g) | sCreatinine (μmol/L) | UPC | urea (mmol/L) | sAlb (g/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SDMA (μmol/L) | | −0.116 | −0.205 | −0.497* | −0.275 | −0.395 | 0.036 | 0.476* | −0.250 | 0.223 | 0.418* |
| RBP/C (mg/g) | | | 0.767* | 0.673* | 0.006 | 0.831* | 0.668* | 0.262 | 0.861* | 0.390* | −0.419* |
| IgG/C (mg/g) | | | | 0.632* | 0.138 | 0.898* | 0.656* | 0.273* | 0.847* | 0.467* | −0.363* |
| KIM-1/C (mg/g) | | | | | 0.073 | 0.748* | 0.601* | −0.043 | 0.691* | 0.149 | −0.815* |
| uromod/C (mg/g) | | | | | | 0.113 | −0.207 | −0.172 | 0.007 | 0.090 | −0.169 |
| uriALB/C (g/g) | | | | | | | 0.476 | 0.055 | 0.902* | −0.031 | −0.521* |
| NAG/C (UI/g) | | | | | | | | 0.468* | 0.658* | 0.389 | −0.586* |
| sCreatinine (μmol/L) | | | | | | | | | 0.222 | 0.594* | −0.024 |
| UPC | | | | | | | | | | 0.508* | −0.476* |
| urea (mmol/L) | | | | | | | | | | | −0.155 |

SDMA – symmetric dimethylarginine, uromod/C – uromodulin-to-creatinine ratio, KIM-1/C - urinary kidney injury molecule-1-to-creatinine ratio, IgG/C - urinary immunoglobulin G-to-creatinine ratio, RBP/C - urinary retinol-binding protein-to-creatinine ratio, NAG/C - N-acetyl-b-D-glucosaminidase-to-creatinine ratio, uriALB/C – urinary albumin-to-creatinine ratio, sCreatinine – serum creatinine concentration, UPC - urine protein to creatinine ratio, sAlb – serum albumin concentration.

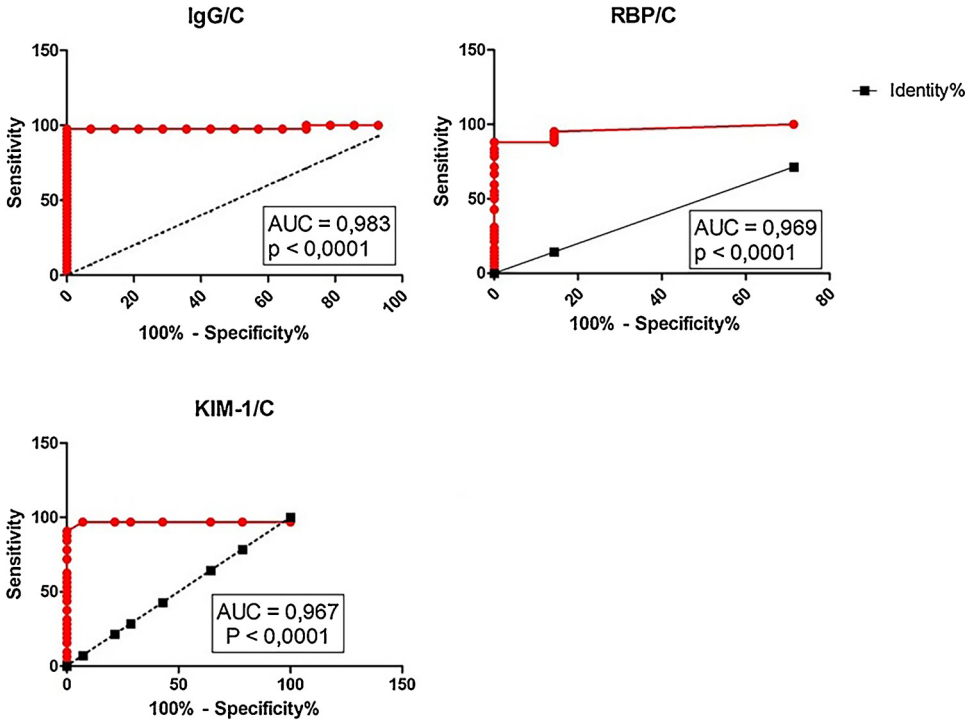


**Fig. 3.** Receiver operating characteristic (ROC) curves for selected urine markers: IgG/C (urinary immunoglobulin G-to-creatinine ratio), RBP/C (urinary retinol-binding protein-to-creatinine ratio) and KIM-1/C (urinary kidney injury molecule-1-to-creatinine ratio). AUC – area under curve.

**Table 5**
Receiver operating characteristic (ROC) curve analyses for selected urine markers.

| | AUC ± SE (95% CI) | Cut-off point | Sensitivity (%) (95% CI) | Specificity (%) (95% CI) | P value |
|---|---|---|---|---|---|
| **KIM-1/C (mg/g)** | 0.967 ± 0.03 (0.91 – 1.03) | > 0.019 | 90.63 (74.98 – 98.02) | 100 (76.84 – 100) | < 0.001 |
| **IgG/C (mg/g)** | 0.983 ± 0.02 (0.95 – 1.02) | > 2.225 | 97.56 (87.14 – 99.94) | 100 (76.84 – 100) | < 0.001 |
| **RBP/C (mg/g)** | 0.969 ± 0.02 (0.93 – 1.01) | > 0.035 | 88.10 (74.37 – 96.02) | 100 (76.84 – 100) | < 0.001 |

KIM-1/C - urinary kidney injury molecule-1-to-creatinine ratio, IgG/C - urinary immunoglobulin G-to-creatinine ratio, RBP/C - urinary retinol-binding protein-to-creatinine ratio, AUC – area under curve, SE – standard error, CI – confidence intervals.

Kidney injury molecule-1 is a type I transmembrane glycoprotein, expressed at low to undetectable levels in the normal kidney, but it increases significantly in proximal tubule cells, following kidney injury (Jin et al., 2017). KIM-1 is considered to be highly specific for the detection of renal proximal tubular injury, and is used primarily to detect AKI. KIM-1 increased significantly in human AKI patients, compared to non AKI patients, showing an excellent diagnostic performance (Yun and Andrew Craig, 2011). In human AKI patients, urine KIM-1 was seen to increase 2 h after kidney injury, and it stayed elevated up to 48 h after injury. A recent study evaluated the sensitivity and specificity of urine KIM-1 for the detection of naturally occurring AKI in dogs and found urine KIM-1/C to elevate in early, non-azotemic AKI (Lippi et al., 2018).

Our results also showed excellent performance of this marker, as it significantly increased in all of 3 groups of dogs with babesiosis compared to healthy dogs. These findings suggest the presence of transitory kidney damage in babesiosis even before the occurrence of any conventional parameter changes (as seen in group A). The value of this marker has also been demonstrated in that it shows strong correlations with other urinary markers, RBP/C, IgG/C, uriALB/C, NAG/C, as well as with the conventional marker such as UPC ($R = 0.691$, $p < 0.001$).

A marker that might be useful in the recognition of distal tubular injury is uromodulin or Tamm-Horsfall protein (MW 100 kDa), a glycoprotein exclusively synthesized in the cells lining the thick ascending limb and distal convoluted tubules (Hokamp and Nabity, 2016). The high metabolic activity of these cells makes them particularly vulnerable to ischemic injury. Uromodulin is one of the major urinary proteins present in healthy dogs and normal urine has high concentrations of uromodulin. Significantly reduced urinary concentrations are seen in dogs and cats with renal diseases, including CKD (Hokamp and Nabity, 2016). Contrary to this, Winiarczyk et al. (2017) found an increased urinary excretion of uromodulin in dogs with babesiosis. In our study, no difference was found between dogs with babesiosis and healthy ones. This could be a consequence of the test's interference with heamoglobinuria, due to which some samples were excluded mainly from groups B and C.

Serum symmetric dimethylarginine is a relatively newly discovered renal function biomarker. Due to its small molecular size, it is freely filtered by in the glomerular filtrate. Therefore, it is an endogenous marker of GFR not influenced by muscle mass, which is the advantage it has over creatinine (Relford et al., 2016). Longitudinal studies of SDMA in dogs and cats with CKD showed that SDMA level increased months earlier than serum creatinine, when there was on average a 40% reduction in GFR, whereas creatinine level increases late, when there is up to as much as a 75% reduction of GFR. Serum SDMA has been included provisionally as part of the IRIS CKD guidelines, as modified in 2015, for staging of both early and advanced CKD (Relford et al., 2016). So far, SDMA has been used successfully to diagnose CKD in dogs and cats. Only one study analysed the relevance of SDMA in dogs with AKI, and showed that SDMA concentrations measured by liquid chromatography-mass spectrometry were significantly higher in dogs with AKI or CKD in comparison with healthy dogs, but could not differentiate between acute and chronic injury (Dahlem et al., 2017).

In our study, we only found a difference between group A and healthy dogs, which showed with a lower concentration of serum SDMA in babesiosis group. This was contrary to what we have expected. Decreased SDMA concentrations might indicate increased GFR or hyperfiltration. Hyperfiltration is hypothesized to be a precursor of intraglomerular hypertension leading to albuminuria. This could be due to changes in systemic arterial pressure and/or changes in efferent and afferent arteriolar resistances (Palatini, 2012). Whether this mechanism is present in babesiosis needs to be further addressed. In addition, serum biomarkers of renal damage are obviously inferior to urinary, and considering complex pathophysiology of babesiosis involving haemostatic and inflammatory disturbances they could be influenced by different mediators and pathways (Goddard et al., 2016; Kuleš et al., 2017a, b). Comparing dogs from group B and healthy dogs we found no difference in SDMA concentrations. SDMA could therefore not differentiate dogs with babesiosis induced transitory kidney damage from healthy dogs in our study.

Of all tested markers, we selected RBP, IgG and KIM-1 for ROC analysis, as they showed the best results performance in differentiating renal damage in dogs with babesiosis. RBP and IgG showed a similar pattern, with significantly increased levels in groups B and C compared to healthy dogs, while group A did not differ from healthy ones. This suggest that these markers are increasing earlier than serum creatinine concentrations, which are within reference range in dogs from group B. Increase of RBP and IgG are strongly correlated with UPC. With a cut-off value of 2.225 mg/g, IgG/C showed excellent specifity and sensitivity, as well as RBP/C with a cut-off of 0.035 mg/g for detection of renal damage in babesiosis. KIM-1 has shown the best differentiation of all investigated markers, as all groups of dogs with babesiosis had higher values compared to healthy group. This indicates that even in dogs with babesiosis without any changes in conventional markers such as serum creatinine and UPC, renal tubular damage is present. Being highly specific and sensitive for renal damage, with a cut-off value of 0.019 mg/g, KIM-1/C was the most promising urinary kidney damage marker. Urinary KIM-1 has attracted attention as novel tool for establishing the diagnosis and prognosis in AKI. A number of characteristics of KIM-1 are attractive in this regard: the absence of KIM-1 expression in the normal kidney; its marked up-regulation with injury; its high expression on the apical membrane of the proximal tubule; the stability of the large cleaved ectodomain excreted in the urine; presence in urine is seen as highly specific for kidney injury; and it has a higher sensitivity than other classical kidney injury markers (Bonventre, 2009).

We can consider small number of animals as a limitation of our study. Merging of predefined groups due to interferences based exclusion contributed to this limitation. Furthermore, the appropriateness of the creatinine concentration of 140 µmol/L as the cut-off for azotemia might be questionable, considering the differences in gender and muscle mass. Another point to consider is the availability of the investigated assays for the routine veterinary practice. While assays on automated biochemical analysers (NAG, uriALB) would be relatively easy to implement to practice, ELISA assays demand more effort in terms of instrumentation, time and labour work.

### 4.1. Conclusions

There is an urgent need for better biomarkers for acute kidney injury in dogs, for its timely diagnosis and the prediction of severity and outcome. Early detection of renal function impairment, before the onset of clinical abnormalities and development of azotemia, may allow initiation of more effective therapy. Different serum and urinary markers, which have already shown their diagnostic value in AKI among human patients, were tested to gain better insight into the detection of transitory kidney damage associated with babesiosis. In addition, assay validation for several markers was successfully performed for the first time in canine samples, showing acceptable analytical characteristics. Increases of uriALB and IgG suggested glomerular damage, and increases of KIM-1, RBP and NAG suggest proximal tubular damage in dogs with babesiosis. These molecules demonstrated clear advantages when compared to conventional parameters by showing earlier changes in detecting renal damage. These findings will be useful for further inclusion of urinary markers in clinical veterinary practice for the detection and monitoring of renal damage in babesiosis, as well as in other similar diseases. For the comprehensive evaluation of renal damage in babesiosis, a panel of biomarkers instead of a single one has clear advantages.

## Conflicts of interest

All authors report no conflicts of interest.

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PRILOG 3

Tablica 1. Proteini sa značajno promijenjenom razinom u urinu (N=213, p<0,05) između pasa s babeziozom (skupina B, N=6) i zdravih pasa (skupina Z, N=6) otkriveni u neobjavljenom preliminarnom proteomskom istraživanju. U istraživanju je korištena metoda LC-MS/MS za otkriće i kvantifikaciju proteina bez obilježavanja. Programi *MaxQuant* i *Perseus* korišteni su za obradu i statističku analizu podataka. Zelenom bojom označeni su proteini sa značajno povećanom razinom u pasa s babeziozom u odnosu na zdrave, a plavom bojom proteini sa značajno smanjenom razinom u pasa s babeziozom u odnosu na zdrave.

| **Pročišćeni FASTA naslovi** | **Omjer količine proteina Z/B** | **Broj jedinstvenih peptida** | **Pokrivenost sekvence (%)** |
|---|---|---|---|
| VASCULAR CELL ADHESION PROTEIN 1 (VCAM1) | 0,0093047 | 27 | 44,9 |
| SERPINA3 | 0,01534279 | 20 | 55,3 |
| AZGP1 (ZINC ALPHA-2-GLYCOPROTEIN 1) | 0,016803169 | 26 | 66,7 |
| CA1 | 0,021069943 | 12 | 70,9 |
| CTSZ | 0,023154497 | 9 | 46,4 |
| FABP3 | 0,024238792 | 5 | 51,1 |
| ALPHA-1-ACID GLYCOPROTEIN (LOC100685620) | 0,028921546 | 23 | 80 |
| SERPIND1 | 0,032994153 | 11 | 22,8 |
| ADIPONECTIN (APM1) | 0,036001062 | 7 | 34 |
| TF | 0,044460391 | 75 | 77,5 |
| BETA-2 MICROGLOBULIN (B2M) | 0,044664127 | 10 | 64 |
| CP | 0,044882452 | 27 | 36,5 |
| APOLIPOPROTEIN A-I (APOA1) | 0,046561134 | 3 | 77,1 |
| CALRETICULIN (CALR) | 0,047285526 | 13 | 50,7 |
| GC | 0,048876258 | 34 | 85 |
| FIBRINOGEN BETA CHAIN (FGB) | 0,051245873 | 17 | 42,8 |
| SERPINA1 | 0,051677623 | 3 | 45,8 |
| CTSB | 0,057424271 | 20 | 56 |
| CDH5 | 0,058092804 | 14 | 23,3 |
| J9P3E7 / F1PQR5 / E2RH05 (NEOKARAKTERIZIRANI PROTEIN) | 0,059861218 | 3 | 37,4 |
| SMPDL3A | 0,061463103 | 14 | 40 |
| VITAMIN D-BINDING PROTEIN | 0,064248207 | 2 | 41,4 |
| LRG1 | 0,065134306 | 21 | 54,2 |
| TTR | 0,066327029 | 9 | 67,3 |
| COTL1 | 0,071152544 | 11 | 75,6 |
| PARK7 | 0,077109645 | 8 | 56,1 |
| SERUM ALBUMIN (ALB) | 0,077653405 | 6 | 90,6 |

| | | | |
|---|---|---|---|
| SUPEROXIDE DISMUTASE [CU-ZN] (SOD1) | 0,077784189 | 1 | 46,4 |
| F1PWR2 / J9PAD1 (NEOKARAKTERIZIRANI PROTEIN) | 0,080705812 | 57 | 48 |
| SERPINC1 | 0,085089314 | 25 | 71,4 |
| LOC608248 / H9GWR8 (NEOKARAKTERIZIRANI PROTEIN) | 0,086286497 | 1 | 89,6 |
| ITIH4 | 0,089602545 | 2 | 30,4 |
| AFM | 0,09019721 | 21 | 48 |
| F1PYR5 (NEOKARAKTERIZIRANI PROTEIN) | 0,090825857 | 5 | 36,6 |
| CYTOCHROME B5A (CYB5A) | 0,097697603 | 4 | 41 |
| GLUTATHIONE PEROXIDASE (GPX3) | 0,108082078 | 8 | 52,3 |
| AGT | 0,110397773 | 19 | 44,4 |
| F1Q184 (NEOKARAKTERIZIRANI PROTEIN) | 0,111124524 | 4 | 24,3 |
| BLVRB | 0,111574045 | 9 | 61,7 |
| FAM3C | 0,111605767 | 10 | 48,9 |
| C3 | 0,116968784 | 63 | 50,7 |
| SERUM ALBUMIN (ALB) | 0,120634243 | 5 | 90,6 |
| HPX | 0,12645486 | 22 | 58,3 |
| RNASET2 | 0,133451495 | 10 | 54,1 |
| APOLIPOPROTEIN A-I | 0,135765123 | 2 | 15,8 |
| LOC608248 / J9NSQ1 (NEOKARAKTERIZIRANI PROTEIN) | 0,138744503 | 2 | 75,4 |
| CLUSTERIN (CLU) | 0,139286772 | 22 | 47,9 |
| SERPINF2 | 0,146873982 | 16 | 37,9 |
| F1PF90 (NEOKARAKTERIZIRANI PROTEIN) | 0,152819787 | 4 | 29,5 |
| LOC612122 / J9P050 (NEOKARAKTERIZIRANI PROTEIN) | 0,157986042 | 2 | 36 |
| PITHD1 | 0,16175392 | 7 | 43,6 |
| L7N0K2 / J9P5C8 (NEOKARAKTERIZIRANI PROTEIN) | 0,171787973 | 2 | 33,9 |
| GSTP1 / J9NYL8 / LOC611366 | 0,174013033 | 6 | 49 |
| E2RCC8 / J9NYW7 (NEOKARAKTERIZIRANI PROTEIN) | 0,181106895 | 14 | 34,9 |
| CFB | 0,183362592 | 38 | 41,4 |
| J9P3L5 (NEOKARAKTERIZIRANI PROTEIN) | 0,183856887 | 2 | 10,9 |
| CD59 | 0,187740279 | 6 | 26,6 |

