UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

PERFIL PROTEÔMICO DO LÍQUIDO CEFALORRAQUIDIANO APÓS TRANSPLANTES INTRATECAL DE CÉLULAS ESTROMAIS MESENQUIMAIS MULTIPOTENTES EM EQUINOS

DENIS JERONIMO SVICERO

Botucatu, SP
Fevereiro - 2019

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Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor.

Orientador: Prof. Ass. Dr. Rogério Martins Amorim

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LISTA DE ABREVIATURAS

2-DE - Eletroforese bidimensional em gel
AD - Tecido adiposo
AD-MSCs - MSCs derivadas do tecido adiposo
AD-G - Grupo tecido adiposo
Apo-B100 - Apolipoproteína B-100
APOA - Apolipoproteínas
BDNF - Fator neurotrófico derivado do cérebro
BHE - Barreira hematoencefálica
BM - Medula óssea
BM-G - Grupo medula óssea
BM-MSCs - MSCs derivadas da medula óssea
CD - Cluster differentiation
CNS - Sistema nervoso central
Control - Grupo controle
CSF - Líquido cefalorraquidiano
DPBS - Solução salina tamponada com fosfato Dulbecco's ou grupo DPBS
EHV-1- Herpesvírus tipo 1
ELISA - Ensaio de imunoabsorção enzimática
EPM - Mieloencefalite protozoária equina
EPM group - Grupo EPM
HGF - Fator de crescimento de hepatócitos
IA- intraarterial
IDO - Indoleamina 2,3-dioxigenase
Ig - Imunoglobulina
IL 6 - Interleucina 6
IT - Via intratecal
IV - Via intravenosa
M0 ou Before - Antes do primeiro tratamento
M90 ou After - 30 dias após o terceiro tratamento

MHC-II- Complexo de histocompatibilidade principal classe II
MSCs - Células estromais mesenquimais multipotentes
MUE - Meningoencefalite de etiologia desconhecida
NCBI - National Center for Biotechnology Information
NGF - Fator de crescimento nervoso
NO - Óxido nítrico
NSE - Proteína enolase neurônio-específica
NSE (ENO-2) - Proteína enolase neurônio-específica de isoforma do tipo γ-enolase
NT-3 - Neurotrofina 3
PFA- Proteínas de fase aguda
PGE 2 - Prostaglandina E2
RIA - Radioimunoensaios
RIFI - Reação de imunofluorescência indireta
SAG - de antígenos de superfície
SVCM - Mielopatia estenótica vertebral cervical
TGF β - Fator de crescimento transformador beta
Treg - Células T reguladoras
UC - Cordão umbilical
UC-MSCs - MSCs derivadas do cordão umbilical
VEGF - Fator de crescimento endotelial vascular
WB - Western blot

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SVICERO, D.J. **Perfil proteômico do líquido cefalorraquidiano após transplantes intratecal de células estromais mesenquimais multipotentes em equinos**. Botucatu, 2019. 118 p. tese (Doutorado) - Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

Estudos com células estromais mesenquimais multipotentes (MSCs) estão em crescente progresso devido às suas propriedades imunomoduladoras, antiinflamatórias, antiapoptóticas e de regeneração tecidual, tornando essa modalidade de terapia celular promissora no tratamento de diversas doenças. Devido à limitada capacidade regenerativa do sistema nervoso central (CNS), causando sequelas funcionais, as MSCs estão sendo investigadas como uma alternativa terapêutica para condições neurológicas inflamatórias, vasculares, traumáticas e degenerativas em diversas espécies animais. A Mieloencefalite protozoária equina (EPM) causada por ambos os protozoários do filo Apicomplexa, *Sarcocystis neurona* e *Neospora hughesi*, permanece como uma importante doença neurológica dos equinos nas Américas, embora a maioria dos casos seja devida à infecção por *S. neurona*. A aplicação da proteômica com sua gama de ferramentas na clínica de equinos pode contribuir significativamente para o entendimento de processos patológicos e facilitar a descoberta de novos alvos terapêuticos ou marcadores diagnósticos. Neste contexto, os objetivos deste estudo foram avaliar o perfil proteômico do líquido cefalorraquidiano (CSF) antes e após múltiplos transplantes intratecal de MSCs em equinos hígidos e o perfil proteômico do CSF de equinos cronicamente afetados pela EPM. Doze cavalos adultos clinicamente saudáveis foram divididos aleatoriamente em três grupos experimentais: grupo DPBS (DPBS ou control; n = 4) onde a solução salina tamponada com fosfato de Dulbecco's (DPBS) foi administrada pela via intratecal; grupo AD-G (AD-G; n = 4), no qual foram realizados transplantes intratecal com MSCs alogênicas de tecido adiposo; e grupo BM-G (BM-G; n = 4), no qual foram realizados transplantes intratecal com MSCs alogênicas de medula óssea. Todos os grupos experimentais receberam três tratamentos seriados (DPBS ou MSCs) com intervalo de 30 dias entre eles. As amostras de CSF foram coletadas dos grupos experimentais imediatamente antes do primeiro tratamento (denominado momento M0 ou Before) e 30 dias após o terceiro tratamento (denominado momento M90 ou After). Ao mesmo tempo, os CSF de 16 equinos clinicamente saudáveis e de 9 equinos cronicamente afetados por EPM foram coletados

da subaracnóide, denominados grupo Control (Control; n = 16) e grupo EPM (EPM; n = 9) respectivamente. Cada uma das amostras de CSF do grupo Control e do grupo EPM foram individualmente ressuspendidas e agrupadas em *pool*, totalizando dois *pool* representativos de cada um dos grupos experimentais e denominados igualmente (Control e EPM) para avaliar o perfil proteômico dos mesmos. De modo similar, os três grupos que receberam transplantes e coletas de CSF nos dois distintos momentos, foram agrupados em seis *pool* representativos para cada grupo e momento. Considerando a plataforma proteômica utilizada, os três grupos foram pareadamente comparados: DPBS After *vs*. Before, AD-G After *vs*. Before e BM-G After *vs*. Before, sendo encontradas 208, 211 e 131 proteínas em cada comparação, respectivamente. Do mesmo modo, na comparação pareada do CSF dos grupos EPM e Control, EPM *vs*. Control, 201 proteínas foram identificadas e 33 proteínas observadas exclusivamente no CSF do grupo EPM. Ademais, também foi observada na comparação pareada dos grupos DPBS, BM-G e EPM, a presença dos três tipos de enolases interagindo entre si (ENO 1, ENO 2 e ENO 3), sendo em DPBS e BM-G como exclusivas do momento After e no grupo EPM como exclusivas dele. Neste contexto, este estudo ao avaliar os perfis proteômico do CSF antes e após múltiplos transplantes intratecal de MSCs em equinos hígidos e do CSF de equinos cronicamente afetados pela EPM permitiu identificar as enolases como potenciais marcadores de lesão neural e/ou de EPM. Novos estudos devem ser realizados para confirmar estes achados e avançar no conhecimento dos efeitos da terapia celular com MSCs pela via intratecal, para o tratamento de lesões neurológicas em equinos.

Palavras-chave: Células estromais mesenquimais multipotentes, terapia celular, proteômica, líquido cefalorraquidiano, Mieloencefalite protozoária equina.

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ABSTRACT

Multipotent mesenchymal stromal cell (MSCs) studies are under increasing progress because of their immunomodulatory, anti-inflammatory, antiapoptotic and tissue regeneration properties, making this modality of cell therapy promising in the treatment of various diseases. Due to the limited regenerative capacity of the central nervous system (CNS), causing functional sequelae, MSCs are being investigated as a therapeutic alternative for inflammatory, vascular, traumatic and degenerative neurological conditions in various animal species. Equine protozoal myeloencephalitis (EPM) caused by both protozoa of the Apicomplexa phylum, *Sarcocystis neurona* and *Neospora hughesi*, remains an important neurological disease in horses in the Americas, although most cases are due to *S. neurona* infection. The application of proteomics with its range of tools in the equine clinic can contribute significantly to the understanding of pathological processes and facilitate the discovery of new therapeutic targets or diagnostic markers. In this context, the objectives of this study were to evaluate the proteomic profiling of cerebrospinal fluid (CSF) before and after multiple intrathecal transplantations of MSCs in healthy horses and the CSF proteomic profiling of horses chronically affected by EPM. Twelve clinically healthy adult horses were randomly divided into three experimental groups: DPBS (DPBS or control; n = 4), in which intrathecal "transplants" with Dulbecco's phosphate buffered saline (DPBS) were performed; group AD-G (AD-Gs; n = 4), in which intrathecal transplants were performed with allogeneic adipose tissue MSCs; and BM-G group (BM-G; n = 4), in which intrathecal transplants were performed with allogeneic bone marrow MSCs. All experimental groups received three serial treatments (DPBS or MSCs) with a 30-day interval between them. CSF samples were collected from the experimental groups immediately before the first treatment (called the M0 or Before) and 30 days after the third treatment (called the M90 or After). At the same time, CSF of 16 healthy horses and 9 horses chronically affected by EPM were collected from the subarachnoid, Control group (Control; n = 16) and EPM group (EPM; n = 9) respectively. Each of the CSF samples from the Control group and from the EPM group were individually

resuspended and pooled, totalizing two representative pools of each one the experimental groups and also named (Control and EPM) to evaluate their proteomic profilings. Similarly, the three groups that received transplants and CSF collections at two different times were grouped into six representative pools for each group and time. Considering the proteomic platform used, the three groups were similarly compared: DPBS After *vs*. Before, AD-G After *vs*. Before and BM-G After *vs*. Before, being found 208, 211 and 131 proteins in each comparison, respectively. Likewise, in the paired comparison of the CSF of the EPM and Control groups, EPM *vs*. Control, 201 proteins were identified, and 33 proteins were observed exclusively in the CSF of the EPM group. In addition, the presence of the three types of enolases interacting with each other (ENO 1, ENO 2 and ENO 3) was also observed in the comparison of the DPBS, BM-G and EPM groups, being exclusives in DPBS and BM-G at time After and in the EPM group as its exclusively. In this context, this study, when evaluating the proteomic profilings of CSF before and after multiple intrathecal transplantations of MSCs in healthy horses and CSF of horses chronically affected by EPM, allowed the identification of enolases as potential markers of neural and / or EPM injury. Further studies should be performed to confirm these findings and to advance the knowledge of the effects of intrathecal cell therapy with MSCs for the treatment of neurological lesions in horses.

Key words: Multipotent mesenchymal stromal cells, cell-based therapy, proteomics, cerebrospinal fluid, Equine protozoal myeloencephalitis.

CAPÍTULO 1

1 INTRODUÇÃO

Estudos com células estromais mesenquimais multipotentes (MSCs) estão em crescente avanço devido às suas propriedades imunomodulatórias, antiinflamatórias e de regeneração tecidual, tornando esta modalidade de terapia celular promissora no tratamento de diversas enfermidades. (BURK et al., 2013; CARRADE et al., 2012; CHAMBERLAIN et al., 2007; GUTIERREZ-NIBEYRO, 2011; NÖTH et al., 2010). Devido a limitada capacidade do sistema nervoso central (CNS) se regenerar, as MSCs estão sendo investigadas como alternativa terapêutica para condições neurológicas inflamatórias, vasculares, traumáticas e degenerativas (KARUSSIS; PETROU; KASSIS, 2013).

Os mecanismos pelos quais as MSCs induzem efeitos positivos no tecido nervoso lesionado ainda não foram completamente elucidados. Alguns dos mecanismos que podem ter um papel importante na neurorregeneração e neuroproteção incluem a secreção de fatores de crescimento neurotróficos, anti-apoptóticos e, assim como, de citocinas antiinflamatórias e proteínas de matriz extracelular (JONES; MCTAGGART, 2018).

Estudos têm demonstrado o potencial terapêutico das MSCs em tratar doenças do sistema nervoso como Alzheimer, esclerose múltipla e lesões de medula espinhal, mostrando efeitos benéficos (CHENG et al., 2015; COHEN, 2013; HYATT et al., 2014; KARUSSIS; PETROU; KASSIS, 2013; LINDVALL; KOKAIA, 2006; MAZZINI et al., 2010; PENHA et al., 2014; UCCELLI et al., 2011).