| | | | |
|---|---|---|---|
| L7N0J5 (NEOKARAKTERIZIRANI PROTEIN) | 0,190896355 | 2 | 9,4 |
| FETUB | 0,191514336 | 20 | 69,9 |
| BOVINE SERUM ALBUMIN (ALB) | 0,199258734 | 47 | 73,8 |
| ALPHA-1-ACID GLYCOPROTEIN | 0,201333915 | 9 | 37,6 |
| EPIDIDYMAL SECRETORY PROTEIN E1 (NPC2) | 0,206531568 | 12 | 50,3 |
| RBP4 | 0,213223664 | 14 | 70,6 |
| GMFG | 0,216228619 | 2 | 17,1 |
| HEPCIDIN (HAMP) | 0,233260294 | 2 | 38,8 |
| PLASMINOGEN (PLG) | 0,243061909 | 22 | 71,3 |
| SERPING1 | 0,262123226 | 9 | 17,9 |
| SERPINA5 | 0,268578811 | 5 | 16,5 |
| APOPTOSIS INHIBITOR OF MACROPHAGE (CD5L) | 0,274735346 | 5 | 18,8 |
| ALPHA-2-HS-GLYCOPROTEIN (AHSG) | 0,285854018 | 5 | 25,6 |
| SERINE PROTEASE INHIBITOR KAZAL-TYPE 5 (SPINK5) | 0,289880314 | 25 | 29,1 |
| RIBONUCLEASE A B1 (RNASE4) | 0,301882958 | 3 | 29 |
| CTGF (CONNECTIVE TISSUE GROWTH FACTOR) | 0,308888005 | 9 | 29,2 |
| FLNC (FILAMIN-C) | 0,331778826 | 20 | 13,3 |
| CAECAM1 | 0,353720456 | 3 | 23,3 |
| F13A1 (COAGULATION FACTOR XIII A CHAIN) | 0,363373239 | 2 | 4,2 |
| IG HEAVY CHAIN V REGION GOM | 0,372736453 | 2 | 31,2 |
| ADAMTS1 | 0,373409152 | 4 | 7,2 |
| IL1R1 (INTERLEUKIN-1 RECEPTOR TYPE 1) | 0,378551697 | 6 | 12,9 |
| ACYL-COA-BINDING PROTEIN (DBI) | 0,38852281 | 5 | 62,1 |
| BTD (BIOTINIDASE) | 0,391318081 | 7 | 17,9 |
| GLUCOSAMINE (N-ACETYL)-6-SULFATASE (GNS) | 0,459967256 | 11 | 28,5 |
| NUCB1 (NUCLEOBINDIN-1) | 1,885744777 | 18 | 43,5 |
| CYR61 | 1,919964094 | 4 | 11,5 |
| INTERCELLULAR ADHESION MOLECULE 1 (ICAM1) | 2,062398737 | 1 | 19 |
| PDCD1LG2 | 2,13143375 | 3 | 15,4 |
| CD55 | 2,224778335 | 6 | 18,4 |
| FRIZZLED-6 (FZD6) | 2,233217457 | 6 | 48,9 |
| COPPER TRANSPORT PROTEIN ATOX1 (ATOX1) | 2,268157931 | 7 | 89,6 |
| COL18A1 (COLLAGEN ALPHA-1(XVIII) CHAIN) | 2,287389014 | 12 | 9,3 |
| AHNAK | 2,356753952 | 28 | 23,2 |
| FLG2 (FILAGGRIN-2) | 2,43793425 | 6 | 4 |
| FOLR2 (FOLATE RECEPTOR BETA) | 2,473197429 | 6 | 36,9 |

| | | | |
|---|---|---|---|
| CD93 | 2,477721444 | 16 | 29,3 |
| EPHRIN-B2 (EFNB2) | 2,485183304 | 8 | 26,7 |
| THROMBOSPONDIN-1 (THBS1) | 2,498524549 | 12 | 13,6 |
| MAJOR PRION PROTEIN (PRNP) | 2,573742002 | 1 | 24,9 |
| TREFOIL FACTOR 3 (TFF3) | 2,616786827 | 6 | 53,8 |
| COLEC12 | 2,650706905 | 6 | 8,1 |
| AKAP12 | 2,74040042 | 16 | 14 |
| HSPG2 (HEPARAN SULFATE PROTEOGLYCAN 2) | 2,755407413 | 14 | 22,9 |
| F2R (COAGULATION FACTOR II RECEPTOR) | 2,862254254 | 3 | 6,1 |
| IGJ (IMMUNOGLOBULIN J CHAIN) | 2,86376893 | 6 | 32,7 |
| TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 5 (CD40) | 2,87832364 | 5 | 24,8 |
| J9P9J6 (NEOKARAKTERIZIRANI PROTEIN) | 2,90171903 | 13 | 60,1 |
| BSG (BASIGIN) | 3,005647444 | 6 | 25 |
| PROTHROMBIN (F2) | 3,038844006 | 22 | 43,8 |
| FSTL1 (FOLLISTATIN-RELATED PROTEIN 1) | 3,039349593 | 11 | 38,8 |
| CLEC14A | 3,073447386 | 13 | 38,7 |
| FLNA (FILAMIN-A) | 3,09624823 | 24 | 14,4 |
| CD99L2 | 3,120998167 | 4 | 21,4 |
| SERPINI1 | 3,17285011 | 15 | 37,6 |
| VTN (VITRONECTIN) | 3,266486126 | 17 | 37 |
| GLYCOGEN PHOSPHORYLASE, MUSCLE FORM (PYGM) | 3,371874516 | 61 | 61,6 |
| IGFBP2 (INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN 2) | 3,379569385 | 11 | 70 |
| LYVE1 | 3,425898156 | 9 | 25 |
| CUBILIN (CUBN) | 3,430010197 | 1 | 21,1 |
| MASP2 | 3,439646394 | 6 | 9,6 |
| C17ORF37 | 3,499535775 | 5 | 45,2 |
| CPE (CARBOXYPEPTIDASE E) | 3,574421441 | 10 | 34,2 |
| LTBP1 | 3,578073868 | 10 | 9,7 |
| BETA-2-GLYCOPROTEIN 1 (APOH) | 3,651257914 | 15 | 56,2 |
| FBLN5 (FIBULIN-5) | 3,736195642 | 12 | 26,6 |
| LRP2 (LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 2) | 3,742080776 | 82 | 22,3 |
| LPHN1 (ADHESION G PROTEIN-COUPLED RECEPTOR L1) | 3,775880783 | 16 | 14 |
| CDSN (CORNEODESMOSIN) | 3,797734656 | 4 | 7 |
| COL5A2 (COLLAGEN ALPHA-2(V) CHAIN) | 3,806397177 | 8 | 6,1 |
| MYOSIN-9 (MYH9) | 3,819087189 | 34 | 24,9 |
| CDH2 (CADHERIN-2) | 3,984825015 | 16 | 26,9 |
| GRN (GRANULIN) | 4,047490059 | 7 | 20,4 |

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|---|---|---|---|
| TNXB (TENASCIN-X) | 4,122397818 | 34 | 16,8 |
| IGSF8 | 4,130482803 | 7 | 14,6 |
| PANTETHEINASE (VNN1) | 4,171466971 | 2 | 29,2 |
| LMNA (LAMIN-A/C) | 4,227952877 | 10 | 18,3 |
| GPA33 | 4,236156017 | 3 | 9,9 |
| MPZL1 | 4,247832339 | 4 | 10,1 |
| FIBRONECTIN (FN1) | 4,258156678 | 58 | 36 |
| TOLLIP | 4,293909376 | 2 | 8,9 |
| J9P317 (NEOKARAKTERIZIRANI PROTEIN) | 4,333562579 | 2 | 13,7 |
| PRO-EPIDERMAL GROWTH FACTOR (EGF) | 4,380130386 | 2 | 31,9 |
| F11R (JUNCTIONAL ADHESION MOLECULE A) | 4,514388768 | 14 | 51,5 |
| SCUBE2 | 4,668811053 | 4 | 6,3 |
| PIP (PROLACTIN-INDUCIBLE PROTEIN) | 4,68127432 | 7 | 56,6 |
| FBN1 (FIBRILLIN-1) | 4,79453534 | 36 | 15,1 |
| GUCA2B (UROGUANYLIN) | 4,797298765 | 4 | 44,3 |
| LAMA5 (LAMININ SUBUNIT ALPHA-5) | 4,798234357 | 7 | 9,5 |
| CRTAC1 | 4,810067083 | 8 | 17 |
| NECTIN 4 (PVRL4) | 4,818266913 | 9 | 29,4 |
| GPC3 (GLYPICAN-3) | 4,877074744 | 7 | 11,6 |
| MXRA8 | 4,94579762 | 15 | 31,4 |
| PCOLCE | 4,946871174 | 9 | 27,1 |
| HEG1 | 4,999814669 | 4 | 6,5 |
| DLST | 5,119351845 | 9 | 20,2 |
| SYNDECAN (SDC1) | 5,141394552 | 2 | 10,6 |
| DERMATOPONTIN (DPT) | 5,250625987 | 8 | 50,7 |
| VIL1 (VILLIN-1) | 5,307965061 | 6 | 9,2 |
| LAMC1 (LAMININ SUBUNIT GAMMA-1) | 5,465655882 | 3 | 2,4 |
| ADAMTSL4 | 5,524113894 | 5 | 6,2 |
| IGFBP7 | 5,57623714 | 15 | 53,2 |
| NOV (CCN3) | 5,587454142 | 14 | 48,7 |
| FBLN2 (FIBULIN-2) | 5,595711544 | 13 | 15,8 |
| APLP1 (AMYLOID-LIKE PROTEIN 1) | 5,641498765 | 3 | 7,8 |
| VSIG4 | 5,684173914 | 5 | 27,5 |
| TNC (TENASCIN-C) | 5,707275175 | 42 | 32,1 |
| APOLIPOPROTEIN C-III (APOC3) | 5,740454211 | 3 | 35 |
| F1PTI2 (NEOKARAKTERIZIRANI PROTEIN) | 5,786516882 | 1 | 20 |
| PIK3IP1 | 5,803913779 | 6 | 27,6 |
| THROMBOMODULIN (THBD) | 5,807862983 | 12 | 38,9 |
| KALLIKREIN (KLK1) | 5,976443498 | 13 | 81,8 |
| ITM2B | 6,065833014 | 2 | 7,6 |
| HMCN1 (HEMICENTIN-1) | 6,068235322 | 9 | 2,1 |

| | | | |
|---|---|---|---|
| LTBP4 | 6,210360141 | 26 | 23,3 |
| APLP2 (AMYLOID-LIKE PROTEIN 2) | 6,364928201 | 7 | 10,7 |
| PPP1R21 | 6,52729182 | 2 | 3,8 |
| AMYLOID BETA A4 PROTEIN (APP) | 6,566272119 | 8 | 13,3 |
| RELT | 6,606400188 | 2 | 8 |
| LAYN (LAYILIN) | 6,992503258 | 4 | 7,4 |
| EFEMP2 | 7,005646011 | 7 | 17 |
| PECAM1 (CD31) | 7,154299935 | 7 | 7,9 |
| CATHELICIDIN (CAMP) | 7,211173391 | 9 | 37,8 |
| CSTA (CYSTATIN-A) | 7,529405641 | 8 | 84,7 |
| NGFR (NERVE GROWTH FACTOR RECEPTOR) | 7,670174848 | 1 | 13,1 |
| PSCA (PROSTATE STEM CELL ANTIGEN) | 7,696255245 | 2 | 21,8 |
| UROMODULIN (UMOD) | 7,735440282 | 32 | 48,4 |
| CATHEPSIN L1 (CTSL) | 7,803009106 | 6 | 25,2 |
| BETA-DEFENSIN 1 (CBD1) | 7,846785958 | 4 | 43,5 |
| CD248 (ENDOSIALIN) | 8,699268625 | 13 | 24,8 |
| COL12A1 (COLLAGEN ALPHA-1(XII) CHAIN) | 8,748432259 | 50 | 20,9 |
| DMKN (DERMOKINE) | 8,815819936 | 3 | 29,8 |
| MMRN2 (MULTIMERIN-2) | 8,828424282 | 10 | 14,8 |
| PENK (PROENKEPHALIN) | 8,976976174 | 4 | 20,1 |
| PLAU (UROKINASE-TYPE PLASMINOGEN ACTIVATOR) | 9,188907314 | 13 | 25,4 |
| CD84 | 9,234306341 | 6 | 16,5 |
| NBL1 (NEUROBLASTOMA SUPPRESSOR OF TUMORIGENICITY 1) | 9,381953044 | 4 | 39,4 |
| TREFOIL FACTOR 2 (TFF2) | 9,3928125 | 2 | 71,4 |
| MYH14 (MYOSIN-14) | 9,419283485 | 5 | 5 |
| AXL (TYROSINE-PROTEIN KINASE RECEPTOR AXEL) | 9,856779192 | 8 | 13,7 |
| RNASE6 / RAD1 (RIBONUCLEASE A D1) | 10,12228374 | 6 | 39,5 |
| LY6D (LYMPHOCYTE ANTIGEN 6D) | 10,1683165 | 4 | 52,3 |
| TREFOIL FACTOR 1 (TFF1) | 10,60046823 | 4 | 56,8 |
| DEOXYRIBONUCLEASE-1 (DNASE1) | 10,75257042 | 10 | 46,5 |
| CD44 ANTIGEN (CD44) | 11,17176678 | 4 | 15,7 |
| SCGB1A1 (UTEROGLOBIN) | 12,25773313 | 5 | 55,1 |
| LTBP2 (LATENT-TRANSFORMING GROWTH FACTOR BETA-BINDING PROTEIN 2) | 12,46133572 | 11 | 6,3 |

| | | | |
|---|---|---|---|
| ESAM (ENDOTHELIAL CELL-SELECTIVE ADHESION MOLECULE) | 13,0486214 | 6 | 22,4 |
| HMCN2 (HEMICENTIN-2) | 13,15927445 | 13 | 6,9 |
| LCNL1 (LIPOCALIN-LIKE PROTEIN 1) | 14,40304761 | 11 | 55,7 |
| GAA (LYSOSOMAL ALPHA-GLUCOSIDASE) | 15,38909207 | 14 | 21,7 |
| FBLN7 (FIBULIN-7) | 15,51398177 | 11 | 26,1 |
| LOC611675 | 18,79853089 | 2 | 7,5 |
| COL14A1 (COLLAGEN ALPHA-1(XIV) CHAIN) | 21,35205466 | 12 | 9,1 |
| SLURP1 | 24,70988381 | 3 | 49 |
| LYPD2 | 34,95606322 | 2 | 25,4 |

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# Serum proteome profiling in canine idiopathic dilated cardiomyopathy using TMT-based quantitative proteomics approach

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ABSTRACT

Idiopathic dilated cardiomyopathy (iDCM) is a primary myocardial disorder with an unknown aetiology, characterized by reduced contractility and ventricular dilation of the left or both ventricles. Naturally occurring canine iDCM was used herein to identify serum proteomic signature of the disease compared to the healthy state, providing an insight into underlying mechanisms and revealing proteins with biomarker potential. To achieve this, we used high-throughput label-based quantitative LC-MS/MS proteomics approach and bioinformatics analysis of the *in silico* inferred interactome protein network created from the initial list of differential proteins. To complement the proteomic analysis, serum biochemical parameters and levels of know biomarkers of cardiac function were measured. Several proteins with biomarker potential were identified, such as inter-alpha-trypsin inhibitor heavy chain H4, microfibril-associated glycoprotein 4 and apolipoprotein A-IV, which were validated using an independent method (Western blotting) and showed high specificity and sensitivity according to the receiver operating characteristic curve analysis. Bioinformatics analysis revealed involvement of different pathways in iDCM, such as complement cascade activation, lipoprotein particles dynamics, elastic fibre formation, GPCR signalling and respiratory electron transport chain.

*Significance:* Idiopathic dilated cardiomyopathy is a severe primary myocardial disease of unknown cause, affecting both humans and dogs. This study is a contribution to the canine heart disease research by means of proteomic and bioinformatic state of the art analyses, following similar approach in human iDCM research. Importantly, we used serum as non-invasive and easily accessible biological source of information and contributed to the scarce data on biofluid proteome research on this topic. Bioinformatics analysis revealed biological pathways modulated in canine iDCM with potential of further targeted research. Also, several proteins with biomarker potential have been identified and successfully validated.

## 1. Introduction

Dilated cardiomyopathy (DCM) is a myocardial disorder affecting 1 in 2500 human individuals and represents the most frequent cause of heart transplantation [1]. It is characterized by dilation and impaired contraction of the left or both ventricles and carries a poor prognosis with progression to congestive heart failure or fatal arrhythmias [2]. Pathologic mechanisms underlying DCM are not understood, especially regarding the idiopathic form (iDCM), for which the primary cause is unknown. If there is some evidence/suspicion of the aetiology, DCM is not considered idiopathic, but can be caused by different contributing factors, such as genetics, nutritional deficiencies, metabolic disorders, immune system abnormalities, infectious diseases or intoxication [1,3]. Taking into account DCM is the second most common heart disease of dogs, understanding its mechanisms can lead to the advances in companion animal veterinary medicine. Also, due to the similarities between the dog and human cardiovascular system on anatomical, physiological and molecular level [4], naturally occurring canine iDCM is a suitable model for studying the human counterpart.

Diagnosis of iDCM is based on echocardiography [5]. Radiography and clinical examination findings, ECG examination, genetic tests and certain biomarkers circulating in blood, such as cardiac troponin I (cTnI) and N-terminal pro-B-type natriuretic peptide (NT-proBNP), have an added value in both human and canine iDCM diagnostics.

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Although serum cTnI and NT-proBNP concentrations are increased in humans and dogs with iDCM, they are not exclusive biomarkers of this disease and therefore have limited clinical utility [6].

With the development of new high-throughput proteomic technologies, there is a growing interest in study of new protein biomarkers which could help to diagnose and treat iDCM, but also unravel underlying pathologic mechanisms. Plasma or serum are especially promising sources of new easily accessible protein biomarkers since the blood proteome reflects systemic changes that happen upon organ dysfunction. Proteomic research on both blood and heart tissue can reveal different aspects of the same condition, encompassing systemic and local changes taking place during the course of the disease. While there are some recent studies on myocardial tissue transcriptome and proteome in human iDCM [7,8], large-scale proteomic analyses of blood which could identify proteins with biomarker potential in both human and canine iDCM are lacking. To the authors´ knowledge, there is only one proteomic study of serum of dogs with DCM, where label-free quantitative LC-MS/MS approach was used [9].

Therefore, we have performed a study of serum proteome changes in iDCM compared to the healthy state, using naturally occurring canine iDCM as a model, by means of an isobaric tandem mass tag (TMT) label-based high-resolution quantitative proteomic approach. This was followed by validation of several biomarker candidates using Western blot. The TMT label-based approach enables multiplex identification and relative quantification of proteins between samples in an experimental set by LC-MS/MS. Functional bioinformatics analysis was also conducted on the acquired dataset in the interest of deeper understanding of mechanisms involved in iDCM. To complement these analyses, serum biochemical parameters and serum cTnI and NT-proBNP levels were measured.