Há diversas enfermidades neurológicas que afetam os equinos, cujos tratamentos preconizados não são totalmente eficientes e acarretam em sequelas, como na mieloencefalite protozoária equina (EPM) e a mielopatia estenótica vertebral cervical (CVSM) (DIRIKOLU; FOREMAN; TOBIN, 2013; REED; GRANT; NOUT, 2008). Neste contexto a terapia com MSCs poderia trazer benefícios no tratamento destas enfermidades. Contudo para que esta biotecnologia saia da bancada dos laboratórios para a rotina clínica, faz-se necessário estudos clínicos de segurança e eficácia, levando-se em consideração a enfermidade a ser tratada, o tipo celular, a via de transplante, a dose e a frequência dos tratamentos.

Já existem dados indicando que tanto as MSCs autólogas quanto as alogênicas são bem toleradas em equinos quando administradas pelas vias intravenosa (IV), intra-

articular, intra/perilesional e intratecal (IT) (BARBERINI, 2017; CARRADE et al., 2011a; MAIA et al., 2015; PIGOTT et al., 2013). As MSCs alogênicas são ideais para o tratamento de lesões agudas onde a expansão celular de MSCs autólogas em cultura é uma limitação para o uso imediato destas células na terapia.

A ausência da expressão do complexo de histocompatibilidade principal classe II (MHC-II) na superfície das MSCs é uma importante característica imunomodulatória (BARBERINI et al., 2014). Esta ausência fornece às MSCs o potencial de escapar do reconhecimento das células T, fazendo com que a terapia alogênica seja possível (BORJESSON; PERONI, 2011; CARRADE et al., 2011b; RYAN et al., 2005; SOLE et al., 2012). Por outro lado, estudos têm revelado que as MSCs alogênicas quando transplantadas podem elicitar uma resposta humoral no hospedeiro, assim como expressar MHC-II na presença de interferon gama, acarretando em diminuição do tempo de sobrevida celular (SCHNABEL et al., 2014; PEZZANITE et al., 2015).

Inúmeros estudos descrevem aspectos morfológicos, de capacidade de proliferação, diferenciação celular, imunofenotipagem e propriedades terapêuticas das MSCs provenientes de diferentes fontes teciduais. Contudo, até o momento não está estabelecido um consenso de qual é a melhor fonte tecidual para obtenção das MSCs em equinos com finalidades terapêuticas (BARBERINI et al., 2015).

Pelo nosso conhecimento não existem estudos sobre o perfil proteômico do líquido cefalorraquidiano (CSF) de equinos após múltiplos transplantes intratecal de MSCs alogênicas provenientes do tecido adiposo (AD-MSCs) e MSCs alogênicas provenientes da medula óssea (BM-MSCs). Além disso, desconhecemos a existência de estudos sobre o perfil proteômico do CSF de equinos acometidos por EPM.

A análise proteômica do CSF de equinos, sadios ou com EPM, que receberam múltiplos transplantes intratecal de MSCs poderá revelar quais vias metabólicas e/ou potenciais biomarcadores estão diferencialmente expressos antes e após os transplantes celulares.

Os resultados obtidos com esta abordagem poderão contribuir tanto com o entendimento dos mecanismos de ação das MSCs no processo de neuroregeneração do CNS dos equinos, como com o desenvolvimento de um protocolo de terapia celular seguro e eficiente

2 REVISÃO DE LITERATURA

2.1 Células estromais mesenquimais multipotentes (MSCs)

As MSCs são células progenitoras multipotentes, não hematopoiéticas, facilmente isoladas a partir de diversos tecidos adultos, e caracterizadas por sua extensa habilidade proliferativa e de diferenciação *in vitr*o em diversas linhagens mesenquimais em resposta a um estímulo apropriado, incluindo osteoblastos, adipócitos, condrócitos, tenócitos e miócitos (CHAMBERLAIN et al., 2007; NIXON et al., 2008; NÖTH et al., 2010).

Para a caracterização imunofenotípica das MSCs em equinos, a aderência ao plástico, a diferenciação nas linhagens osteogênica, condrogênica e adipogênica, a expressão dos marcadores CD90, CD44, CD105, CD29, e a não expressão dos marcadores CD34, CD14, CD79α e MHC-II, são as propriedades disponíveis mais confiáveis para a caracterização das MSCs equinas (BARBERINI et al., 2014; CARVALHO et al., 2011; DE SCHAUWER et al., 2012).

Diversas fontes podem ser utilizadas para obtenção de MSCs, como sangue do cordão umbilical (BARTHOLOMEW et al., 2009), tecido adiposo (AD) (CARVALHO et al., 2011; MAMBELLI et al., 2009), medula óssea (BM) (BOURZAC et al., 2010; RANERA et al., 2011), tecido do cordão umbilical (UC) (HOYNOWSKI et al., 2007), membrana amniótica (LANGE-CONSIGLIO et al., 2013), sangue periférico (KOERNER et al., 2006) e tendão (BURK et al., 2013). Sendo que, a BM e o AD são as fontes mais comuns de obtenção de MSCs (GUTIERREZ-NIBEYRO, 2011; KOERNER et al., 2006; RICHARDSON et al., 2007).

Normalmente, as MSCs são obtidas dos próprios pacientes (autólogas) (RICHARDSON et al., 2007), porém, sua utilização para o tratamento possui limitações, como em lesões agudas, pois a expansão das MSCs através da cultura pode demorar de 10 a 21 dias (CARRADE et al., 2011a), e em pacientes idosos, pois há uma diminuição na quantidade, na proliferação e no potencial de diferenciação das MSCs proporcionalmente à idade (ALT et al., 2012).

Estudos realizados em diversas espécies demonstram que o uso alogênico de MSCs para aplicações clínicas é possível, indiferentemente do tecido de origem, pois estas células possuem baixa imunogenicidade *in vitro*, e podem escapar do processo normal de reconhecimento de aloantígenos (CARRADE et al., 2011b; RYAN et al., 2005). Devido a esta característica, as MSCs têm sido denominadas de imuno-

privilegiadas. Contudo, alguns estudos têm questionado este conceito (BERGLUND et al., 2017). A disponibilidade do uso de MSCs alogênicas em equinos, por meio de um banco de células, oferece a vantagem de se iniciar rapidamente o tratamento em casos de lesões agudas (BORJESSON; PERONI, 2011; BURK et al., 2013; CARRADE et al., 2011a, 2011b; PERONI; BORJESSON, 2011).