## 2. Materials and methods

### 2.1. Animals and heart function examination

Two groups of dogs were enrolled in the study in the period between March 2015 and March 2016: 8 clinically healthy dogs (used as controls) and 8 dogs diagnosed with iDCM. The study was approved by the Committee on the Ethics of the University of Zagreb, Faculty of Veterinary Medicine (Permit Number: 640–01/14–305/16, 251–61-01/139–14-28). Healthy dogs were admitted to the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia, while diseased dogs to the Small Animals Clinic, Department of Clinical Veterinary Science, Vetsuisse Faculty, University of Bern, Switzerland. All dogs of the control group underwent clinical examination, haematological and biochemical serum analyses, as well as cardiac function evaluation performed in unsedated dogs, which included a 1-min 6 lead ECG (ASPEL, AsCard Mr. Silver) and transthoracic echocardiography using Esaote MyLab40 Vet machine and a 5 MHz sector transducer. Dogs diagnosed with iDCM were enrolled based on the concomitant presence of the two major criteria for DCM [5]: a) enlarged left ventricular M-mode systolic (LVDs) and diastolic (LVDd) dimensions defined according to weight-adjusted values [10] and b) left ventricular M-mode fractional shortening of < 20%. Exclusion criteria were evidence of any other disease than iDCM based on history, clinical examination, laboratory results or imaging. The cardiac evaluation of dogs with iDCM included physical examination, thoracic radiographs evaluated by a board-certified radiologist, a 1-min 6 lead ECG (Schiller AT 101) and transthoracic echocardiography performed by a board-certified cardiologist (AK). Echocardiography was performed using an Aloka ProSound Alpha 5SV machine and a 5-MHz sector transducer in unsedated dogs. Echocardiography was performed in a standard manner [11]. Diseased dogs were classified according to the International Small Animal Cardiac Health Council (ISACHC) classification system [12]. All procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments, as well as subject to informed owner consent.

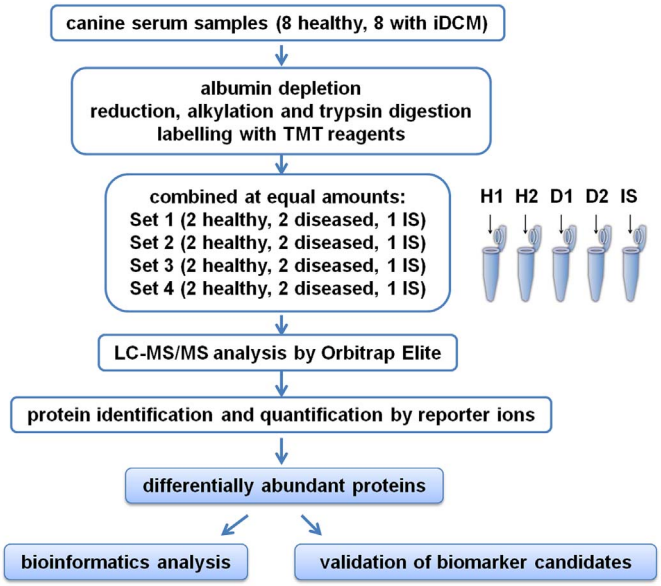


**Fig. 1.** TMT study design for identification of proteins with biomarker potential and pathways involved in canine iDCM (H1, H2 – samples from different healthy dogs; D1, D2 - samples from different dogs with iDCM, IS – internal standard sample).

### 2.2. TMT study

Scheme of the TMT study design is shown in Fig. 1. Serum samples collected from 8 dogs with iDCM and 8 healthy dogs (controls) were all processed and analysed at the same time in order to reduce inconsistencies. Highly abundant albumin was depleted from all 16 samples followed by protein concentration determination. An internal standard (IS) was made as a pool of equal protein amount from all 16 albumin-depleted samples as a reference for normalization. Equal amount of each protein sample and IS was reduced, alkylated, digested with trypsin and labelled with TMT reagents. Samples were combined at equal amounts into 4 sets (each consisting of 1 IS, 2 healthy and 2 diseased canine samples) and each set was analysed by LC-MS/MS. Acquired data was processed statistically to find differentially abundant proteins, some of which were validated as potential biomarkers of iDCM using immunoblotting. Also, bioinformatics analysis was performed in order to find pathways modulated in canine iDCM.

### 2.3. Serum samples and albumin depletion

Serum was obtained from all 16 dogs by centrifugation of completely clotted blood at 3500*g* for 10 min at room temperature. Samples were aliquoted and stored at −80 °C until analysed. All samples were collected during a one-year period and thawed just once upon completion of collection, immediately before any analysis. Serum of dogs with iDCM was collected at the time of initial diagnosis and before any treatment. Before proteomic analysis, serum samples were depleted by removal of highly abundant albumin using a salt-ethanol precipitation protocol, described by Colantonio et al. [13]. Briefly, 100 µL of each serum sample was processed by adding 10 µL of 1 M sodium chloride solution (VWR, Pennsylvania, USA) to yield a final concentration of 0.1 M and incubated with rotation for 1 h at 4 °C. Cold ethanol absolute (≥ 99.8%, VWR, Pennsylvania, USA) was then added to yield a final concentration of 42% and again incubated for 1 h at 4 °C. Samples were centrifuged at 16000*g* for 45 min at 4 °C. First pellets were retained and supernatants were further processed. The pH of the supernatants was lowered to 5.7 by adding cold 0.6 M sodium acetate buffer (Sigma-Aldrich) of pH 5.6, and incubated for 1 h at 4 °C. Supernatants were then

centrifuged as described above to yield the second pellet. Supernatants (containing albumin) were removed and the first and second pellets were combined to yield albumin-depleted samples. Pellets were re-suspended in 100 mM triethylammonium bicarbonate buffer (TEAB) (Sigma-Aldrich) containing 1% SDS (Sigma-Aldrich), compatible with further proteomic analysis. Treated samples were resolved by 1-D SDS PAGE in order to test the efficiency of albumin depletion procedure for each sample. First, protein concentration was measured in duplicate using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) per manufacturer's guidelines. Samples were then mixed with Laemmli Sample Buffer containing 355 mM 2-mercaptoethanol (Bio-rad) and heated on a heating block for 5 min at 95 °C. The first well of gel was loaded with 8 μL of PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) and others with 10 μg of protein per sample. Proteins were separated by 1-D electrophoresis using Criterion TGX precast gels 4–15% (Bio-rad). Gels were run at 300 V for 20 min in Tris-Glycine-SDS running buffer (Bio-rad). In order to visualise protein bands, gels were stained with Coomassie Brilliant Blue R-250 solution (Bio-rad), de-stained in the destaining solution over night and scanned using UMAX PowerLook III scanner.

### 2.4. TMT labelling

Amine-reactive Tandem Mass Tags (TMT) isobaric reagents (Thermo Scientific) were used to label samples in order to multiplex quantification of serum proteins by mass spectrometry (MS). Samples were grouped into 4 experimental sets in a way that each set contained albumin-depleted serum sample of 2 healthy dogs, 2 diseased dogs and an internal standard sample. Internal standard (IS) was used to allow for normalization of data and comparison of biological replicates between 4 individual pentaplex TMT experiments. One hundred micrograms of each protein sample and IS samples was prepared and labelled at peptide level with TMT reagents according to manufacturer's instructions. In brief, samples were reduced with 200 mM DTT (Sigma-Aldrich), alkylated with 375 mM iodoacetamide (Sigma-Aldrich) and precipitated with ice-cold acetone (VWR, Pennsylvania, USA) for 4 h. Samples were then centrifuged at 8000 g for 10 min and acetone was decanted. Pellets were resuspended with 100 μL of 100 mM TEAB buffer and digested with trypsin (Promega) overnight at 37 °C (2.5 μg of trypsin per 100 μg of protein). Thereafter, IS peptide samples were labelled with TMT-126 reagent, while other 16 peptide samples were labelled randomly with TMT-127, TMT-128, TMT-129 and TMT-130 reagents to avoid possible labelling preference. TMT label reagents were equilibrated to room temperature, dissolved in anhydrous acetonitrile, LC-MS grade (Thermo Scientific) for 5 min with occasional vortexing and added to each sample (41 μL of the reagent to 100 μg sample). Labelling reaction was incubated for 1 h at room temperature and then quenched by adding 5% hydroxylamine (Thermo Scientific) for 15 min. Samples were then combined at equal amounts into 4 pentaplex sets (as described above) and 6 μg of each mixed sample set was placed in a well of a microplate. Samples were vacuum-dried for 15 min and stored at −20 °C before further LC-MS/MS analysis.

### 2.5. LC-MS/MS analysis

The LC−MS/MS analysis was performed on Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberley, UK) and Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). TMT-labelled peptide mixtures were reconstituted in buffer A (2% acetonitrile in 0.1% formic acid). An amount of 3 μg was loaded on the trapping column C18 PepMap100 (5 μm, 100 A, 300 μm × 5 mm) and then separated using C18 RSLC PepMap ID column (15 cm × 75 μm) with linear gradient 5–35% buffer B (0.1% formic acid in 80% acetonitrile) over 135 min at a flow rate of 300 nL/min. Eluate from the column was introduced to the Orbitrap Elite MS. The ionisation voltage was set to 1.7 kV and the ion transfer tube temperature to 220 °C. MS was operating in positive ion mode using collision-induced dissociation/higher energy collisional dissociation CID/HCD fragmentation methods for MS2. Full scan Fourier transform-based mass spectrometry (FTMS) spectra were acquired in range from $m/z$ 380.0 to 1800.0 with resolution of 60,000. The maximum injection time for FTMS full scan was set as 200 ms reaching an automatic gain control (AGC) target value of $1 \times 10^6$. Three most intense peaks from MS spectrum were selected for each fragmentation mode. Ions with the charge state $1^+$ were excluded from the fragmentation list. The HCD MS/MS scan was fixed to start from $m/z$ 100.00 with resolution of 15,000 using MS2 AGC target of $5 \times 10^4$. The collision energy was set as 40% normalized collision energy (NCE). Isolation window of ± 1.5 Da was applied to isolate precursor ions with dynamic exclusion of 20 s. Every precursor ion was repeated twice within duration time of 30 s and was excluded for 20 s. Ion trap mass spectrometry CID MS/MS scan spectra were acquired with 35% NCE and an AGC target of $1 \times 10^4$.

### 2.6. MS/MS data analysis

Acquired MS/MS spectra were analysed for protein identification and quantification using Proteome Discoverer software 2.1 (Thermo Fisher Scientific). Protein identification was performed using the Mascot algorithm against the *Canis lupus familiaris* protein database from NCBInr (version 04/05/2016; 41,195 sequences), with a precursor mass tolerance of 10 ppm and fragment ion mass tolerance of 0.8 Da. Set modifications were addition of TMT 6-plex labels to lysines and N-termini, carbamidomethylation of cysteine as fixed modification and methionine oxidation as variable modification. Two missed cleavages for the trypsin digestion were permitted. Identified peptides were filtered with a cut-off criterion of a q-value of 0.01, corresponding to a 1% false-discovery rate (FDR) for highly confident peptide hits and a q-value of 0.05 (5% FDR) for peptide hits with moderate confidence. Quantification was performed using abundances of reporter ions based on signal to noise ratio values or intensity. Normalization was carried out based on total peptide amount and scaling on channels average. Abundances of reporter ions from only unique and razor peptides were used to estimate the abundances of proteins. Identification and quantification data were exported from Proteome Discoverer to Microsoft Excel software. Abundances ratios were obtained for each protein by comparing with values of corresponding internal standard and then used to calculate average fold change ratio between healthy and iDCM groups. Internal standard-normalized protein abundances ratios were used in statistical analysis to detect significant differences between the studied groups.

### 2.7. Validation of proteomics results

Validation of proteomics results was performed by Western blotting using the non-depleted serum samples of the same patients as in proteomic analysis. In brief, 25 μg of proteins of each serum sample (8 healthy controls and 8 iDCM) was boiled at 95 °C for 5 min in SDS-loading buffer and separated over 4–10% polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes (Amersham Protran, GE Healthcare) for 2 h at 50 mA in 20% methanol (Sigma) transfer buffer at 4 °C using Biostep electro blotting module. After the transfer, gels were stained with Coomassie Brilliant Blue G-250 (Amresco) in order to verify equal transfer and to use protein load as a reference for protein quantity normalization. The membranes were blocked for 1 h with 5% skim milk (VWR, Pennsylvania, USA) in Tris buffered saline (pH = 7.6) containing 0.05% Tween20 (Sigma) at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies to ITIH4 (dilution 1:5000, kindly provided by F. Lampreave group, University of Zaragoza), ITIH3 (1:200, Santa Cruz Biotech, sc-21979), MFAP4 (1:500, Aviva Systems Biology, ABIN2776850), TfR1 (1:500, Covalab, pab75255), APOA4 (1:250, Biorbyt, orb5708) and AGT (1:500, Aviva Systems Biology, ABIN2781494). Specific polyclonal

rabbit antiserum against the purified canine ITIH4 was raised by F. Lampreave group [14]. The ITIH3, MFAP4, TfR1, APOA4 and AGT antisera were not raised towards corresponding canine proteins, but had predicted cross-reactivity based on the immunogen amino acid sequence homology (as stated by the manufacturers). After washing, membranes were incubated for 1 h at room temperature with appropriate secondary antibody (dilution 1:5000, donkey anti-goat IgG, sc-2020 and goat anti-rabbit sc-2004, Santa Cruz Biotech) conjugated with horseradish peroxidase. Immunostained proteins were detected by incubation with Western blotting luminol reagent (Santa Cruz Biotech) for 8 min and recorded in chemiluminesence mode using Li-Cor Odyssey Fc (Li-Cor, Inc). Acquired figures were analysed using ImageJ software (US National Institutes of Health, Bethesda, Maryland, USA) and differences between healthy and diseased dogs determined using unpaired *t*-test, with $p$ value $< 0.05$ considered statistically significant.

### 2.8. Serum biochemical parameters

One serum aliquot was used for measurement of biochemical parameters using commercial reagents (Beckman Coulter) per manufacturer's instructions in an automatic analyser (Olympus AU640, Japan). The following parameters were measured: serum urea, creatinine, bilirubin, glucose, proteins, albumin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, alkaline phosphatase, creatine kinase, lactate dehydrogenase, C-reactive protein, alpha-amylase, lipase, cholesterol, triglycerides, calcium, magnesium, phosphates.

### 2.9. Measurement of cTnI and NT-proBNP blood concentration

Cardiac troponin I (cTnI) was measured in serum samples of control ($N = 8$) and iDCM (N = 8) group using commercially available ADVIA Centaur TnI-Ultra assay, which is a high-sensitivity immunoassay validated for use in both humans and dogs [15]. Analysis was performed in Dubrava Clinical Hospital, Department of clinical diagnostics (Zagreb, Croatia) using Siemens Advia Centaur XP according to manufacturer's instructions. Samples which had serum concentration of cTnI below the lower level of detection of the assay were allocated a value of 0.01 μg/L. Analysis of NT-proBNP concentration in all 16 samples was performed in Vet Med Labor GmbH, reference IDEXX Laboratory (Germany) using IDEXX Cardiopet® proBNP test.

### 2.10. Statistics

Statistical analyses were performed using the R software version 3.3.1 [16]. Statistical differences in the age, sex, protein abundances ratios acquired in proteomic analysis, serum biochemical parameters and cTnI and NT-proBNP levels between healthy and iDCM groups were determined using nonparametric Mann-Whitney test. In order to compare healthy group, iDCM ISACHC class II and iDCM ISACHC class IIIA, the nonparametric Kruskal-Wallis and Dunn *post hoc* test were used. For all statistical comparisons, $p < 0.05$ was considered statistically significant. In order to test sensitivity and specificity of potential protein biomarkers according to proteomic analysis, receiver operating characteristic (ROC) curve analysis was performed and area under the curve (AUC) computed using MedCalc for Windows, version 18.0 (MedCalc Software, Ostend, Belgium). The required sample size for the comparison of the area under a ROC curve with a null hypothesis value was calculated using following parameters: null hypothesis value = 0.5, type I error (alpha, significance) = 0.05, type II error (beta, 1-power) = 0.2.

### 2.11. Bioinformatics analysis

Proteins with significantly differential abundances observed by proteomic and serum biochemical analyses (in total $N = 15$) were further functionally analysed using bioinformatics tools. As human database is more complete than canine one (reviewed proteins in UniprotKB/Swiss-prot release 2018_01: humans 20,259, dogs 817), dog's proteins were converted to human proteins by performing BLAST (protein-protein BLAST) analysis of 15 identified canine proteins using human protein database (UniprotKB/Swiss-prot release 2017_10). The best matching protein (ID score, query coverage and *E*-value) was considered as ortholog for each canine protein in the initial list. Then, an enriched network of proteins was built based on the list of the initial 15 proteins, adding a maximum of 40 best interactors (using the following combination of *Homo sapiens* database: IntAct, Reactome, and String-database) with the Cytoscape (v3.5.1) application Cluepedia (v1.3.5). All types of interactions were considered. Clusters of interacting proteins were calculated by the Cytoscape application MCODE (v1.4.2), with node cutoff at 0.3 and K-core at 4. Thereafter, pathway analysis of each cluster was performed using the Cytoscape (v3.5.1) plugin ClueGO (v2.3.5). Ontology used was Reactome-Pathways (21/10/2017). *P*-value of each term was corrected with Bonferroni step-down. Pathway terms groups were defined according to the kappa score (threshold at 0.6) and sharing group percentage at 50%. Pathways leading term of each group was determined by its *p*-value. After determining Pathway terms for each cluster, terms were merged if they were identical or one term had a higher hierarchical position than another term. For the latter, the upper hierarchical Pathway term was considered as the leader term. Hierarchical orders were accessed using the Reactome.org database. Complete networks of each identified Pathway terms (available on Reactome.org with all proteins/genes and chemicals) were merged to constitute the *in silico* inferred interactome network of molecular processes from the initial 15 proteins. Using this *in silico* inferred interactome network, pathways and cellular compartment analyses were performed. Pathways analyses used the Reactome-Pathways (21/10/2017) with following parameters: evidence codes used “All_Experimental”, Kappa score threshold 0.4, number of genes = 30, minimum percentage = 70, *p*-value correction used = Bonferroni step down. Cellular compartment analyses used the GO-CellularComponent-EBI (27/10/2017), with following parameters: evidence codes used “All_withoutIEA”, Kappa score threshold 0.5, number of genes = 20, minimum percentage = 15, minimum GO level = 3, Maximum GO level = 10, p-value correction method used = Bonferroni step down.