2.2 Utilização das MSCs para o tratamento de lesões do CNS

Lesões nervosas são particularmente incapacitantes, tanto para a espécie humana como nos animais domésticos. Por causa da capacidade regenerativa limitada do tecido neural, o potencial terapêutico do transplante de MSCs tem recentemente sido estudado em várias condições patológicas do CNS, como condições neurológicas degenerativas e inflamatórias crônicas, assim como de dano neuronal agudo (KARUSSIS; PETROU; KASSIS, 2013). Existem evidências de que as MSCs transplantadas no CNS migram para o tecido danificado e se diferenciam em células neurais, melhorando a função neurológica (CHEN et al., 2001; VAQUERO et al., 2006).

Embora as interações entre MSCs e a barreira hematoencefálica ainda não sejam completamente elucidadas, a utilização de MSCs pode desempenhar um papel importante na regulação das doenças inflamatórias de equinos, devido às suas atividades tróficas e sua capacidade em estimular angiogênese, reduzir inflamação, recrutar células progenitoras residentes e minimizar a fibrose (PERONI; BORJESSON, 2011; STEWART, 2011), podendo regenerar ou fornecer um suporte para a sobrevivência das células existentes, parcialmente danificadas (KARUSSIS; PETROU; KASSIS, 2013).

Vários distúrbios neurológicos têm levantado interesse particular para a terapia celular, de forma que muitos estudos em modelos experimentais e clínicos foram conduzidos (em ratos, camundongos, coelhos, cães e em humanos), tais como a doença de Parkinson, acidente vascular cerebral, doença de Huntington, esclerose lateral amiotrófica, doença de Alzheimer, esclerose múltipla e lesões da medula espinhal, mostrando efeitos benéficos (COHEN, 2013; KARUSSIS; PETROU; KASSIS, 2013; LINDVALL; KOKAIA, 2006).

2.2.1 Mecanismos de ação das MSCs

As MSCs possuem um potencial terapêutico enorme para enfermidades neurológicas, devido aos seus efeitos imunomodulatórios, relacionado com a secreção

do fator de crescimento de hepatócitos (HGF), fator de crescimento transformador beta (TGFβ), prostaglandina E2 (PGE2), indoleamina 2,3-dioxigenase (IDO), óxido nítrico (NO), fator de crescimento endotelial vascular (VEGF) e interleucina 6 (IL-6) (CARRADE; BORJESSON, 2013; KOTA et al., 2017), antiinflamatórios e de neuroproteção (RAVANIDIS et al., 2015; ROBINSON et al., 2017). Promovem o crescimento neuronal endógeno, diminuem a apoptose, reduzem os níveis de radicais livres, incentivam a conexão sináptica de neurônios danificados e regulam a inflamação, principalmente através de ações parácrinas (AL DELFI et al., 2016; JOYCE et al., 2010; KARUSSIS; PETROU; KASSIS, 2013). A secreção de fator neurotrófico derivado do cérebro (BDNF), fator de crescimento nervoso (NGF) e neurotrofina 3 (NT-3) são importantes efeitos terapêuticos das MSCs que promovem neurogênese e conexões sinápticas (CHUNG et al., 2016; SONG et al., 2015).

Foram relatadas que as células T reguladoras (Treg) possuem um papel importante além da imunomodulação, elas também promovem a diferenciação de oligodendrócitos e a (re) mielinização (DOMBROWSKI et al., 2017), uma descoberta importante visto que as MSCs agem estimulando as células Treg (LU et al., 2013; MELIEF et al., 2013).

Existem evidências de que as MSCs transplantadas no CNS migram para a área lesada, como áreas de hipóxia, apoptóticas, inflamadas, degeneradas e se diferenciam em células neurais, melhorando a função neurológica (CHEN et al., 2001; LEE et al., 2003; LIU et al., 2008; PAUL; ANISIMOV, 2013; VAQUERO et al., 2006; YANO et al., 2005). Embora a diferenciação das MSCs em células da linhagem neural não seja o fator principal na recuperação funcional após uma lesão cerebral ou medular (MALTMAN; HARDY; PRZYBORSKI, 2011; PARR; TATOR; KEATING, 2007), uma abordagem interessante seria a transdiferenciação das MSCs antes do transplante, aumentando assim o benefício terapêutico do tratamento, contribuindo para a remielinização de axônios lesados e, assim, na recuperação funcional da medula espinhal (CHELLUBOINA; DINH; VEERAVALLI, 2015).

Vários estudos têm demonstrado a segurança da aplicação intratecal com MSCs autólogas (CHEN et al., 2015; MAIA et al., 2015; SATTI et al., 2016; ZEIRA et al., 2015) e alogênicas (LIANG et al., 2009; VILLANOVA; BACH, 2015), mostrando efeitos benéficos. No entanto, um relato do caso de uma mulher de 27 anos de idade com mielite transversa aguda, demonstrou um episódio de encefalite e neurite óptica, seguido da doença desmielinizante encefalomielite disseminada aguda após transplantes de MSCs provenientes da MO (KISHK; ABOKRYSHA; GABR, 2013). Outro relato de

caso, de um homem de 66 anos de idade que sofreu múltiplas infusões comerciais de células-tronco (alogênicas, embrionárias e mesenquimais) demonstrou o desenvolvimento de uma neoplasia altamente proliferativa resultante da administração de células-tronco pluripotentes (SAAD et al., 2016).

2.2.2 Vias de transplante

Diversas vias de transplante de MSCs têm sido utilizadas para atingir o CNS, dentre elas a via intravenosa (IV), intra-arterial (IA) e intratecal (IT), intralesional e intranasal (GALEANO et al., 2018).

Em comparação com a administração sistêmica, as vias de transplante tópicas (local, intralesional) possuem a vantagem de que as MSCs chegam diretamente ao tecido alvo com pouca perda durante a migração. As MSCs administradas por via IV são mais facilmente presas em pequenos capilares pulmonares por causa de seu tamanho e expressão de moléculas de adesão celular, este aprisionamento pulmonar diminui o número de células entregues aos tecidos alvo e pode resultar em um tratamento ineficaz (ZHANG et al., 2015).

A distribuição das MSCs após a aplicação sistêmica pela via IV em camundongos demonstrou que em animais jovens as MSCs migraram para os pulmões, linfonodos axilares, sangue, rim, medula óssea, baço fígado, coração e córtex cerebral; em animais idosos, somente no córtex cerebral (FABIAN et al., 2017; SCHREPFER et al., 2007). Além disso, utilizando MSCs oriundas de animais idosos, a biodistribuição foi apenas em baço e sangue, o transplante em animais doentes (modelos transgênicos de doença de Alzheimer APP / PS1 (camundongos transgênicos que sobre-expressam proteína precursora amilóide humana (APPKM670 / 671NL) e presenilina-1 (PS1L166P) sob o controle do promotor Thy-1) teve uma distribuição neuronal grande no cérebro, sendo encontradas no córtex, cerebelo, hipocampo, bulbo olfatório e tronco encefálico; além de fígado, medula óssea e pulmão. Portanto, o envelhecimento do receptor e das MSCs afeta drasticamente a eficiência do transplante e da migração pós-transplante (FABIAN et al., 2017).

Outro trabalho em cães com sinais neurológicos multifocais (meningoencefalite de etiologia desconhecida (MUE)) comparando o uso das vias IT utilizada em conjunto com a via IA , e via IT em conjunto com a via IV; demonstrou que os transplantes por via IT e via IA associados possuem maior possibilidade de atingir o CNS do que por via IV, induzindo efeitos neurotróficos e neuroprotetores (ZEIRA et al., 2015).

Comparando as vias intraespinhal (centro da lesão) e IT (intratecal), um trabalho demonstrou que as MSCs transplantadas via intraespinhal podem promover a regeneração em longo prazo, enquanto que as MSCs transplantadas pela via IT possuem efeito através do mecanismo parácrino, que não exige que as células estejam presentes no tecido (AMEMORI et al., 2015).

Em um modelo de trauma cerebral, foram comparadas as vias IT lombar e IV, sendo que a IT demonstrou resultados mais eficientes (LIU et al., 2008). Outros trabalhos recentes também utilizam a via IT (HARRIS; VYSHKINA; SADIQ, 2016; VAQUERO et al., 2017).

2.2.3 Fontes de obtenção de MSCs e doses

Ainda não se sabe a melhor fonte ou dose para se utilizar na terapia celular visando efeitos benéficos no sistema nervoso.

Um estudo em pacientes com esclerose múltipla utilizou MSCs derivadas da medula óssea (BM-MSCs), e foram realizadas entre 2 e 5 transplantes pela via IT de doses crescentes. Com base na escala progressiva da dose, uma dose final de 2 a 10 milhões de células a cada 3 meses foi bem tolerada sem eventos adversos significativos (HARRIS; VYSHKINA; SADIQ, 2016).

No entanto, outro estudo com BM-MSCs em camundongos como modelo de esclerose múltipla, demonstrou que três transplantes pela via IT com intervalo de uma semana resultaram em melhora significativa dos sintomas da doença em comparação com uma única aplicação (HARRIS et al., 2012).

Para lesão em medula espinhal, um estudo com BM-MSCs em humano utilizou cinco transplantes a cada 3 e 4 meses pela via IT lombar, demonstrando melhorias clínicas significativas (JAROCHA et al., 2015).

Em cães, foi utilizado um único transplante de BM-MSCs na dose de 2 milhões de células pela via IT, 4 milhões pela via IA e de 500 mil células/kg pela via IV para meningoencefalomielite de causa desconhecida. Todos os cães apresentaram melhora em suas condições gerais e neurológicas, com efeito especial na dor cervical (ZEIRA et al., 2015).

Há ainda estudos com fontes de tecidos fetais, como Geléia de Wharton, relatando ser melhor que BM-MSCs *in vitro* (DRELA et al., 2016) e sangue do cordão umbilical (CHUNG et al., 2016), este último indicando o início do tratamento com 3 dias após a lesão na medula espinhal em ratos e que um transplante adicional após 3

semanas poderia induzir maiores efeitos protetores. Em camundongos, foram utilizadas MSCs derivadas do cordão umbilical (UC-MSCs) na dose de 2 milhões de células pela via IV para um modelo de doença de Alzheimer, demonstrando efeitos benéficos (CUI et al., 2017).

Em equinos foram utilizadas BM-MSCs para um estudo de segurança do transplante pela via IT através do espaço atlanto-occipital, na dose de 1 milhão de células por animal em uma única aplicação (MAIA et al., 2015).

2.3 Enfermidades neurológicas em equinos

As doenças neurológicas são muitas vezes fatais e são uma das razões mais comuns para a eutanásia em cavalos (RECH; BARROS, 2015). Diversas enfermidades neurológicas podem acometer os equinos, como a leucoencefalomalácia, EPM, CVSM, doença do neurônio motor, Herpesvírus tipo 1 (EHV-1) entre outras, que muitas vezes não possuem um tratamento efetivo e frequentemente os animais apresentam sequelas neurológicas. Neste contexto optamos por investigar um potencial marcador de lesão neural em equinos cronicamente afetados pela EPM, visto que é uma enfermidade comum no Brasil e que gera inúmeros prejuízos na indústria equina (LINS; FEIJÓ; NOGUEIRA, 2012; PEIXOTO et al., 2003; STELMANN; AMORIM, 2010).

2.3.1 Mieloencefalite protozoária equina (EPM)

A EPM é uma síndrome neurológica que acomete o CNS dos equinos, sendo causada pelos protozoários *Sarcocystis neurona* e *Neospora hughesi* (DUBEY et al., 2001; HOWE; MACKAY; REED, 2014; PUSTERLA et al., 2014). É uma doença infecciosa não contagiosa, endêmica nas Américas, tendo os equinos como hospedeiros acidentais, sendo de grande importância econômica (DIRIKOLU; FOREMAN; TOBIN, 2013; REED et al., 2016; WITONSKY et al., 2008).