## 3. Results

### 3.1. Animals and heart function

The control group consisted of 8 healthy dogs with normal cardiac function, aged from 5 to 12.5 years of following breeds: 2 Belgian shepherds, 1 Border collie, 1 Beauceron, 1 Flat-coated retriever, 1 Labrador retriever and two mixed-breed dogs (25 and 38 kg). Three dogs in the control group were male, 1 was male castrated and 4 were female spayed. During the 13 months of the study, 8 adult dogs were diagnosed with iDCM at the co-authors´ (AK) institution. In the diseased group there were dogs of either sex, aged between 3 and 7 years. These were 1 mixed breed dog (40 kg) and 7 dogs from 5 different breeds (2 German shepherds, 1 Doberman, 1 Cane Corso Italiano, 1 Leonberger, 1 Bouvier des Flandres and 1 Great Dane). Two dogs were female, 2 female spayed, 1 male und 3 male castrated. There was no statistical difference in the age between the control (median; interquartile range: 8.5 years; 5.5–10.75) and iDCM (7 years; 7–7.75) group. If the neutering is disregarded, there was also no difference in the sex between the groups (each group consisted of 4 males and 4 females). At initial physical examination all dogs of iDCM group were considered in heart failure based on elevated respiratory rate and effort, signs of interstitial or interstitial-alveolar lung pattern and absolutely dilated pulmonic veins on the thoracic radiographs. Four dogs were classified as ISACHC class II and four dogs as ISACHC class IIIA. In accordance with the

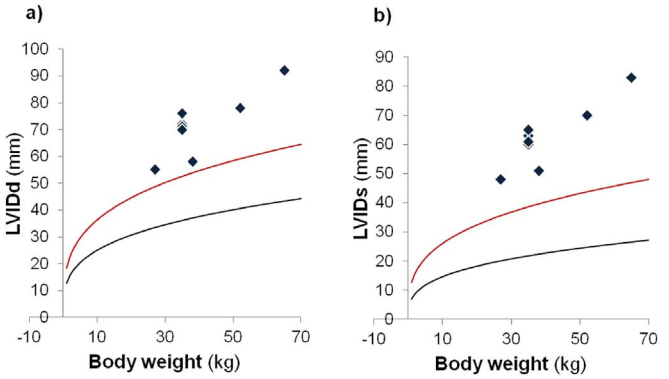


**Fig. 2.** Scatter plots showing the echocardiographic parameters in 8 dogs diagnosed with iDCM: a) left ventricular end diastolic diameter (LVIDd) and b) left ventricular end systolic diameter (LVIDs). All dogs are above the upper reference values (red lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ISACHC classification system, dogs in class II group presented with a mild to moderate heart failure, while dogs in class IIIA presented with an advanced heart failure. A grade II-VI left-sided systolic heart murmur was identified in all diseased dogs. Six dogs were in atrial fibrillation with a ventricular heart rate 150–240/min. Two of those dogs had rare ventricular premature complexes (VPC) and one dog a left bundle branch block (LBBB). Two dogs showed a sinus rhythm 150/min respec. 160/min. According to the inclusion criteria, echocardiographic examination showed abnormal left ventricular end diastolic (Fig. 2a) and end systolic diameter (Fig. 2b) and reduced fraction shortening (8–16%). The left atrial/aorta ratio (LA:Ao) was enlarged (1.8–3) in all dogs and all showed some functional mitral regurgitation, while 5 dogs an additional functional tricuspidal regurgitation.

### 3.2. Albumin depletion

The most abundant serum protein albumin, which could mask identification and quantification of low abundant proteins in downstream proteomic analysis, was removed with good reproducibility and efficiency from all 16 canine serum samples using salt-ethanol precipitation protocol, verified by 1-D SDS PAGE. Fig. 3 shows that the intensity of the dominant band corresponding to albumin (~68 kDa) in non-depleted canine serum decreased remarkably in both healthy and diseased serum samples after the depletion procedure.

### 3.3. Protein identification and quantification using TMT approach

In this study, 4 pentaplex experimental sets containing 16 different samples (plus IS samples) were analysed using TMT label-based quantification approach. Since an IS was used in each set, it was possible to use it as a normalization reference and compare protein quantities detected in more than one set across different TMT runs. In total, 358 proteins were identified with high and medium confidence by combining all data from 4 sets. These proteins were grouped by the Proteome Discoverer software into 134 groups, with the top-ranking protein of the group listed as the master protein. Of 134 master proteins, 129 were identified with high (1% FDR) and 5 with medium (5% FDR) confidence, while quantification values were obtained for 131 proteins. Only highly confident master proteins (1% FDR), identified and quantified by 2 or more unique peptides and detected in at least 4 biological replicates were selected for the quantification analysis, which included 76 proteins (list provided as Supplemental Table 1). For these proteins the average abundance fold change (iDCM/healthy ratio) ranged from 0.5 to 2. There was one protein (microfibril-associated glycoprotein 4, MFAP4) where exception was made in terms of criterion for the number of unique peptides. Although MFAP4 was identified and

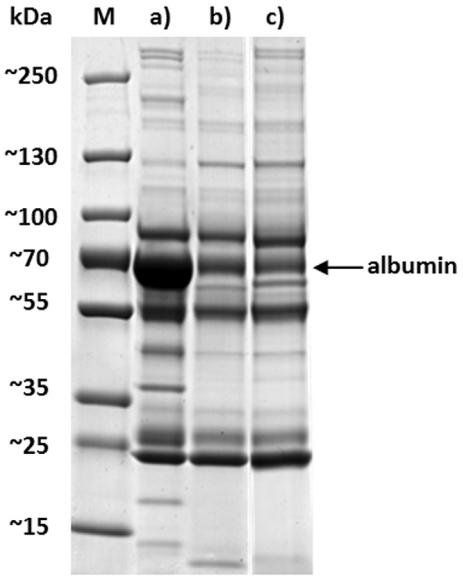


**Fig. 3.** Albumin depletion from canine serum using salt-ethanol procedure. 1-D SDS PAGE of a) non-depleted canine serum; b) depleted serum sample of a dog with iDCM; c) depleted serum sample of a healthy dog (Notes: a), b) and c) are sera of different dogs; marker and samples a) and b) were run on one gel and sample c) on another gel).

quantified by only one unique peptide, there was a remarkable fold change (1.6) so it was included in the statistical analysis, and its differential abundance between the groups was later tested by Western blotting. When nonparametric Mann-Whitney test was applied, 12 proteins showed significantly different levels ($p < 0.05$) in iDCM *versus* control serum with the fold changes ≥ 1.2 or ≤0.8 (Table 1). Of those 12 proteins, microfibril-associated glycoprotein 4 (MFAP4), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and apolipoprotein A-IV (APOA4) were also significantly different ($p < 0.05$) between the controls, iDCM ISACHC II and iDCM ISACHC IIIA groups (Fig. 4).

### 3.4. Validation of proteomics results

In order to verify differences in serum protein abundances observed by proteomic analysis, 5 selected proteins were validated by Western blotting using total protein load as normalization reference. Consistent with TMT based proteomics results, the relative abundances of ITIH4, ITIH3 and MFAP4 were significantly increased in iDCM group compared to controls, while those of TfR1 and APOA4 were decreased (Fig. 5, $p < 0.05$). Angiotensinogen (AGT) relative abundance increase according to proteomic analysis was also tested by imunoblotting, but there was no significant difference between the groups (data not shown). We were not able to confirm our observation possibly due to the existence of AGT glycoprotein forms in canine plasma [17], requiring different method of validation, such as 2-D Western blotting.

### 3.5. Serum biochemistry

Values of serum biochemical parameters which were significantly different between dogs with iDCM and healthy dogs are presented in Table 2. C-reactive protein (CRP), urea and triglycerides concentrations, as well as creatine kinase (CK) and lactate dehydrogenase (LDH) activities, were significantly increased in serum of dogs with iDCM compared to healthy dogs. There were no significant differences in other analysed parameters: creatinine, bilirubin, glucose, proteins, albumin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, alkaline phosphatase, alpha-amylase, lipase, cholesterol, calcium, magnesium and phosphates.

**Table 1**
Proteins with significantly differential abundances between healthy dogs and dogs with iDCM identified in serum using TMT approach.

| GI accession number[a] | Description | N of unique peptides | Mascot score | Fold change (iDCM/healthy) | p-Value (iDCM versus healthy) | N of detections[b] |
|---|---|---|---|---|---|---|
| Upregulated | | | | | | |
| 928186325 | Inter-alpha-trypsin inhibitor heavy chain H4 | 21 | 3170 | 2 | 0.0003 | 16 |
| 73956164 | Microfibril-associated glycoprotein 4 | 1 | 369 | 1.6 | 0.0043 | 12 |
| 928186331 | Inter-alpha-trypsin inhibitor heavy chain H3 | 3 | 126 | 1.6 | 0.0286 | 8 |
| 545494757 | Angiotensinogen | 5 | 866 | 1.2 | 0.0207 | 16 |
| 928150787 | Complement C4-A | 52 | 16701 | 1.2 | 0.0379 | 16 |
| 545496317 | Complement component C9 | 2 | 245 | 1.2 | 0.0379 | 16 |
| 928159887 | Plasma protease C1 inhibitor | 4 | 2194 | 1.2 | 0.0499 | 16 |
| Downregulated | | | | | | |
| 345799905 | Apolipoprotein A-IV | 19 | 3621 | 0.6 | 0.0019 | 16 |
| 17066524 | Immunoglobulin gamma heavy chain A | 5 | 7053 | 0.6 | 0.0499 | 16 |
| 924859480 | Apolipoprotein C-III precursor | 4 | 1521 | 0.7 | 0.0379 | 16 |
| 50978812 | Transferrin receptor protein 1 | 2 | 141 | 0.8 | 0.0379 | 16 |
| 928133662 | Apolipoprotein A-I | 26 | 45231 | 0.8 | 0.0499 | 16 |

[a] Accession number from NCBI protein database for *Canis lupus familiaris*.
[b] Number of samples in which the protein was detected.

### 3.6. Measurement of cTnI and NT-proBNP blood concentration

Concentrations of serum cTnI, as well as plasma NT-proBNP, were significantly increased in dogs with iDCM when compared to the control group (Fig. 6a and b, respectively).

### 3.7. Bioinformatics analysis

Fig. 7 displays the workflow of the bioinformatics analysis. All 15 differentially abundant canine proteins (according to proteomic ($N = 12$) and serum biochemistry ($N = 3$) analyses) were successfully mapped to *Homo sapiens* protein orthologs (Supplemental Table 2). Then, enrichment added 334 proteins/genes to the initial 15 proteins, with best interactors matching defined criteria. All 15 initial proteins were connected to the network. In this network, 10 different clusters were defined (Supplemental Table 2). All clusters were defined by at least 1 Pathway term. A group of 135 nodes was not able to form a cluster and therefore named Non-clustered, and was characterized by 4 Pathways terms. Since different clusters exhibit same Pathways terms or terms belong to the same upper hierarchical order term, some terms were merged. After merging terms from clusters, 19 pathways were identified (Supplemental Table 2). All 19 Pathways terms were merged to establish the inferred interactome network, with 1341 nodes (1230 genes/proteins and 111 chemicals). All Pathways terms were connected to the network, with the exception of some parts of the Chemokine receptors bind chemokines term. Finally, Reactome Pathways terms analysis was performed on the whole inferred network, resulting with 15 identified terms (Table 3) which were grouped according to their roles into 4 groups (Fig. 8). These groups were Signal transduction GPCR signalling, Immune system/Platelet, Vesicles transport and Metabolism. Furthermore, GO Cell localization terms analysis of the whole inferred network identified 17 mostly associated cell localizations terms (Table 4).

### 3.8. The performance of biomarkers

In order to test the discriminatory power of the 12 differentially abundant proteins between the groups, ROC curves were constructed and AUC calculated, but only 4 proteins fulfilled the criterion of the required sample size (ITIH4, ITIH3, MFAP4 and APOA4). The ROC curves, AUC values, $p$ values, sensitivity and specificity with corresponding criterion for those 4 proteins are shown in the Fig. 9. All 4 proteins yielded high AUC values (above 0.9), as well as sensitivity and specificity indicating their good performance as potential biomarkers of iDCM in dogs.

## 4. Discussion

Using the TMT label-based relative quantification proteomics approach, we have found 12 differentially abundant serum proteins between dogs with iDCM and healthy dogs. Identified differences indicate involvement of several physiological pathways in studied disease, such

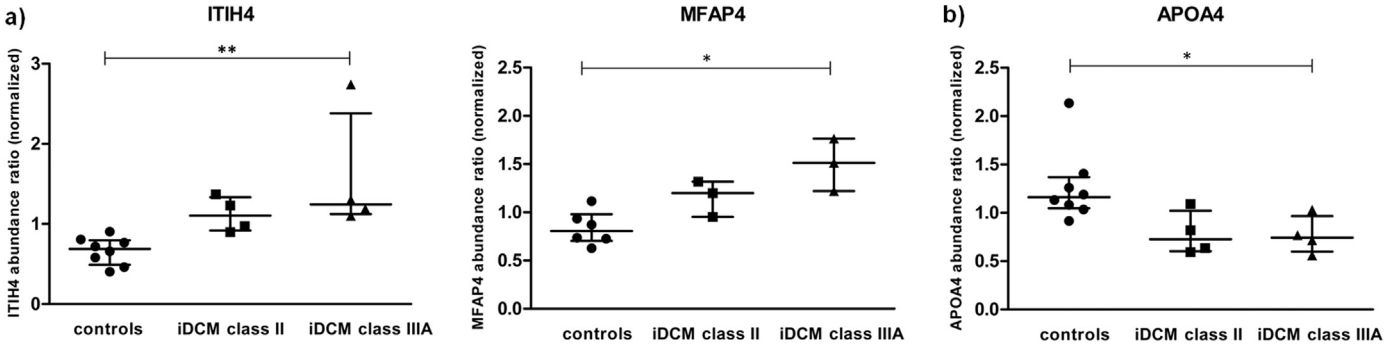


**Fig. 4.** Comparison of normalized protein abundances ratios between healthy controls, iDCM ISACHC class II and class IIIA.
a) ITIH4 and MFAP4 are up-regulated in iDCM ISACHC class IIIA compared to controls. b) APOA4 is down-regulated in iDCM ISACHC class IIIA compared to controls. Results are expressed as median and interquartile range, $*p < 0.05$, $**p < 0.01$. Note that for ITIH4 and APOA4 there are $N = 8$ controls, $N = 4$ iDCM class II, N = 4 iDCM class IIIA and for MFAP4 $N = 6$ controls, $N = 3$ iDCM class II and N = 3 iDCM class IIIA.

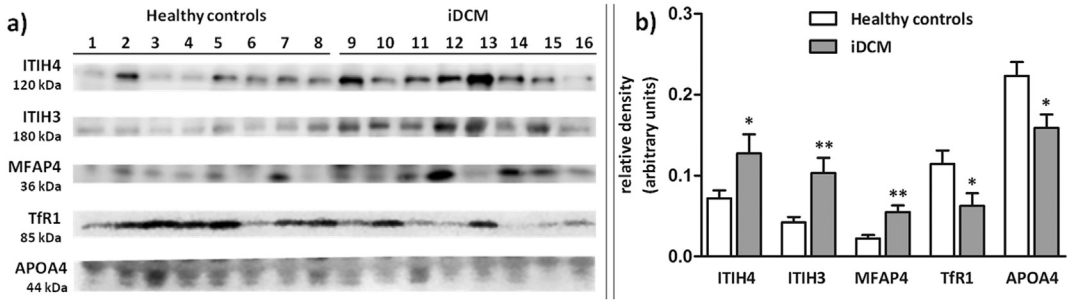


**Fig. 5.** Validation of proteomics results:
a) Representative figures of Western blotting of serum ITIH4, ITIH3, MFAP4, TfR1 and APOA4 from healthy controls and dogs with iDCM (figures of individual membranes were cropped to show the band of interest). b) Relative density comparison of the five proteins between healthy controls and iDCM; data are shown as mean with SEM, *p < 0.05, **p < 0.01.

**Table 2**
Serum biochemical parameters with significantly differential values between healthy dogs and dogs with iDCM.

| Parameter | Healthy group | iDCM group | Reference range |
|---|---|---|---|
| C-reactive protein (mg/L) | 0 (0–0.3) | 9.1 (8.5–20.1)** | 0–10.7 |
| Creatine kinase (U/L) | 75 (69–123) | 163 (132–460)* | 0–160 |
| Lactate dehydrogenase (U/L) | 64 (37–68) | 141 (106–228)* | 45–233 |
| Urea (mmol/L) | 5.2 (4.4–5.6) | 8.4 (6.2–17.9)* | 3.3–8.3 |
| Triglycerides (mmol/L) | 0.6 (0.3–0.7) | 0.9 (0.6–1.5)* | 0.2–1.3 |

The results are expressed as median and interquartile range.
Outliers were excluded based on box plot (CRP, LDH and CK - 1 outlier in each group).
*$p < 0.05$, **$p < 0.01$ (compared to the control group).

as complement activation, acute phase response, lipoprotein particles dynamics and tissue remodelling. Furthermore, bioinformatics analysis revealed an impact on some metabolic and signalling processes, such as "The citric acid cycle and respiratory electron transport" and "Signal transduction GPCR signalling".

Several components of the innate immune system response were found to be up-regulated by proteomic analysis in iDCM compared to healthy patients: complement C4-A, complement component C9, plasma protease C1 inhibitor (C1-INH) and newly recognized acute-phase response proteins inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3) and H4 (ITIH4). Components of the complement system C4-A and C9, as well as complement regulatory protein C1-INH, were significantly increased in dogs with iDCM, but with the average fold change (iDCM/healthy ratio) of only 1.2, which suggests the activation of the complement cascade in dogs with iDCM is present, at least in some of the patients studied. Complement protein C4 is an early component of the cascade activated through the classical or lectin pathway, while complement protein C9 is one of the components of the membrane attack complex (MAC) in the terminal pathway [18].

Up-regulation of positive acute-phase response (APR) proteins ITIH4 and ITIH3 detected in serum of dogs with iDCM suggests there is an activation of APR in the setting of heart tissue injury. Acute phase response (APR) is an immediate, nonspecific and complex defensive reaction which develops in an animal upon any tissue injury (caused by infection, inflammation, neoplasia, trauma or other causes) in order to restore homeostasis. During the APR, pro-inflammatory cytokines stimulate the production of positive acute-phase proteins (APPs) in hepatocytes leading to increase in their plasma concentration [19]. ITIH3 was shown to be up-regulated in human liver upon inflammation mediated by interleukin-1 and interleukin-6 [20]. ITIH4 is a major APP in pigs which is specifically induced in hepatocytes by interleukin-6 [21,22]. ITIH4 was also elevated in serum of cows with both experimentally induced and naturally occurring mastitis [23,24]. Recently, it was found to be a new positive APP in dogs where major surgery was used as an inflammatory model [14]. Both ITIH3 and ITIH4 bind covalently to hyaluronan, thereby promoting its stabilization. Hyaluronan is one of the main components of extracellular matrix in vertebrates which is involved in cell migration and tissue repair upon injury [25]. Increased levels of ITIH3 and ITIH4 found in our study could be due to the need of extracellular matrix stabilization in heart tissue of diseased dogs. Additionally, ITIH4 could have anti-inflammatory effects since it may have a role in complement suppression [25].

Our finding of significantly increased concentration of C-reactive protein (CRP) in iDCM group compared to healthy (measured by serum biochemistry test) also provides evidence for activation of APR in iDCM. CRP is a well known major positive APP in both humans and dogs, used as sensitive, but nonspecific biomarker of systemic inflammation [26]. This study contributes to the rare reports of serum CRP concentration evaluation in dogs with iDCM, but with limitation of a small sample size and remark that some values in iDCM group didn't exceed the upper limit of the normal reference range. Similar results were observed in a study of dogs with chronic valvular disease (CVD), the most common acquired heart disease of the dog, where CRP concentration was significantly higher in dogs with CVD compared to healthy dogs, but with a large degree of overlap between the two groups [27]. Increased plasma CRP concentrations were also found in dogs with congestive heart failure due to mitral valve disease or dilated

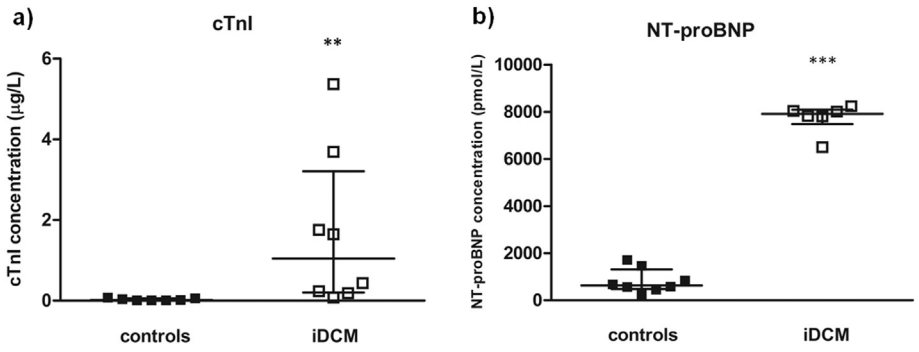


**Fig. 6.** Comparison of a) cTnI and b) NT-proBNP blood concentrations between healthy dogs and dogs with iDCM. The results are expressed as median and interquartile range.
Outliers were excluded before statistical analysis based on grubbs' test for outliers and are not shown in the figure (cTnI – 1 outlier in the control group, no outliers in the iDCM group; NT-proBNP – no outliers in the control group, 2 outliers in the iDCM group).
**p < 0.01, ***$p < 0.001$ (compared to the control group).

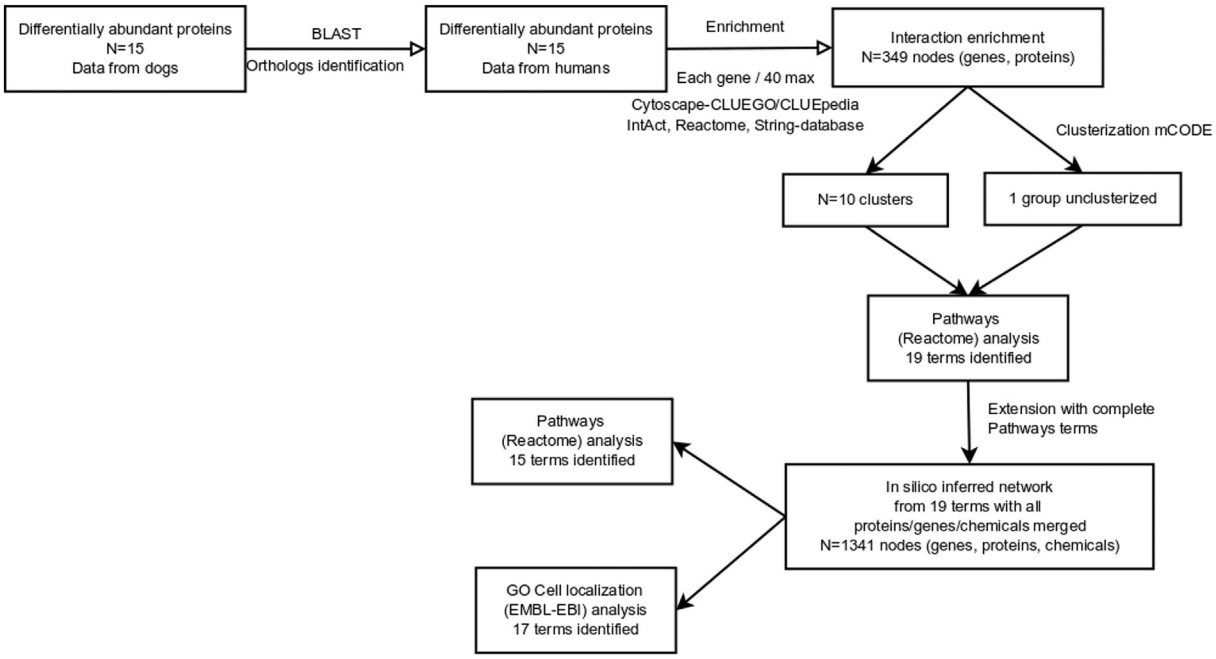


**Fig. 7.** Design of bioinformatics analysis based on initial list of differential proteins between healthy dogs and dogs with iDCM.

**Table 3**
Pathways terms (Reactome) identified on the whole *in silico* inferred network.

| Pathways term | Term P-value | Network nodes |
|---|---|---|
| G alpha (i) signalling events | 8.23E-196 | 223 |
| The citric acid (TCA) cycle and respiratory electron transport | 6.91E-95 | 131 |
| Clathrin-mediated endocytosis | 4.45E-103 | 123 |
| Response to elevated platelet cytosolic Ca2+ | 5.75E-102 | 120 |
| PPARA activates gene expression | 3.72E-91 | 107 |
| Anchoring of the basal body to the plasma membrane | 6.66E-71 | 86 |
| Clathrin derived vesicle budding | 8.46E-66 | 70 |
| Plasma lipoprotein assembly, remodelling, and clearance | 2.69E-54 | 63 |
| Complement cascade | 6.89E-44 | 52 |
| Chemokine receptors bind chemokines | 2.71E-42 | 46 |
| G alpha (z) signalling events | 1.55E-33 | 41 |
| Retinoid metabolism and transport | 1.77E-33 | 39 |
| Formation of Fibrin Clot (Clotting Cascade) | 1.43E-33 | 37 |
| Elastic fibre formation | 5.29E-26 | 35 |
| Class C/3 (Metabotropic glutamate/pheromone receptors) | 1.36E-24 | 31 |

cardiomyopathy compared to controls, but only 5 dogs with DCM were included [28]. In a study of human patients with iDCM, serum CRP levels were increased compared to controls [29].

While there is widening evidence of role of immune processes in a portion of human DCM cases [30], such findings are poorly documented in canine DCM research. However, in a study of Buse et al. [31] there were significantly higher levels of serum auto-antibodies against myosin heavy chain and α-cardiac actin in dogs with DCM than in controls, while Day [32] found anti-mitochondrial antibodies in one third of examined English Cocker Spaniels with DCM. Results of our study support the notion that immunological processes are also involved in the pathopshysiology of canine iDCM, although sequence of events leading to their involvement in the disease cannot be clearly elucidated.

Apolipoproteins A1 (APOA1), A4 (APOA4) and C3 (APOC3), which are lipid-binding proteins involved in the transport of lipids in plasma, were found to be down-regulated in iDCM compared to control serum in our study. Lower plasma APOA1 levels were also found in human patients with iDCM compared to controls [29,33]. APOA1 is a major constituent of high-density lipoprotein particles which was found to have anti-inflammatory properties since it may interfere with the assembly of complement C9 of the MAC of complement cascade [34]. APOA4 is a protein found free in plasma or as a component of different lipoprotein particles, which was shown to have anti-atherogenic and antioxidative properties. Lower levels of APOA4 were found in human patients with cardiovascular disease compared to controls and low APOA4 levels predicted the risk for sudden cardiac death in patients with high risk [35]. It is possible that reduced levels of APOA1 and APOA4 detected in dogs with iDCM contribute to the development or advancement of the disease and could be potentially used as a risk biomarker.

Interesting finding of increased serum microfibril-associated glycoprotein 4 (MFAP4) levels in dogs with iDCM could have origin in remodelling processes taking place in dilated myocardium. MFAP4 is an extracellular matrix (ECM) glycoprotein expressed in various elastic tissues which has a role in elastic fibre organization [36]. Increased serum levels of MFAP4 were proposed to be a potential biomarker in pathologies characterized by ECM remodelling, such as liver fibrosis in hepatitis C patients [37]. MFAP4 was also found to be elevated in plasma of human patients with congestive heart failure [38]. In a proteomic study of serum glycoproteins in canine model of dyssynchronous heart failure, MFAP4 was upregulated when compared to control dogs [39]. It is well known that, in the setting of both human and canine iDCM, heart tissue is undergoing fibrosis which involves ECM degradation and remodelling [40,41]. We show herein that serum level of MFAP4 could serve as biomarker of heart tissue remodelling in iDCM.

Transferrin receptor protein 1 (TfR1) is a membrane glycoprotein which has a role in transport of iron from plasma to cell and whose expression on the surface of cells is dependent on tissue iron status. Serum TfR1 (sTfR1) represents the soluble extracellular portion of TfR1 whose level reflects TfR1 density on cells (*i.e.* iron status) and the number of cells expressing TfR1 (*i.e.* mostly cells with erythropoietic activity). Levels of sTfR1 are used as biomarker in iron deficiency and anaemia of chronic disease in humans [42]. We detected slightly decreased levels of sTfR1 in diseased dogs compared to healthy, which

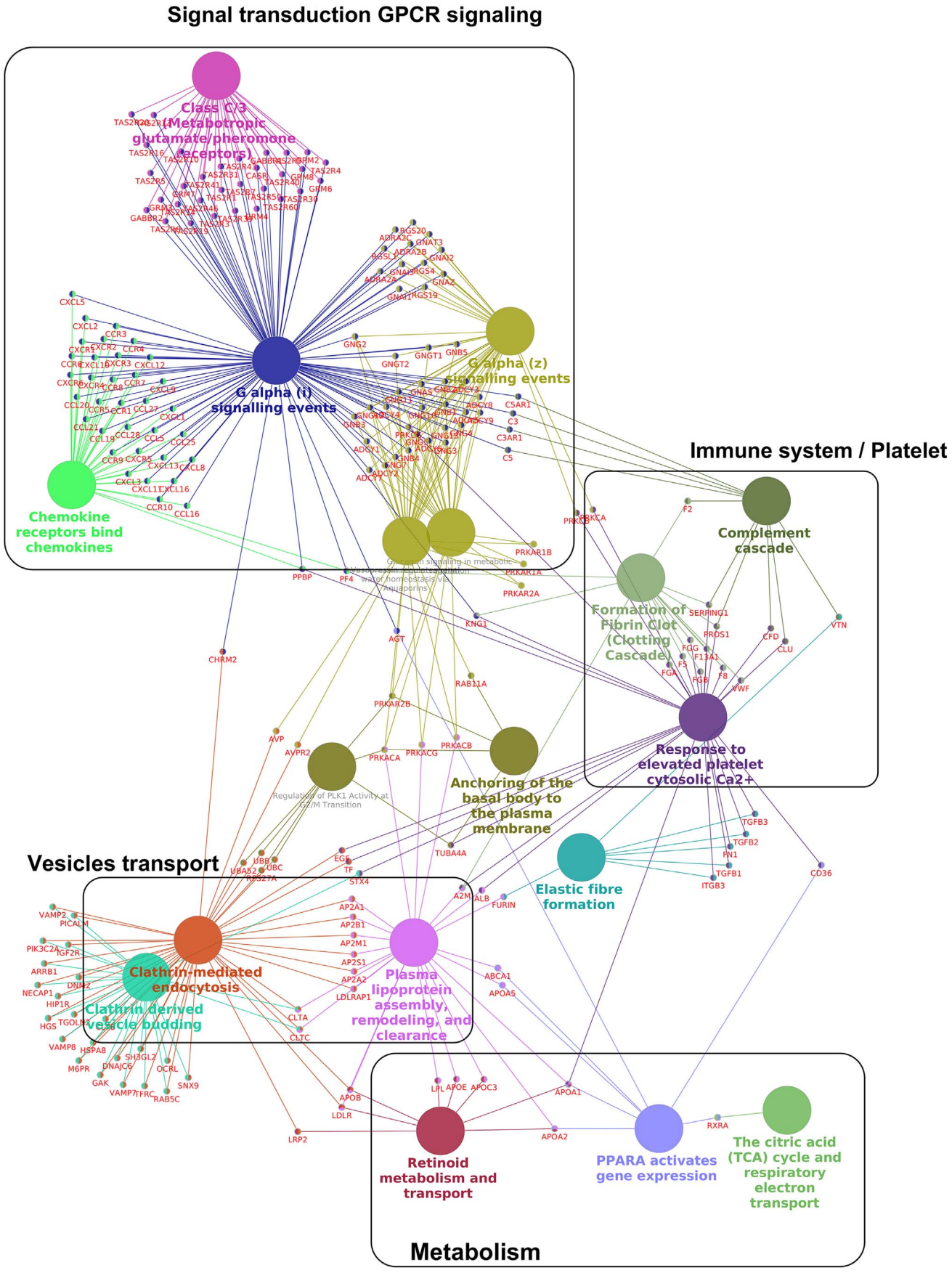


**Fig. 8.** *In silico* inferred interactome protein network of identified Pathways terms involved in canine iDCM. Only nodes interacting with at least 2 terms are represented. Organic layout was applied.

**Table 4**
GO Cell localization terms of the *in silico* inferred network.

| GO Cell localization term | Term P-value | Network nodes |
|---|---|---|
| Cytoplasmic vesicle part | 7.99E-56 | 257 |
| Membrane protein complex | 6.11E-53 | 167 |
| Lysosome | 2.79E-20 | 114 |
| Endosome | 1.33E-13 | 108 |
| Cell surface | 4.18E-16 | 107 |
| Platelet alpha granule | 9.05E-75 | 81 |
| Inner mitochondrial membrane protein complex | 5.43E-43 | 67 |
| Secretory granule membrane | 1.02E-07 | 52 |
| Plasma membrane protein complex | 5.69E-13 | 49 |
| Trans-Golgi network | 1.41E-07 | 38 |
| Early endosome | 8.19E-05 | 36 |
| Microtubule organizing center part | 1.95E-07 | 31 |
| Proton-transporting two-sector ATPase complex | 1.62E-24 | 30 |
| Azurophil granule | 9.37E-05 | 28 |
| Clathrin-coated pit | 1.23E-23 | 28 |
| Lysosomal lumen | 2.46E-07 | 25 |
| Phagocytic vesicle | 5.66E-06 | 21 |

could suggest a change of erythropoietic activity or iron status in iDCM. Although anaemia is not common finding in dogs with iDCM, it is present in certain number of diseased dogs [43,44].

Increased serum abundances of angiotensinogen (AGT) in dogs with iDCM are not a surprising finding since AGT is a part of the renin-angiotensin-aldosterone system (RAAS), which is known to be activated in dogs with DCM, although only in those with clinical signs (the New York Heart Association (NHYA) class III and IV) [45,46]. AGT production in dogs is in some minor extent stimulated by positive feedback by its final product angiotensin II at physiological conditions [47]. Dogs with iDCM included in our study were classified to the NHYA class II and III, which could explain for only slight elevation of serum AGT in comparison to healthy dogs. While serum AGT elevation in the diseased group in our study indicates activation of the RAAS, increase of specifically measured plasma NT-proBNP herein also points out to the natriuretic system stimulation in dogs with iDCM. NT-proBNP was found to be elevated in both humans and dogs with DCM [48]. Elevated NT-proBNP concentrations are detected in blood as a response to different factors, such as volume overload, hypertrophy and hypoxia. Actions of natriuretic system lead to natriuresis and vasodilation, acting in contrary to the RAAS, which induces sodium retention and vasoconstriction. It is known that activities of both systems contribute to the congestive heart failure development [49]. Another known biomarker of myocardium damage is cardiac troponin I (cTnI), which is released from cardiac myocytes upon their injury. It is used as a blood biomarker of acute myocardial infarction in humans, but it also has prognostic value in patients with chronic heart disease [50]. While cTnI concentrations are increased in dogs with iDCM, there are limitations in its utility since it is not specific to the myocardial injury cause, can be of normal level in animals with mild disease and is falsely elevated if kidney injury is present [6]. We observed significant increase of serum cTnI in dogs with iDCM compared to healthy, as expected.

Bioinformatics analysis of the *in silico* inferred protein network enabled us to generate more information from the experimental results, even providing data on intracellular pathways, not normally captured by serum proteome analysis. The strength of the *in silico* inferred network analysis is reflected in the detection of pathways known to be involved in iDCM, which indicates validity of the *in silico* constructed data. Also, recognition of these pathways has an importance in the possibility of further targeted research.

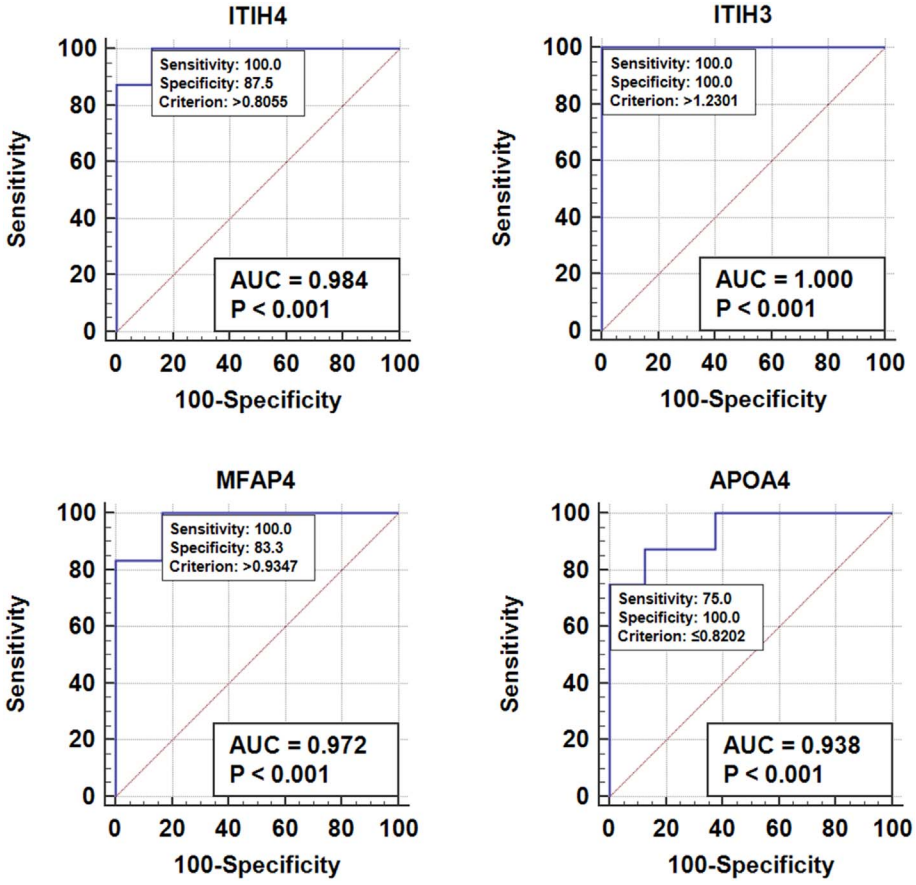


**Fig. 9.** ROC curves of potential protein biomarkers of iDCM in dogs. Note that there were N = 8 controls, N = 8 iDCM values for ITIH4 and APOA4 analysis; N = 6 controls, N = 6 iDCM for MFAP4 analysis and N = 4 controls, N = 4 iDCM for ITIH3.

Based on the *in silico* network, we were able to detect "The citric acid cycle and respiratory electron transport" as pathway implicated in the disease. This result is consistent with GO cell localization analysis of the network, which detected "inner mitochondrial membrane protein complex" and ¨proton-transporting two-sector ATPase complex¨ GO terms. Mitochondria, organelles which main function is energy production, are believed to have an important role in aetiology and/or progression of heart dysfunction pathologies taking into account myocardium high energy demand [51]. Interestingly, in several studies on heart tissue of both human and canine iDCM there was a finding of perturbations in mitochondrial electron transport activity [7,52,53]. Also, when Heinke et al. [54,55] performed proteomic studies on left ventricular tissue of dogs with pacing-induced heart failure, model state resembling iDCM, they found impairment of mitochondrial energy production. Furthermore, Lopes et al. [56] found that most of the altered mitochondrial proteins of heart tissue of dogs with induced or naturally occurring iDCM, compared to controls, were involved in respiratory electron transport chain.