A EPM induz lesões multifocais inflamatórias no CNS, os sinais clínicos podem ser agudos ou crônicos e desenvolvem-se de acordo com o local anatômico da infecção. Normalmente, há manifestações assimétricas da medula espinhal, atrofia muscular, ataxia, disfagia, fraqueza muscular, tropeço e inclinação da cabeça (DUBEY et al., 2001, 2015; REED et al., 2016). As lesões macroscópicas aparecem como focos necróticos vermelhos e descoloridos (hemorrágicos), as lesões microscópicas afetam tanto a substância cinzenta quanto a branca com necrose, hemorragia e reação

inflamatória consistindo de linfócitos, macrófagos, neutrófilos, eosinófilos e algumas células gigantes multinucleadas. Os merozoítos intracelulares podem ser observados em neurônios, células gigantes, neutrófilos ou macrófagos ou extracelularmente em cistos dentro do neurópilo (RECH; BARROS, 2015).

Nas últimas duas décadas, diversos testes sorológicos tornaram-se disponíveis para ajudar no diagnóstico de EPM causada por *S. neurona*, incluindo Western blot (WB), reação de imunofluorescência indireta (RIFI) e ensaio de imunoabsorção enzimática (ELISA) de antígenos de superfície (SAG). Todos podem ser realizados no soro e no CSF e nenhum é considerado o padrão ouro. A sensibilidade e especificidade de cada método, respectivamente, são de aproximadamente 89% e 80% para WB, 92% e 99% para RIFI, 88% e 96% para SAG ELISA (REED et al., 2016).

Para o tratamento da EPM, recomenda-se terapia com fármaco anti-protozoário (ponazuril, diclazuril ou sulfadiazina e pirimetamina) por tempo prolongado, sendo no mínimo de 28 dias, porém, a maioria dos cavalos com EPM são tratados por um período de 6 a 8 semanas ou mais, dependendo da resposta clínica (DUBEY et al., 2015; REED et al., 2016).

Um estudo mostrou que 65,2% dos cavalos com EPM que foram tratados melhoraram após o tratamento. A maioria dos animais com déficits neurológicos leves (74,1%) melhoraram após o tratamento, ao passo que um número menor de cavalos com moderados (57,8%) ou graves (50%) déficits neurológicos melhoraram após o tratamento (SAVILLE et al., 2000). Os cavalos podem apresentar recidiva clínica da EPM, mesmo após tratamento prolongado, com sinais clínicos semelhantes ao primeiro episódio. A causa desta recidiva ainda é desconhecida (DUBEY et al., 2015).

2.4 Proteômica

Compreender a função proteica é um objetivo primário da era de sequenciamento pós-genoma (HUBBARD, 2002). Para ser de maior valor, esse entendimento deve ser holístico e aplicar-se a uma variedade de configurações fisiopatológicas. A forte sensibilidade ao contexto do comportamento proteico requer conhecimento de redes de proteínas e sua dinâmica tempo-espacial *in vivo*, além de compreender as propriedades das proteínas individuais isoladamente. Deste modo, para identificar e entender essas diferenças é fundamental conhecer o conjunto de proteínas codificadas e expressas pelo genoma, denominado assim como proteoma (APPEL; BAIROCH, 2004; HUBBARD, 2002; JANSSEN, 2003).

O termo proteômica refere-se ao estudo do proteoma, e este não é apenas a soma dos produtos traduzidos a partir das sequências genômicas, mas inclui também as proteínas resultantes de processos pós-transcricionais; bem como os complexos formados por essas biomoléculas (JENSEN, 2004). Além de sua grande complexidade, o proteoma é extremamente dinâmico e seu perfil se altera nas células, nos tecidos e nos fluidos corporais em condições e/ou momentos distintos, de acordo com o estado fisiológico do organismo, enquanto o genoma de um organismo permanece relativamente estável ao longo de sua vida; assim, um único genoma pode gerar um número muito grande de proteomas (HUBBARD, 2002).

Técnicas poderosas tornam possível a triagem rápida da expressão de mRNA; no entanto, muitas vezes existe uma fraca correlação entre a concentração de mRNA e a quantidade de proteínas funcionais correspondentes. A proteômica é um campo científico emergente que envolve identificação, caracterização e quantificação de proteínas em uma célula, tecido ou fluido corporal. A ampla aplicação da proteômica nas ciências básicas e clínica médica, com sua gama de ferramentas, irá acelerar nossa compreensão dos processos de doenças e poderá facilitar a descoberta de novos alvos de fármacos e marcadores de diagnóstico (GYGI et al., 1999; IDEKER et al., 2001).

Estima-se que 20000 genes codificadores de proteínas são responsáveis pela presença de mais de 1 milhão de proteínas encontradas em matrizes biológicas. A quantificação dessas proteínas, comumente nas amostras de plasma, soro, urina, saliva e tecidos, forneceu avanços significativos na ciência médica através do desenvolvimento de ensaios diagnósticos e prognósticos para pacientes que apresentam ou estão sob risco de uma grande quantidade de doenças (ISRAR; HEANEY; SUZUKI, 2018).

A proteômica clínica, definida como a aplicação da proteômica no campo da medicina, tem o potencial de influenciar a prática clínica diária no fornecimento de ferramentas para diagnóstico ou prognóstico, definindo estados de doença, avaliando os perfis de risco e resultados, estabelecendo estratégias terapêuticas individuais. Com esse objetivo, a maioria das aplicações clínicas de proteômica foca em biomarcadores de sangue (séricos ou plasmáticos), embora outros fluidos corporais possam ser usados. Ao contrário das células ou tecidos, o sangue e outros fluidos corporais não possuem um genoma, tornando as técnicas proteômicas uma das poucas opções para descobrir biomarcadores (GUO; FU; VAN EYK, 2007). A descoberta efetiva de biomarcadores, mesmo em amostras biológicas simples, requer uma combinação de sub-fracionamento e separação, ou enriquecimentos diretos de proteínas ou peptídeos, antes da identificação e caracterização dos marcadores por espectrometria de massas, por

exemplo. A tecnologia apropriada ou a combinação de tecnologias para correlacionar as questões biológicas a serem respondidas devem ser cuidadosamente escolhidas para permitir a cobertura máxima do "subproteoma" selecionado e maximizar a interpretação e a utilidade e curso dos dados (SCHIESS; WOLLSCHEID; AEBERSOLD, 2009; ZHOU; PETRICOIN III; LONGO, 2012).