Very extensive group of the *in silico* network analysis was the "Signal transduction GPCR signalling", which included "G alpha (i) and (z) signalling events", "Chemokine receptors bind chemokines" and "Class C/3 (Metabotropic glutamate/pheromone receptors)" pathways. G protein-coupled receptors (GPCRs) signalling is involved in various physiological pathways and activated by most of the known neurotransmitters, hormones and chemokines [57]. GPCRs in turn trigger downstream signalling events, among them G alpha (i) signalling, reported to be increased in human hearts affected by iDCM, where it seems to mediate myocardium contractility defects [58].

Two pathways implicated in iDCM based on the *in silico* protein network are related to haemostasis – "Formation of Fibrin Clot" and "Response to elevated platelet cytosolic $Ca^{2+}$". Activation of coagulation system was found present in humans and dogs with iDCM, possibly resulting from changes in blood flow due to dilated cardiac chambers and low cardiac output or as a consequence of activation of the RAAS system [59–61]. Our bioinformatics results are in accordance with the finding of procoagulant state in dogs with iDCM.

Furthermore, bioinformatics analysis confirmed our observation of immune system involvement, plasma lipoproteins remodelling and elastic fibre formation in iDCM physiopathology. One important finding of our study is the supporting evidence of immunological processes in canine iDCM, which could target the development of new treatment strategies.

## 5. Conclusions

Limitations of this study are small sample size and utilization of samples of the same patients for validation of proteomics results by Western blot. Therefore, this work can be considered preliminary and our findings need further validation using different samples and larger sample size. Nevertheless, we can highlight three putative biomarkers validated herein which could be relevant for underlying iDCM processes, taking into account the fold change ratio and high specificity and sensitivity resulting from the analysis of ROC curves. We propose ITIH4 to be connected with extracellular matrix changes happening upon tissue injury, which also supports its recently identified role as acute phase protein in dogs [14]. Furthermore, serum MFAP4 could serve as a biomarker of fibrosis in dogs, whose utility should be tested in different settings of canine fibrosis in both cardiac and non-cardiac diseases. Also, low levels of serum APOA4 could be tested as a risk factor for development or worsening of canine iDCM. Since the data acquired herein could be translational to human iDCM, these biomarkers may also be of relevance to human patients. In conclusion, label-based high-resolution quantitative proteomics analysis and bioinformatics approach used herein represent a valid tool for elucidating complex iDCM pathophysiology and uncovering disease relevant proteins with biomarker potential.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.03.007.

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PRILOG 5

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# Serum proteome profiling in canine chronic valve disease using a TMT-based quantitative proteomics approach

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Biomarker

ABSTRACT

Chronic valve disease (CVD) is the most common clinically significant heart disease of dogs, affecting 20 to 40% of dogs. The aim of this study was to evaluate the serum protein profile of healthy and CVD affected dogs, by means of an isobaric tandem mass tag (TMT) label-based high-resolution quantitative proteomic approach. Additionally, conventional cardiac biomarkers were measured in the serum, functional bioinformatics analysis was employed for elucidating molecular mechanisms and pathways associated with CVD, and validation of proteomic results was performed by immunoassays and Western blotting. Of 290 identified and quantified proteins, 15 proteins showed significantly different abundances ($p < .05$), including antithrombin-III, alpha-2-antiplasmin, tetranectin, apolipoprotein M, adiponectin, inter-alpha-trypsin inhibitor heavy chain H1, gelsolin and apolipoprotein B-100. The identified proteins with differently abundances are involved in a number of pathways, such as complement and coagulation cascades, haemostasis, regulation of actin cytoskeleton, lipid metabolism and transport. We found comparative similarities with human disease in terms of identified proteins and GO pathways, which confirmed similar pathophysiology of this disease, but also differences, mainly in lipid metabolism.

*Significance:* There have been few investigations of canine serum proteome despite the potential for biomarker discovery and comparative disease analysis. Establishing serum proteomic signatures in healthy dogs and dogs with CVD will benefit for understanding the aetiology of disease in dogs, identify putative biomarkers and provide models of comparative human disease. Circulating biomarkers are important for understanding of the mechanisms of cardiovascular disease and high incidence of CVD in dogs prioritizes the search for novel biomarkers.

## 1. Introduction

Veterinary proteomics is an evolving field which holds great promise not only for fundamental and applied discoveries regarding biology and pathology of domestic species, but can also be implemented in comparative applications of human diseases research [1]. The heart in dogs and humans is similar in many characteristics on both the organ and cellular levels. Canine heart rate, body weight, and heart weight are more comparable to humans than the other animals such as mice, rabbits, and rats. The opportunity to use dog as model animal for comparative disease studies is based on the sequencing of the canine genome and the increasing availability of canine specific biological tools and reagents [2]. Chronic valve disease (CVD) is the most common heart disease in dogs and the most frequent cause of congestive heart failure in this species, representing approximately 75% of all heart disease in the dog [3]. CVD is pathologically identical in humans and dogs, suggesting a common pathogenesis in these species, and creating an increasing interest in the canine CVD as a model for the human medicine. Various other names for the disease are used and include endocardiosis, valvular regurgitation, valvular insufficiency, mitral regurgitation, myxomatous degeneration of the valve, degenerative mitral valve disease, senile nodular sclerosis, mucoid degeneration, chronic mitral valve fibrosis [4].

Chronic valve disease is characterised by a chronic progression,

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from mild, clinically silent disease to severe disease with signs of congestive heart failure. Myxomatous valvular degeneration most often leads to lesions of the mitral valve (62% of cases), sometimes both atrioventricular valves (33% of cases) and infrequently the tricuspid valve (1% of cases) [5].

Reported risk factors associated with progression of disease or death in dogs with CVD include age, gender, intensity of heart murmur, degree of valve prolapse, severity of valve lesions, the degree of mitral valve regurgitation, degree of left atrial enlargement, severity of eccentric hypertrophy, rupture of chordae tendinae and increased concentration of natriuretic peptides. The vast majority of the breeds at elevated risk of CVD are small or toy breeds, males are 1.5 times more represented than females, and the disease is rare before the age of 4 years [3,4,6].

The aim of this study was to evaluate the serum protein profile of healthy and CVD affected dogs, by means of an isobaric tandem mass tag (TMT) label-based high-resolution quantitative proteomic approach. Additionally, conventional cardiac biomarkers were measured in the serum, functional bioinformatics analysis was employed for elucidating molecular mechanisms and pathways associated with CVD, and validation of proteomic results was performed by immunoassays and Western blotting. Only one previous study has been performed to identify serum proteins that were differentially expressed in healthy Cavalier King Charles Spaniel and those affected by CVD in mild to severe stages, using two-dimensional gel electrophoresis separation and analysis by MALDI-TOF-MS for protein identification [7]. To the authors´ knowledge, this is the first proteomic study of serum of dogs with CVD, where label-based quantitative LC-MS/MS approach was used.

## 2. Materials and methods

### 2.1. Animals

Two groups of dogs were enrolled in the study in the period between June 2015 and May 2017: 8 clinically healthy dogs (used as controls) and 8 dogs diagnosed with CVD. The study was approved by the Committee on the Ethics of the University of Zagreb, Faculty of Veterinary Medicine (Permit Number: 640–01/14–305/16, 251–61-01/139–14-28). Healthy dogs were admitted to the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia, while diseased dogs were admitted to the Small Animals Clinic, Department of Clinical Veterinary Science, Vetsuisse Faculty, University of Bern, Switzerland. Serum of dogs with CVD was collected at the time of initial diagnosis and before any treatment.

All dogs of the control group underwent clinical examination, haematological and biochemical serum analyses, as well as cardiac function evaluation performed in unsedated dogs, which included a 1-min 6 lead ECG (ASPEL, AsCard Mr. Silver) and transthoracic echocardiography using Esaote MyLab40 Vet machine and a 5 MHz sector transducer. Dogs were diagnosed with CVD-MV if the history, clinical exam results and the results of an ultrasound imaging were confirmative and other acquired heart disease so as congenital heart diseases were ruled out [8]. The cardiac evaluation of dogs included physical examination, thoracic radiographs evaluated by a board-certified radiologist, a 1-min 6 lead ECG (Schiller AT 101) and transthoracic echocardiography performed by a board certified cardiologist (AK). Echocardiography was performed using an Aloka ProSound Alpha 5SV machine and a 5-MHz sector transducer in unsedated dogs. Echocardiography was performed in a standard manner [8]. Diseased dogs were classified according to the American College of Veterinary Internal Medicine (ACVIM) consensus guidelines for the diagnosis and treatment of myxomatous mitral valve disease in dogs [9].

All procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments, as well as subject to informed owner consent.

Serum was obtained from all dogs by centrifugation of completely clotted blood at 3500*g* for 10 min at room temperature. Supernatants were collected, aliquoted and stored at −80 °C until analyses. All samples used for repetitive analysis were frozen in aliquots and only vials needed for each assay run were used, to avoid possible changes caused by repetitive thawing and freezing.

### 2.2. Serum biochemistry and cardiac biomarker analyses

One serum aliquot was used for measurement of biochemical parameters using commercial reagents (Beckman Coulter) per manufacturer's instructions in an automatic analyser (Olympus AU 640, Hamburg, Germany). The following parameters were measured: serum urea, creatinine, bilirubin, glucose, proteins, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (AP), creatine kinase (CPK), lactate dehydrogenase (LDH), C-reactive protein (CRP), alpha-amylase, lipase, cholesterol, triglycerides, calcium, phosphates.

Cardiac troponin I (cTnI) was measured using a commercially available ADVIA Centaur TnI-Ultra assay, which is a high-sensitivity immunoassay validated for use in both humans and dogs [10]. Analysis was performed in Dubrava Clinical Hospital, Department of clinical diagnostics (Zagreb, Croatia) using Siemens Advia Centaur XP according to manufacturer's instructions. Samples which had serum concentration of cTnI below the lower level of detection of the assay were allocated a value of 0.01 μg/L. Analysis of N-terminal pro b-type natriuretic peptide (NT-proBNP) concentration in all samples was performed in Vet Med Labor GmbH, reference IDEXX Laboratory (Germany) using IDEXX Cardiopet® proBNP test.

### 2.3. Proteomic analysis by LC-MS/MS

Proteomic analysis of canine serum samples was performed by TMT-based quantitative approach as described previously [11]. In brief, after total protein concentration determination using Bradford assay (Thermo Scientific, Rockford, USA), 35 μg of total proteins from samples and internal standard (a pool of equal protein amount from all samples used as a reference for normalization) were diluted to a volume of 50 μL using 0.1 M triethyl ammonium bicarbonate (TEAB, Thermo Scientific, Rockford, USA), reduced by adding 2.5 μL of 200 mM DTT (60 min, 55 °C) (Sigma Aldrich, St. Louis, MO, USA), alkylated by adding 2.5 μL of 375 mM IAA (30 min, room temperature in the dark) (Sigma Aldrich, St. Lois, MO, USA) and acetone-precipitated (addition of 300 μL, overnight, −20 °C). Protein pellets were collected subsequently by centrifugation (9000 *g*, 4 °C), dissolved in 50 μL of 0.1 M TEAB and digested using 1 μL of trypsin (1 mg/mL, Promega; trypsin-to-protein ratio 1:35, at 37 °C overnight).

TMT sixplex reagents (Thermo Scientific, Rockford, IL, USA) were prepared according to the manufacturer's procedure and 19 μL of the appropriate TMT label was added to each sample used for the labelling reaction (60 min, room temperature) which was quenched by 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA). Five TMT-modified peptide samples were combined with the internal standard (labeled with TMT *m/z* 126) into the new tube, aliquoted, dried and stored at −20 °C for further analysis. A total of 16 samples led to 4 individual TMT experiments with the inclusion of internal standards in each experiment but 2 samples were repeated using the same internal standard because of low labelling efficiency.

High resolution LC-MS/MS analysis of TMT-labeled peptides was carried out using an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were dissolved in loading solvent (1% ACN, 0.1% formic acid) and loaded onto the trap column (C18 PepMap100, 5 μm, 100A, 300 μm × 5 mm), desalted for 12 min at the flow rate of 15 μL/min and separated on the analytical column (PepMap™ RSLC C18, 50 cm × 75 μm) using a linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min,

45% to 90% for 2 min, held at 80% for 2 min and re-equilibrated at 5% B for 20 min at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid in water. Ionisation was achieved using nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) containing a 10 μm-inner diameter SilicaTip emitter (New Objective, USA). The MS operated in positive ion mode using DDA Top8 method. Full scan MS spectra were acquired in range from $m/z$ 350.0 to $m/z$ 1800.0 with a resolution of 70,000, 110 ms injection time, AGC target $1 \times 10^6$, a ± 2.0 Da isolation window and the dynamic exclusion 30 s. HCD fragmentation was performed at step collision energy (29% and 35% NCE) with a resolution of 17,500 and AGC target of $2 \times 10^5$. Precursor ions with unassigned charge state, as well as charge states of +1 and more than +7 were excluded from fragmentation.

Acquired MS/MS spectra were analysed for protein identification and quantification using the SEQUEST algorithm implemented into Proteome Discoverer (version 2.0., ThermoFisher Scientific). Database search against *Canis lupus* FASTA files (downloaded from NCBI database on 14/10/2016, 41,787 sequences) was performed according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), deamidation (N,Q) and TMT sixplex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow based on the search results against a decoy database and was set at 1% FDR. At least two unique peptides and 5% FDR were required for reporting confidently identified proteins.

Protein quantification was accomplished by correlating the relative intensities of reporter ions extracted from tandem mass spectra to that of the peptides selected for MS/MS fragmentation. The internal standard was used to compare relative quantification results for each protein between the experiments (sixplexes).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017129.

### 2.4. Bioinformatic analysis

Proteins with significantly differential abundances observed by proteomic analyses were further functionally analysed using bioinformatics tools. As the human database is more complete than the canine one (reviewed proteins in UniprotKB/Swiss-prot release 2018_01: humans 20,259, dogs 817), dog's proteins were converted to converted to human gene official names by the database bioDBnet. The 10 unique proteins have been enriched for their best interacting proteins according to Intact and Reactome databases, using the tool CluePedia (v1.5.2) from Cytoscape (v3.6.1). Maximum number of 10 best interactors has been set.

The resulting interactome was used to determine the enriched GO terms, using ClueGO (v2.5.2) from cytoscape with GO_biologicalprocess (04.09.2018), GO levels from 3 to 12, minimal number of gene = 2, mimimum percentage = 3.0, Kappa score theshold = 0.4, evidence codes used All_without_IEA.

List of GO terms was then submitted to analysis by ReviGO to remove redundant terms and define GO groups based on similarity, with the SimRel semantic similarity measure. Redundant GO terms were then removed from the interactome, realized with Cytoscape. GOterms with a minimum of 2 initial proteins have been considered for the analysis of GO terms related with CVD. Interactome have been designed using the radial layout of Cytoscape.

### 2.5. Validation of proteomic results

Validation of proteomics results was performed by ELISA and Western blotting using the serum samples of the same patients as in proteomic analysis. All samples used for repetitive analysis were frozen in aliquots and only vials needed for each assay run were used, to avoid possible changes caused by repetitive thawing and freezing.

Canine specific ELISA kits were used for apoB-100 (BlueGene Biotech, Shanghai, China), apoD (ABclonal, Woburn, USA) and adiponectin (Wuhan Fine Biotech Co., LTD., Wuhan, China) according to manufacturer's instructions. For analytical performance of the ELISA assays, assay precision and accuracy were calculated [12,13]. For intra-assay precision, pool of samples with different concentrations of analytes were prepared from serum, while for inter-assay precision, pool was divided into aliquots and stored in plastic vials at −20 °C until analysis. Intra-assay coefficient of variation (CV) was calculated after analysis of pool six times in a single assay run. Inter-assay CV was determined by analysing the same samples in five separate runs carried out on different days. The accuracy of the assays was evaluated indirectly by linearity under dilution. Briefly, serum pool was serially diluted with diluent provided with the kit and analysed.

For immunoblotting, the samples (30 μg of total protein) were boiled for 3 min at 95 °C in Laemmli SDS loading buffer and loaded on 10% SDS polyacrylamide gel and after electrophoresis (1 h at 110 V) transferred to a PVDF membrane (Amersham Hybond, 0.45 PVDF, GE Healthcare Lifescience) (2 h at 65 V, 150 mA) in 20% methanol (Sigma) transfer buffer at 4 °C using Biostep electro blotting module. The membranes were blocked for 1 h at room temperature with shaking in blocking buffer (1xTris buffered saline (TBS)/0,1% Tween 20/0,2% I-Block reagent). Subsequently, the membrane was incubated at 4 °C with primary antibody for adiponectin (1:500 in blocking buffer; from Santa Cruz Biotechnology, Heildeberg, Germany) and kininogen-1 (1:500 in blocking buffer; Antibodies-online GmbH, Achen, Germany). Membranes were then washed three times with TBST buffer and incubated with secondary antibody (rabbit anti-mouse from Santa Cruz Biotechnology, 1:2000). Proteins were visualized by chemiluminescence using HRP chemiluminescence blotting substrate (Radiance Plus, Azure Biosystems, USA) on Odyssey Fc (LI-COR, Bad Homburg, Germany). The abundance of the protein of interest was normalized to the total amount of protein in each lane after staining with Ponceau S. Western blots were quantified using ImageJ software (National Institutes of Health). Statistical validation of the data was achieved by Mann-Whitney test, with $P$ value < .05 considered statistically significant.

### 2.6. Statistical analysis

For proteomics, statistical analysis was performed using using R (v3.4.3) under the RStudio environment (v1.0.143) [14,15]. Paired $t$-test was applied to calculate $p$-values to determine statistical significance among healthy dogs and dogs with CVD. For immunoblotting, statistical analysis was performed using the statistical software, GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Differences between healthy and diseased dogs were assessed by Mann-Whitney test. Non-parametric statistics was chosen due to the small sample size. $P$-value < .05 were considered statistically significant for all performed tests.