De modo geral, as abordagens genômicas atuais oferecem informações úteis e relativamente rápidas sobre o que as proteínas podem estar "fazendo" *in vivo*, mas, para garantias mais concretas, muitas vezes serão necessárias abordagens moleculares complementares. A análise em larga escala dos níveis de metabólitos (metabolômica) está ganhando espaço como um meio útil para elucidar as funções das proteínas refratárias às abordagens baseadas em expressão. Essa estratégia revelou com sucesso impressões digitais funcionais para genes metabólicos "silenciosos" que não produziram ou demonstravam nenhum fenótipo quando excluídos. No entanto, a análise global de proteínas em seu contexto natural (proteômica) atualmente é a arma mais promissora do arsenal de "caça de função" (RAAMSDONK et al., 2001).

Deste modo, a análise do perfil proteômico é definida como sendo o conjunto de metodologias analíticas empregadas para caracterizar qualitativamente e quantitativamente um proteoma, ou seja, um estudo em larga escala das proteínas que estão sendo expressas; usualmente por métodos bioquímicos para separação, identificação e então quantificação das proteínas, tais como eletroforese, cromatografia, espectrometria de massas e auxílio da bioinformática, tratando-se de uma área interdisciplinar da ciência (AEBERSOLD; MANN, 2003; TYERS; MANN, 2003).

O sinergismo de tamanha interdisciplinaridade faz-se necessário em um cenário onde se pretende estudar a função e o comportamento dos genes com base nos proteomas presentes. Este cenário, alavancou o desenvolvimento de novas tecnologias para o estudo e a análise do perfil proteômico. Estas ferramentas passaram então a serem denominadas instrumentos da proteômica (SADYGOV; COCIORVA; YATES, 2004; TYERS; MANN, 2003).

2.4.1 Proteômica clínica ou translacional na medicina humana e veterinária

Os avanços em abordagens proteômicas baseadas em espectrometria de massas e suas combinações contribuíram para perfilar sistematicamente peptídeos ou componentes de proteínas de misturas complexas, vias celulares e bioquímicas, permitindo a avaliação qualitativa e quantitativa comparada de estados ou momentos

fisiológicos de um organismo; presença e/ou não de doença.

A proteômica funcional, focada em níveis de expressão e atividade proteica, contribui para uma compreensão mais completa dos processos biológicos e não apenas a pura identificação de proteínas (VAN GOOL; HENDRICKSON, 2012).

A proteômica química é multidisciplinar, integrando bioquímica, biologia celular, síntese orgânica e espectrometria de massas. Tipicamente, envolve três etapas principais: (1) imobilização de uma molécula, (2) incubação da molécula/estado sólido com um extrato de tecido/lisado celular para permitir a ligação de proteica e (3) eluição de proteínas interagentes seguidas de análise usando espectrômetro de massas. Esta abordagem oferece a oportunidade de pesquisar múltiplos potenciais biomarcadores e alvos de fármacos (VAN GOOL; HENDRICKSON, 2012).

A proteômica clínica, ou a proteômica translacional, visa a descoberta e compreensão do papel das proteínas nas enfermidades, tais como a identificação de estágios iniciais de doença, a previsão da progressão da doença, a identificação de novos alvos terapêuticos e a avaliação das respostas ao tratamento.

Essa abordagem baseada em espectrometria de massas, fornece uma identificação altamente sensível e específica de centenas a milhares de proteínas relacionadas a regulações fisiológicas e metabolismo energético e, portanto, tem sido usada para compreender e diagnosticar doenças humanas importantes, como câncer (CHAE e GONZALEZ-ANGULO, 2014), distúrbios neurológicos (KROKSVEEN et al., 2011) e várias condições cardiovasculares (EDWARDS et al., 2008). Em medicina veterinária, esta modalidade ainda está no começo; devido à falta de sequências do genoma e descrições incompletas de funções de genes na grande maioria das espécies domésticas.

Os imunoensaios especializados, como o ELISA e os radioimunoensaios (RIAs), foram utilizados para identificar e quantificar proteínas (CELI, 2011; ISLAM et al., 2013; THOMAS et al., 2015), porém, há limitações relacionadas à técnica, custo e tempo de processamento. Além disso, observa-se também uma falta de concordância nos resultados deles e entre eles; devido a variações na estrutura do epítopo entre os indivíduos, saturação de anticorpos evitando a formação de "sanduíches"; o que resulta em uma falsa baixa concentração dos analitos de interesse (THOMAS et al., 2015).

Uma vez que as proteínas são os mediadores da função celular, a natureza do proteoma é dinâmica. As diferenças na expressão das proteínas ou nos graus de modificações pós transcricionais podem representar mudanças na homeostase fisiológica. Portanto, eles são usados como indicadores sensíveis e confiáveis para o

diagnóstico de várias doenças (ECKERSALL e BELL, 2010). A abordagem proteômica baseada em espectrometria de massas pode ser aplicada em uma variedade de amostras biológicas, incluindo urina (ECKERSALL e BELL, 2010), LCR (NOBEN et al., 2006), saliva (GUTIÉRREZ et al. 2014; JACOBSEN et al., 2014; SOUSA-PEREIRA et al., 2015), fluído seminal (DE CANIO et al., 2014), biópsias de tecido (JOHN, 2009), leite (GAGNAIRE et al., 2009; RONCADA et al., 2012; HERNÁNDEZ-CASTELLANO et al., 2016; VERMA; AMBATIPUDI, 2016) e soro ou plasma (HENNING et al., 2014).

As revisões mais recentes de abordagens proteômicas em medicina veterinária concentraram-se na aplicação de proteômica à patogênese e diagnóstico de doenças animais (CECILIANI et al., 2014) e pesquisa de biomarcadores de câncer (KYCKO; REICHERT, 2014; KLOPFLEISCH, 2015; CECILIANI et al., 2016). Alguns trabalhos descreveram análises de proteômica para o diagnóstico de infecções por *Babesia canis canis* em cães, análise das apolipoproteínas (APOA) em cães, composição de proteínas plasmáticas em cadelas prenhes e análise de lágrimas de cães para busca de potenciais marcadores de câncer, entre outros. Em particular, reconheceu-se que a quantificação de um grupo de proteínas séricas chamadas proteínas de fase aguda (PFA) pode auxiliar na avaliação de infecção, inflamação e trauma em animais. Esses avanços estão agora sendo aplicados em laboratórios de bioquímica clínica para o benefício imediato no diagnóstico, prognósticos e monitoramento do tratamento de animais domésticos (ECKERSALL, 2008).