## 3. Results

### 3.1. Animals and heart function

The control group consisted of 6 females (1 spayed) and 2 males, aged between 3 and 10 years, of following breeds: 2 Miniature Schnauzers, Dachshund, Maltese, Border collie and 3 mixed-breed dogs. In the group of dogs diagnosed with CVD there were 3 females (all spayed) and 5 males (of which 3 castrated), aged between 8 and 12 years. These were dogs of 7 different breeds (2 Cavalier King Charles Spaniels, Australian Kelpie, Coton de Tuléar, Borzoi, Miniature Schnauzer, Toy Australian Shepherd and Doberman). At initial physical examination all dogs of CVD group were considered in heart failure

**Table 1**
Serum biochemical parameters measured in serum of dogs with CVD and healthy dogs.

AST - aspartate aminotransferase, ALT - alanine aminotransferase, GGT - gamma-glutamyl transferase, AP - alkaline phosphatase, CPK - creatine kinase, LDH - lactate dehydrogenase, CRP C-reactive protein, cTnI - cardiac troponin I, N-terminal pro b-type natriuretic peptide - NT-proBNP.

| Parameter (unit) | Healthy dogs (median (Q1-Q3) | Dogs with CVD (median(Q1-Q3) | P value |
|---|---|---|---|
| Urea (mmol/L) | 5.55 (4.98–7.40) | 17.05 (12.68–34.78) | 0.003 |
| Creatinine (μmol/L) | 82 (61.5–85.75) | 113.5 (86.25–144) | 0.008 |
| Total protein (g/L) | 59.5 (58.25–65.75) | 74 (66–91) | 0.007 |
| Albumin (g/L) | 32.5 (29.75–35) | 34.50 (34–43.5) | 0.112 |
| Bilirubin (μmol/L) | 3.05 (2.93–3.55) | 3.3 (1.95–5.28) | 0.650 |
| Glucose (mmol/L) | 5.65 (5.33–5.8) | 6.65 (4.98–8.18) | 0.372 |
| AST (U/L) | 23 (21.5–32.25) | 41.50 (25.5–76.75) | 0.091 |
| ALT (U/L) | 42.5 (22–54.75) | 84 (54.5–129.5) | 0.031 |
| GGT (U/L) | 3.5 (3–4.75) | 2.5 (1.75–7.25) | 0.694 |
| AP (U/L) | 29.5 (24.5–46) | 198.5 (74.25–320) | < 0.001 |
| CPK (U/L) | 104 (81.75–134.3) | 164.5 (99.5–391.5) | 0.161 |
| Alpha amylase (U/L) | 599 (362.3–691) | 614.5 (440.3–1079) | 0.279 |
| Lipase (U/L) | 272.5 (146.5–534.8) | 480 (389.3–631.8) | 0.065 |
| LDH (U/L) | 59 (47–74.75) | 217.5 (161.8–361.8) | 0.001 |
| CRP (mg/L) | 0.6 (0.05–6.1) | 23.2 (9.15–44.05) | 0.006 |
| Calcium (mmol/L) | 2.55 (2.5–2.6) | 2.84 (2.66–3.15) | 0.007 |
| Phosphates (mmol/L) | 1.4 (1.2–1.48) | 2.1 (1.72–2.45) | 0.013 |
| Triglycerides (mmol/L) | 0.65 (0.53–0.78) | 1.3 (1.03–1.65) | 0.005 |
| Cholesterol (mmol/L) | 6.85 (5.38–8.65) | 10.6 (9.75–12.8) | 0.021 |
| cTnI (μg/L) | 0.01 (0.01–0.028) | 1.32 (0.11–4.52) | < 0.001 |
| NT-proBNP (pmol/L) | 421 (265.5–651.3) | 2919 (1574–6620) | 0.009 |

based on elevated respiratory rate and effort, signs of interstitial or interstitial-alveolar lung pattern and absolutely dilated pulmonic veins on the thoracic radiographs. All were classified as ACVIM class C. Six dogs were in sinus rhythm. Two of those dogs had rare ventricular premature complexes and one dog rare supraventricular premature beats. Two dogs showed atrial fibrillation with a ventricular rate 190–250/min. Seven of the eight dogs also had a tricuspid endocardiosis, four of which had signs of mild to moderate pulmonary hypertension. Two out of eight dogs showed a slight regurgitation on the aortic valve and pulmonary valves (Supplemental data 1).

### 3.2. Serum biochemistry and cardiac biomarkers

Values of serum biochemical parameters are presented in Table 1. as median and interquartile range, together with *P* value for comparisons of groups. Concentrations of urea, creatinine, total proteins, CRP, Ca, phosphates and cholesterol, as well as activities of ALT, AP and LDH were significantly higher in serum of dogs with CVD compared to healthy dogs. Cardiac biomarkers, cTnI and NT-proBNP, also had significantly higher concentrations in dogs with CVD compared to healthy dogs.

### 3.3. Proteomics

In this study, 290 quantifiable proteins (171 of which were master proteins and 47 master protein candidates, respectively) were identified by label-based quantitative proteomic approach according to set criteria (2 unique peptides and 5% FDR) (Supplemental data 2). In total, there were 15 proteins with significantly differential abundances between healthy and dogs with CVD, and all of them were downregulated in diseased dogs (Table 2).

**Table 2**
Proteins with significantly differential abundances between healthy and dogs with CVD identified and quantified using TMT approach.

| Accession number[a] | Protein | P-value | Fold change |
|---|---|---|---|
| 545,528,321 | Apolipoprotein B-100[b] (apoB-100) | 0.03 | −0.72 |
| 359,320,010 | Antithrombin-III (AT III) | 0.04 | −0.56 |
| 545,518,174 | Gelsolin | 0.04 | −0.56 |
| 57,109,938 | Kininogen-1 isoform X2 | 0.04 | −0.55 |
| 345,796,419 | Kininogen-1 isoform X1 | 0.04 | −0.55 |
| 73,967,363 | Alpha-2-antiplasmin isoform X2[b] | 0.03 | −0.61 |
| 545,512,145 | Alpha-2-antiplasmin isoform X1[b] | 0.03 | −0.61 |
| 928,162,811 | Tetranectin[b] | 0.02 | −0.73 |
| 928,151,046 | Apolipoprotein M isoform X2[b] (apoM) | 0.02 | −0.65 |
| 928,180,090 | Apolipoprotein D[b] (apoD) | 0.04 | −0.64 |
| 15,825,495 | Adiponectin, partial | 0.04 | −0.59 |
| 54,792,748 | Adiponectin precursor | 0.04 | −0.59 |
| 545,553,489 | Adiponectin isoform X1[b] | 0.04 | −0.59 |
| 218,051,927 | Adiponectin | 0.04 | −0.59 |
| 928,186,333 | Inter-alpha-trypsin inhibitor heavy chain H1-like isoform X1[b] (ITIH1) | 0.02 | −0.62 |

[a] Accession number from NCBI protein database for *Canis lupus familiaris*.
[b] proteins predicted in *Canis lupus familiaris*, with no evidence of existence to date at protein, transcript or homology levels.

### 3.4. Bioinformatics

Proteins with significantly differential abundances were used as a starting point for creating protein interacting networks using different softwares. An additional 77 associated proteins have been added to the initial proteins by the enrichment step. From the 87 genes (initial proteins + enriched), 46 (54.8%) were associated to a GO term. Nine of the 10 (90%) initial proteins have been associated with a GO term. Only ITIH1 was not associated with a GO term. After refinement, 12 representative GO terms have been selected (Table 3, Fig. 1).

### 3.5. Validation of proteomic results

In order to verify differences in serum protein abundances observed by proteomic analysis, selected proteins were validated by ELISA assays and Western blotting. All ELISA assays evaluated in the present study showed adequate precision with intra- and inter-assay CVs lower than 15%, the limit of the objective analytic performance standard for precision [16]. For accuracy of the assays linearity under dilution was accomplished by ordinary linear regression analysis comparing the measured concentrations of analyte with the expected levels. Dilution of canine serum samples with different analyte concentrations resulted in linear regression equations with correlation coefficient close to 1.0 ($r > 0.98$ for all assays).

All tested markers, apolipoprotein B-100, apolipoprotein D and adiponectin, showed significant differences between dogs with CVD and healthy dogs (Fig. 2). Adiponectin concentration was significantly lower in serum of dogs with CVD (median, interquartile range: 489.4 ng/mL, 311.5–859.1 ng/mL) compared to healthy dogs (1887 ng/mL, 769.9–2179 ng/mL; $P = .002$), as well as apoB-100 concentration in dogs with CVD (2.9 μg/mL, 2.3–5.3 μg/mL) compared to healthy dogs (5.6 μg/mL, 5.2–6.8 μg/mL; $P = .026$) and apoD concentration in dogs with CVD (608.1 pg/mL, 487–978.1 pg/mL) compared to healthy dogs (1108 pg/mL, 822.9–2257 pg/mL; $P = .050$). In total, all proteins validated with immunoassays confirmed consistence with proteomic results.

Two selected proteins, adiponectin and kininogen-1, were validated by Western blotting using total protein load after Ponceau S staining as a normalization reference. The relative abundance of ADPN was significantly lower in serum of dogs with CVD compared to healthy dogs ($P = .005$) (Fig. 3), while for kininogen-1 there was no statistical

**Table 3**
GO terms selected to be representative of canine CVD.

| GOterm | Total genes | Associated proteins | -log10(p-value) |
|---|---|---|---|
| Wound healing | 17 | 4 | 6.94 |
| Extracellular structure organization | 16 | 3 | 8.25 |
| Negative regulation of response to external stimulus | 12 | 4 | 6.26 |
| Organic hydroxy compound transport | 9 | 3 | 3.74 |
| Regulation of plasma lipoprotein particle levels | 8 | 3 | 5.19 |
| Positive regulation of lipid metabolic process | 8 | 2 | 4.30 |
| Lipid homeostasis | 7 | 2 | 3.73 |
| Retinoid metabolic process | 6 | 2 | 3.21 |
| Plasminogen activation | 5 | 3 | 4.96 |
| Cholesterol homeostasis | 5 | 2 | 2.58 |
| Regulation of smooth muscle cell proliferation | 5 | 3 | 2.14 |
| Renal protein absorption | 2 | 2 | 2.12 |

significance (data not shown). We were not able to confirm kininogen-1 consistence with proteomic data, possibly due to the existence of different KIN1 isoforms in serum.

## 4. Discussion

Using TMT label-based relative quantification proteomics approach, we have found 15 differentially abundant serum proteins between dogs with CVD and healthy dogs. The identified proteins with differently abundances are involved in a number of pathways, such as complement and coagulation cascades, haemostasis, regulation of actin cytoskeleton, lipid metabolism and transport.

One previous study in Cavalier King Charles Spaniel using two-dimensional gel electrophoresis separation and analysis by MALDI-TOF-MS for protein identification identified eight proteins differentially expressed among healthy and dogs with CVD [7]. None of those proteins were identified as differentially abundant in this study, probably due to difference in proteomic approach. However, there are similarities with research in humans, with special emphasis to tetranectin and gelsolin as novel biomarkers of cardiovascular disease [17].

One of the proteins we found differentially expressed in CVD dogs was adiponectin, a major adipocyte-secreted protein (adipokine), a key component that mediates the cross-talk between adipose tissue, cardiac cells and the vasculature. Several studies in humans and rodents

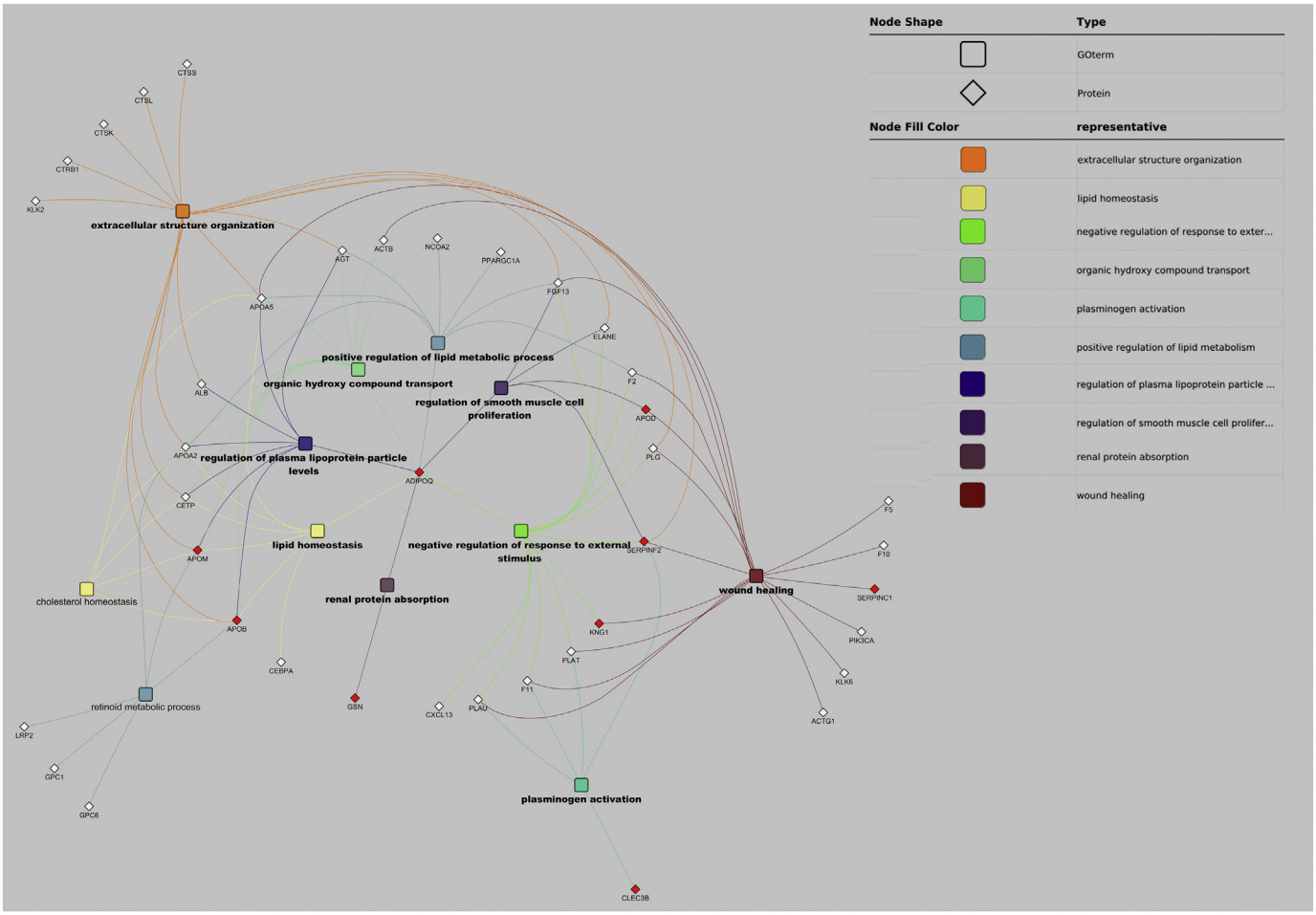


**Fig. 1.** In silico inferred interactome network of identified GO terms over-represented in canine CVD (healthy versus dogs with CVD). Differentially expressed proteins interacting with at least 1 term were added. Radial layout was applied and the GO group leader terms are in black text.

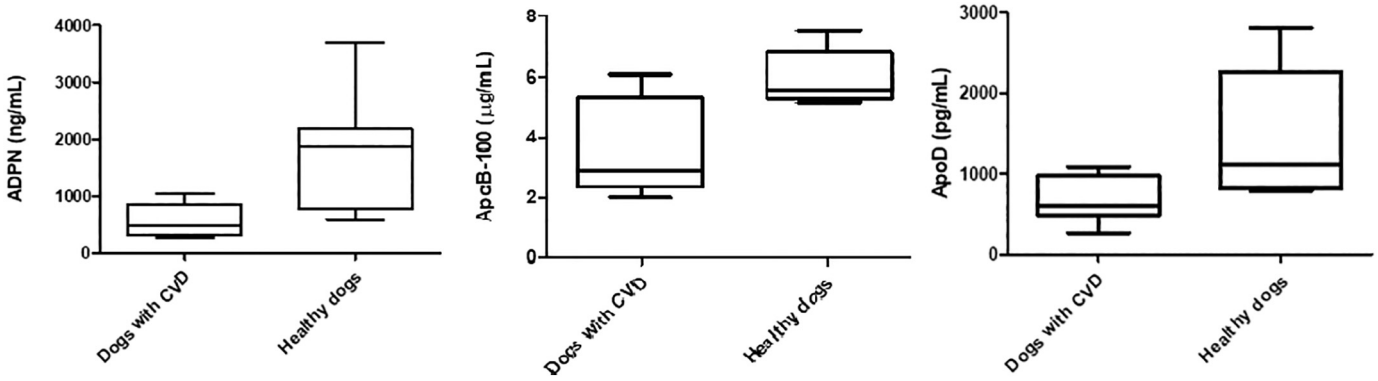


**Fig. 2.** Concentrations of adiponectin (ADPN), apolipoprotein B-100 and apolipoprotein D in serum of dogs with CVD and healthy dogs derived from ELISA, presented in a box and whisker plot (median is marked with a vertical line inside the box, the box spans the interquartile range, whiskers min-max).

showed that adipokines affect cardiovascular functions, as well as many other physiological processes including regulation of energy metabolism, immune function, and inflammation [18]. In a recent study adiponectin concentrations were found significantly lower in dogs with CVD [19], while in another study increased adiponectin concentration was found in dogs with dilated cardiomyopathy compared to healthy dogs and to dogs with CVD in dogs [20]. Identification of mRNAs encoding both adiponectin receptors in cardiac tissues of dogs confirms that circulating adiponectin directly affects cardiomyocytes in dogs with CVD [20].

High adiponectin concentrations in healthy humans are associated with low cardiovascular risk and adiponectin concentration is associated with slowing the progression of cardiovascular diseases such as cardiac hypertrophy, ischemic injury, and atherosclerosis in humans [21,22].

In our study we found lower abundances of this protein in dogs with CVD compared to controls, confirmed with both, immunoassay and immunoblotting, validation method, suggesting its role in modulating cardiovascular function by acting with an anti-inflammmatory effect, reducing oxidative stress and promoting endothelial repair during vascular dysfunction.

Another identified protein is inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1), previously shown to play a particularly important role in inflammation and carcinogenesis, and demonstrated to be both positive and negative acute phase protein under various conditions [23]. In our study, lower protein abundances were found in CVD group. The most consistent histopathologic finding in CVD is accumulation of glycosaminoglycans, mainly hyaluronic acid, in the extracellular matrix of the mitral valve [24,25]. Decrease of ITIH1 could be due to the impairment of extracellular matrix stabilization in cardiac tissue of diseased dogs. Additionally, reduced ITIH1 levels in the serum may contribute to reduced protease inhibitor activity and excess protease-mediated tissue injury in CVD group. This is also suggestive of an inflammatory component underlying this disease, as rapid consumption of protease inhibitors, such as ITIH1, are evidenced in pathophysiology of inflammation in order to prevent excess activation of proteases and limit the potential injurious actions of protease activation on endothelial and epithelial tissues [26].

Gelsolin, the actin-scavenging protein, had lower protein abundances in CVD group compared to controls. Actins are released into the systemic circulation after disruption of the cell membrane as a result of necrosis. The release of actin into the systemic circulation in response to injury or illness-associated necrosis results in adverse pathophysiologic consequences including increase of blood viscosity and disturbances in microvascular flow, activation of platelets with resulting platelet aggregation, and microvascular thrombosis, all contributing to tissue injury due to the high toxicity of actin [27]. In a majority of diseases, decline of gelsolin precedes, and therefore might predict, tissue and organ injury, and can be a predictor of critical care complications; and these alterations are primarily associated with actin scavenging and anti-inflammatory features of gelsolin. A compelling number of animal studies also demonstrate a broad spectrum of beneficial effects mediated by gelsolin, suggesting therapeutic utility for extracellular recombinant gelsolin [27]. Similar mechanism of this actin-scavenging

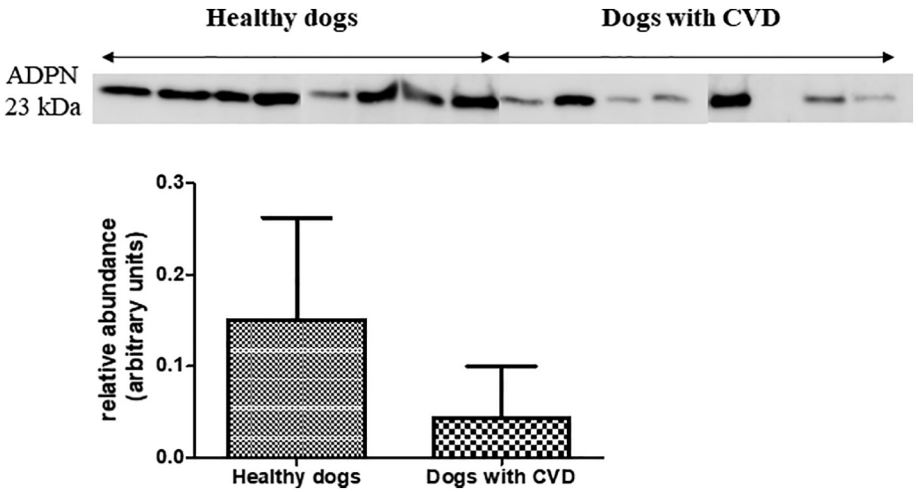


**Fig. 3.** Western blotting and relative density comparison of serum adiponectin from healthy dogs and dogs with CVD (figures of individual membranes were cropped to show the band of interest; data are shown as median with range, $P = .005$).

protein could be proposed in canine CVD, due to lower protein abundances found in dogs with CVD.

Cardiovascular diseases are associated with alteration of haemostasis. We found lower protein abundances of antithrombin III (AT III), alpha-2-antiplasmin, tetranectin and kininogen-1 in dogs affected with CVD compared to healthy dogs.

In humans, low antithrombin is established risk factor for thrombosis and the risk of cardiac events was positively correlated to fibrinogen and negatively correlated to antithrombin III activity measurements [28]. In previous studies of canine CVD leading to congestive heart failure (CHF), AT III were decreased in dogs with CVD [29]. Natural anticoagulant mechanisms are amplified to prevent excessive thrombin generation. AT III, as part of the most important anticoagulant pathway, inhibit fibrinogen conversion into plasmin by creating thrombin-antithrombin complexes. Low AT III levels are caused by excessive thrombin generation and thereby increased consumption of the inhibitor leads to hypercoagulability in CVD.

Alpha-2-antiplasmin is a major inhibitor and regulator of fibrinolysis and one of the essential factors involved in haemostasis. It is a member of the serine proteinase inhibitor (serpin) family and inhibits proteases in general, including trypsin, chymotrypsin, plasma kallikrein, but its main physiological activity is very rapid inhibition of plasmin by forming a stable complex with this proteinase [30]. Lower protein abundances of alpha-2-antiplasmin found in CVD group may suggest fibrinolysis inhibitors consumption and increased fibrinolytic activity due to hypercoagulable state present in disease. Reported alterations in haematologic parameters would shift an overall haemostatic balance toward a more hypercoagulable state in the dogs with CVD.

Another regulator of fibrinolysis, tetranectin, was found to be decreased in our study in dogs with CVD compared to controls. Tetranectin is a C-type lectin and an adhesion molecule found on endothelial cells and platelets that specifically binds to the plasminogen kringle 4 domain, thereby enhancing plasminogen activation and inhibits the proliferation of endothelial cells [31]. A recent proteomics study discovered that the serum level of tetranectin was among the predictors of atherosclerotic cardiovascular disease after adjusting for established risk factors, with tetranectin levels inversely correlated with the risk of atherosclerotic cardiovascular disease [17]. Population studies have shown that decreased plasma tetranectin levels are also associated with coronary artery disease [32] and acute myocardial infarction [33]. These studies in humans are in agreement with the result obtained in this research, suggesting that changes of coagulation and fibrinolysis system play a vital role in pathophysiology of CVD. It is possible that the downregulation of tetranectin is due to its fibrinolytic property in thrombus breakdown.

Kininogen-1, component of a coagulation system, is the precursor protein to high-molecular-weight kininogen (HMWK), low-molecular-weight kininogen (LMWK), and bradykinin. Kinins are generated from HMWK and LMWK by kininogenases such as plasma and tissue kallikrein. A local kallikein-kinin system exists in the heart, which enables it to synthesize and release kinins [34]. Kinins released locally may act as autocrine/paracrine hormones, regulating cardiac function. The contact activation system of the intrinsic pathway of coagulation consists of four plasma proteins: factor XII (FXII), factor XI (FXI), prekallikrein (PK) and HMWK. Previous study showed increased concentrations of FXI, HMWK and PK in patients with a history of myocardial infarction as compared to controls, suggesting that high PK plasma levels may favor contact activation resulting in increased generation of activated FXII and FXI, leading to enhanced activation of the intrinsic pathway of coagulation (FIX) and subsequent thrombin formation [35]. These data indicate that possibly due to increased cleavage of kininogen-1 in activated coagulation system during progression of CVD, we found decreased levels of this precursor.

Lipoproteins in blood play an important physiological role transporting cholesterol, lipids and lipid-soluble substances to the different organs of the body. Diseases such as atherosclerosis, cardiovascular disease and stroke are associated with defects in lipoprotein metabolism. Apolipoproteins are the best lipid-related predictors to cardiovascular diseases [36]. Measurements of apolipoproteins are internationally standardized, automated, cost-effective and more convenient and precise than those for LDL cholesterol. Apolipoproteins, especially apoB, could also replace the standard 'lipid profile' as a target for therapy in at-risk patients [36].

Apolipoprotein B-100 (apoB) is the chief protein component constituent of the atherogenic very-low-density lipoprotein (VLDL), of intermediate-density lipoprotein (IDL) and of LDL particles, each particle including one apoB molecule [37]. ApoM is predominantly found in the HDL fraction and to a smaller extent in LDL, VLDL and chylomicrons. ApoM levels were shown to be dramatically reduced in patients with sepsis and systemic inflammatory response syndromes (SIRS) acting as a negative acute phase protein [38]. Apolipoprotein D (apoD) is a component of HDL.

In our study, protein abundances of all 3 apolipoproteins found (apoB-100, apoM and apoD) were lower in CVD group compared to healthy dogs, which was further validated by independent immunoassay for apoB-100 and apoD. These data were not consistent with findings in human medicine. Possible explanation for this is significant variations of plasma lipoprotein profiles among different animal species. In dogs and cats, HDL is the predominant lipoprotein and major cholesterol-carrying particle, with additionally quite different distribution than in humans. Dogs have five to six times as much HDL as LDL, whereas in humans LDL is 2 to 3 times as much as HDL [39,40]. In case of apoM, which has been suggested as novel negative acute phase protein, its decrease could be contributed to possible underlying inflammatory process in CVD. In affected canine valves several inflammatory cytokine genes were up-regulated suggesting valve endothelium as a source of inflammatory mediators [25].

To review the current status of proteomic biomarkers associated with cardiovascular diseases in humans, comprehensive meta-analysis was recently conducted, summarizing original research articles using proteomics technologies [41]. Identified proteins associated with cardiovascular disease represented pathways in inflammation, wound healing and coagulation, proteolysis and extracellular matrix organization, handling of cholesterol and LDL. Our proteomic and bioinformatics analysis resulted in interactome with enriched GO terms showing high consistency with results of this meta-analysis. The analysis of interaction biological processes by GO terms has identified the processes most affected by CVD were wound healing and extracellular structure organization. Lipid metabolism and transport are also important, as they were presented with different related GO terms like lipid homeostasis, cholesterol homeostasis, regulation of plasma lipoprotein particle levels, retinoid metabolic process. Adiponectin was associated with multiple GO terms, suggesting his importance in this disease.

A prospective study in cardiovascular disease initiative using discovery and targeted proteomic studies, identified single protein biomarkers and panel of proteins that were associated with risk of myocardial infarction or atherosclerotic cardiovascular diseases ASCVD [17]. Two proteins from our list, tetranectin and gelsolin, were also highlighted as novel biomarkers of new-onset cardiovascular disease. Furthermore, another large study using a proteomic platform identified tetranectin as protein biomarker that predicts cardiovascular outcomes and all-cause mortality [42].

In addition to the proteomic analysis, two potential biomarkers of cardiac function in dogs (cTnI and NT-proBNP) were tested herein. Both cTnI and NT-proBNP were found to be prognostic markers for dogs with CVD at the highest risk of death [43,44]. Cardiac troponin I is a protein specifically produced in the cardiomyocytes which is being rapidly released from the injured heart cells into the bloodstream. Since it is heart-specific, has low basal plasma concentration and persists in the circulation during the injury, it is commonly used as a biomarker of

myocardial injury in humans [45]. Serum cTnI levels were found to be significantly increased in dogs with CVD in our study, which was already demonstrated before [46,47]. Limitations of cTnI as a biomarker for CVD include its lack of specificity for the cardiac disease, elevation if kidney damage is present and possibly normal levels when the disease is mild [48]. As well as cTnI, NT-proBNP is gaining interest as a biomarker of canine heart diseases over the past few years. B-type natriuretic peptide (BNP) is a hormone secreted from cardiomyocytes due to myocardial overload and strain of the cells, in order to promote natriuresis, diuresis and vasodilatation. Once secreted, BNP is cleaved by serum proteases and gives rise to NT-proBNP, a stable biomarker of myocardium overload [49]. In several studies of dogs with CVD, there were increased levels of NT-proBNP compared to controls [50–52], which was also confirmed in our study. Nevertheless, new studies show that biologic variability should be taken into account when interpreting changes in both cTnI and NT-proBN*P* values in dogs with CVD [52,53].

Proteins validated with immunoassays and Western blotting showed results consistent to proteomics. Differences in values of fold changes between the immuno-based measurements and the fold changes found by proteomics could be related to the sensitivity of TMT-based LC-MS technology. Numerous studies have been conducted in order to compare the performance of immunoassays and LC-MS methodology, favoring the LC-MS in terms of accuracy and less false results. However, the results of those methodologies are reported as positively correlated [11]. As there are commercially available ELISA kits for some of the proteins listed as potential biomarkers for CVD, further evaluation of their prognostic value would be of interest. As limitations of the study, we can address limited number of cases and differences regarding sex, breed and age between healthy dogs and dogs with CVD. Low number of animals could be justified by demand for complete diagnostic investigation, while inadequate crossmatching is also a result of disease occurring in dogs at an advanced age.

## 5. Conclusions

Label-based high-resolution quantitative proteomics analysis and bioinformatics approach used herein represent a valid tool for elucidating complex pathophysiology of canine CVD and unveiling disease relevant proteins with biomarker potential. Early diagnosis and therapeutic intervention may prevent severe complications in dogs with CVD. Therefore, it is crucial to identify biomarkers that can be used in clinical practice. We found comparative similarities with human disease in terms of identified proteins and GO pathways, which confirmed similar pathophysiology of this disease, but also differences, mainly in lipid metabolism. Proteins associated with extracellular structure organization, haemostasis pathway and lipoprotein metabolism were identified as potential markers in canine CVD.

## Author contributions

JK wrote the paper and participated in the MS data analysis and statistical analysis.

PB participated in the development or design of experiments and methodology.

AH participated in the MS data analysis and statistical analysis and formal analysis.

AK participated in the development or design of experiments and wrote the paper.

NG participated in the MS data analysis and statistical analysis and formal analysis.

BBLJ participated in the laboratory analysis.

AG participated in the laboratory analysis.

IJ participated in the development experiments.

MT participated in the development experiments.

IR participated in the development experiments.

PDE participated in the editing of the paper.

VM participated in the conceptualization, methodology and writing – review and editing.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103825.

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PRILOG 6

Tablica 2. Potencijalni biomarkeri za poremećaj funkcije bubrega otkriveni u serumu i urinu pasa s babeziozom te mehanizmi s kojima su povezani. Kratice: TMT-LC-MS/MS - relativna kvantifikacija proteina pomoću TMT izobarnih privjesaka i vezanog sustava tekućinske kromatografije i tandemne spektrometrije masa; S – serum, U – urin (strelica označava povećanu ili smanjenu razinu u oboljelih pasa u odnosu na zdrave; A/Z – skupina A pasa s babeziozom vs. zdravi, B/Z – skupina B pasa s babeziozom vs. zdravi, C/Z - skupina C pasa s babeziozom vs. zdravi, BAB/Z – psi s babeziozom vs. zdravi; *-statistički značajna razlika, NS -neznačajna razlika.

| PROTEIN | METODA | UZORAK | A/Z | B/Z | C/Z | MEHANIZAM |
|---|---|---|---|---|---|---|
| leucinom-bogati alfa-2-glikoprotein 1 | ▪ TMT-LC-MS/MS | S↑ | * | * | * | imunosni odgovor |
| | | U↑ | * | * | * | |
| alfa-1-kiseli glikoprotein | ▪ TMT-LC-MS/MS<br>▪ ELISA | S↑ | * | * | * | imunosni odgovor |
| | | U↑ | * | * | * | |
| retinol-vezujući protein | ▪ TMT-LC-MS/MS<br>▪ ELISA | S↓ | * | * | * | imunosni odgovor/ metabolizam retinoida |
| | | U↑ | NS | * | * | |
| albumin | ▪ TMT-LC-MS/MS<br>▪ turbidimetrija | U↑ | * | * | * | imunosni odgovor |
| N-acetil-glukozaminidaza | ▪ enzimatska kolorimetrija | U↑ | * BAB/Z | | | lizosomska razgradnja |
| imunoglobulin G | ▪ TMT-LC-MS/MS<br>▪ ELISA | U↑ | * | * | * | imunosni odgovor |
| vitamin D-vezujući protein | ▪ TMT-LC-MS/MS | U↑ | * | * | * | transport vitamina D |
| beta-2-mikroglobulin | ▪ TMT-LC-MS/MS | U↑ | * | * | * | imunosni odgovor |
| uromodulin | ▪ TMT-LC-MS/MS | U↓ | * | * | * | imunosni odgovor |
| molekula bubrežne ozljede-1 | ▪ ELISA | U↑ | * | * | * | imunosni odgovor |
| lipokalin udružen s neutrofilnom gelatinazom | ▪ TMT-LC-MS/MS<br>▪ ELISA | U↑ | * | * | * | imunosni odgovor |

| PROTEIN | METODA | UZORAK | A/Z | B/Z | C/Z | MEHANIZAM |
|---|---|---|---|---|---|---|
| adipocitni protein koji veže masne kiseline | ▪ TMT-LC-MS/MS | U↑ | NS | * | * | metabolizam lipida/odgovor na oksidativni stres |
| jetreni protein koji veže masne kiseline | ▪ TMT-LC-MS/MS<br>▪ ELISA | U↑ | * | * | * | metabolizam lipida/odgovor na oksidativni stres |
| srčani protein koji veže masne kiseline | ▪ TMT-LC-MS/MS | U↑ | * | * | * | metabolizam lipida/odgovor na oksidativni stres |

PRILOG 7

Tablica 3. Potencijalni biomarkeri za poremećaj funkcije srca otkriveni u serumu pasa te mehanizmi s kojima su povezani. Kratice: TMT-LC-MS/MS - relativna kvantifikacija proteina pomoću TMT izobarnih privjesaka i vezanog sustava tekućinske kromatografije i tandemne spektrometrije masa; S – serum (strelica označava povećanu ili smanjenu razinu u oboljelih pasa u odnosu na zdrave; iDKM – idiopatska dilatativna kardiomiopatija; KBSZ – kronična bolest srčanih zalistaka.

| PROTEIN | METODA | UZORAK | BOLEST SRCA | MEHANIZAM |
|---|---|---|---|---|
| protein povezan s mikrofibrilima 4 | ▪ TMT-LC-MS/MS<br>▪ Western blot | S↑ | iDKM | remodeliranje izvanstaničnog matriksa |
| apolipoprotein A4 | ▪ TMT-LC-MS/MS<br>▪ Western blot | S↓ | iDKM | metabolizam lipoproteina |
| teški lanac 3 inter-alfa-tripsin inhibitora | ▪ TMT-LC-MS/MS<br>▪ Western blot | S↑ | iDKM | imunosni odgovor/ remodeliranje izvanstaničnog matriksa |
| teški lanac 4 inter-alfa-tripsin inhibitora | ▪ TMT-LC-MS/MS<br>▪ Western blot | S↑ | iDKM | imunosni odgovor/ remodeliranje izvanstaničnog matriksa |
| teški lanac 1 inter-alfa-tripsin inhibitora | ▪ TMT-LC-MS/MS | S↓ | KBSZ | imunosni odgovor/ remodeliranje izvanstaničnog matriksa |
| adiponektin | ▪ TMT-LC-MS/MS<br>▪ ELISA<br>▪ Western blot | S↓ | KBSZ | imunosni odgovor |
| tetranektin | ▪ TMT-LC-MS/MS | S↓ | KBSZ | regulacija hemostaze |
| gelsolin | ▪ TMT-LC-MS/MS | S↓ | KBSZ | remodeliranje citoskeleta |
| apolipoprotein B-100 | ▪ TMT-LC-MS/MS<br>▪ ELISA | S↓ | KBSZ | metabolizam lipoproteina |
| apolipoprotein D | ▪ TMT-LC-MS/MS<br>▪ ELISA | S↓ | KBSZ | metabolizam lipoproteina |

# 6. ŽIVOTOPIS AUTORA

Petra Bilić (djevojački Nižić) rođena je u 02.06.1989. u Zagrebu, gdje je završila osnovnoškolsko i srednjoškolsko gimnazijsko obrazovanje. Godine 2008. upisala je Preddiplomski studij molekularne biologije na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu, kojeg završava 2011. godine. Diplomski studij molekularne biologije na istom fakultetu završila je 2013. godine, stekavši akademski naziv magistre molekularne biologije. Tijekom studija bila je dobitnica Rektorove nagrade za studentski znanstveni rad (2012.) te više stipendija (Stipendije Ministarstva znanosti, obrazovanja i sporta, Stipendije Nacionalne zaklade za izvrsne studente te Stipendije Grada Zagreba za izvrsnost).

Svoju profesionalnu karijeru započela je 2014. godine u tvrtki *Primevigilance* d.o.o. kao suradnica za medicinske informacije u farmakovigilanciji. Od 2015. do 2021. godine bila je zaposlena kao viša stručna suradnica u sustavu znanosti i visokom obrazovanju na Veterinarskom fakultetu Sveučilišta u Zagrebu. U akademskoj godini 2014./2015. upisuje Poslijediplomski studij biologije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu. Tijekom rada na Veterinarskom fakultetu aktivno je sudjelovala u provedbi znanstveno-istraživačkih projekata u području proteomike, sudjelovala na međunarodnim konferencijama te objavljivala izvorne znanstvene i pregledne radove. Objavila je 11 znanstvenih radova citiranih u bazi Web of Science, od toga 9 radova u časopisima s čimbenikom odjeka Q1/Q2. Radovi su prema bazi Web of Science citirani ukupno 187 puta uz h-indeks 8. Objavila je i 12 kongresnih priopćenja. Primila je službeno Priznanje Veterinarskog fakulteta za znanstveni rad s prvim autorstvom objavljen u časopisu s najvišim čimbenikom odjeka u akademskoj godini 2017./2018. Znanstveno usavršavanje u inozemstvu ostvarila je kroz tromjesečni istraživački boravak na Sveučilištu u Glasgowu (UK), uz potporu stipendije zaklade *British Scholarship Trust* te kroz dvomjesečni boravak na Sveučilištu veterinarske medicine i farmacije u Košicama (Slovačka).

Od 2022. godine zaposlena je u Ministarstvu unutarnjih poslova, u Ravnateljstvu civilne zaštite unutar Sektora za radiološku i nuklearnu sigurnost. Njezin rad u Sektoru obuhvaća stručne poslove vezane uz radiološku i nuklearnu sigurnost, uključujući praćenje stanja te primjenu mjera zaštite.

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