2.4.2 Proteômica do líquido cefalorraquidiano (CSF)

O CSF o fluido intersticial incolor e geralmente denominado como um dialisado do plasma e que flui no espaço subaracnóideo ao redor do cérebro, sistema ventricular e a medula espinhal, sendo produzido no plexo coróide dos ventrículos cerebrais. Dentre as suas funções, estão a de proteger o tecido nervoso mecanicamente, transporte de produtos celulares secretados, biossintetizados e metabolizados dentro ou fora do CNS. A barreira hematoencefálica (BHE) separa o sistema circulatório periférico do CNS e confere permeabilidade seletiva as macromoléculas e assim contribui na manutenção da homeostase iônica e metabólica de seu fluido (GOEHRING et al., 2006).

As proteínas do CSF podem ser derivadas diretamente de células neurais ou serem ativamente transportadas por pinocitose através da BHE. O CSF é um repositório altamente específico de subprodutos celulares, metabólitos, neurotransmissores e fragmentos proteolíticos. O CSF também desempenha um papel mais ativo como meio

de transporte para sinalização de proteínas e peptídeos; deste modo, não um mero e simples "ultrafiltrado" do plasma. Por tanto, o CSF pode ser considerado um "compartimento" biomarcador rico em informações, no qual os peptídeos e proteínas biomarcadoras de seu proteoma representam o estado fisiológico do tecido neural afetado e produzem informações valiosas sobre os processos patológicos e regenerativos em curso (BROCCARDO et al., 2013).

A busca de marcadores biológicos de doenças é um enorme desafio para os pesquisadores com o objetivo de caracterizar proteínas celulares, proteínas segregadas e peptídeos e fragmentos proteolíticos. Neste contexto, modelos de animais domésticos, principalmente grandes animais, apresentam-se como uma oportunidade única para investigar o CSF devido à facilidade de se obter volumes maiores de amostras e à possibilidade de coletas sucessivas.

A análise clínica laboratorial tradicional ou rotineira do CSF concentra-se no exame físico-químico e citológico. Um dos desafios de caracterização do proteoma do CSF é a baixa abundância de proteínas, peptídeos e fragmentos proteolíticos em comparação com o soro sanguíneo. A introdução de análise de microescala/molecular levou a métodos de imunoensaios (e.g. WB), focalização isoelétrica, 2-DE (letroforese bidimensional em gel) e espectrômetro de massas para análise de CSF. O 2-DE foi utilizado inicialmente para identificar mais de 500 bandas proteicas em CSF (BROCCARDO et al., 2013).

As proteínas mais abundantes em CSF humano são a albumina sérica, imunoglobulinas (Igs) e transferrina. Entre as menos abundantes, estão as apolipoproteinas, alfa-1 antitripsina, transtirretina, proteína ligante de retinol, prostaglandina D2 sintetase (ou beta-traço), cistatina C, plasminogênio, gelsolina, beta-2 microglobulina, hemopexina, fibrinogênio, ubiquitina, componentes e fatores do complemento, calicreína, alfa-1-beta glicoproteína, alfa-2-HS glicoproteína, beta-amilóide e fragmentos de beta-amilóide (HU et al., 2005).

A concentração de proteínas no CSF é muito menor do que a concentração de proteínas no plasma devido a BHE. A maioria das proteínas altamente abundantes no plasma também são abundantes no CSF, com exceção das proteínas com taxa de difusão muito baixa para o CSF. A apolipoproteína B-100 (Apo-B100) e a hemoglobina são consideradas apenas do sangue e ausentes no CSF. Outras proteínas, como a cistatina C e a prostaglandina D2 sintetase são mais abundantes no CSF em comparação com ao sangue. A albumina e as imunoglobulinas foram relatadas para representar cerca de 50% e 15%, respectivamente, da quantidade total de proteínas do CSF. As

imunoglobulinas encontradas no CSF são normalmente derivadas do plasma sanguíneo. No entanto, as infecções e inflamações envolvendo o CNS, como esclerose múltipla, comumente apresentam síntese intratecal de imunoglobulinas no CNS (CHAMOUN et al., 2001).

A dinâmica das proteínas originadas exclusivamente no CNS foi investigada usando a taxa de fluxo do CSF no CNS influenciada pela concentração da albumina através do quociente de sua concentração no CSF e no Soro sanguíneo (CSF:Soro). Dois grupos diferentes podem ser discriminados: proteínas de neurônios ou células gliais (e.g., proteína tau, enolase neurônio-específica e proteína S-100) que entram no LCR principalmente no espaço ventricular e cisternal. A sua concentração diminui entre o CSF ventricular e lombar normais em comparação com as proteínas sanguíneas em humanos. As proteínas cerebrais com origem principalmente leptomeníngea (e.g., proteína beta-traço e cistatina C) mostram uma concentração crescente entre o CSF ventricular e lombar normal (REIBER e PETER, 2001).

Neste contexto, com as emergentes abordagens, combinações de técnicas quantitativas e a abordagem proteômica específica em modificações pós transcricionais, tem-se revelado características moleculares de "redes" celulares complexas; o que contribuirá para uma melhor compreensão dos mecanismos moleculares multifacetados das doenças neurodegenerativas. A combinação de neuroimagem, dados clínicos e abordagem proteômica pode melhorar a precisão do diagnóstico de doenças neurodegenerativas e aumentar a validação de candidatos biomarcadores. Além disso, o estudo detalhado das vias metabólicas deste possíveis biomarcadores ou proteínas/peptídeos de interesse facilitará em muito a aquisição e a interpretação dos dados na rotina clínica (VAN GOOL; HENDRICKSON, 2012).

2.4.2.1 Proteômica do CSF em animais domésticos

A análise proteômica do CSF de bovinos acometidos pela encefalopatia espongiforme bovina (BSE) detectou primeiramente 11 proteínas como potenciais "marcadores específicos" da enfermidade, além de outras proteínas altamente abundantes também descritas como presentes no CSF bovino, tais como albumina, alfa-1antitripsina, apolipoproteína A-I, apolipoproteína E, apolipoproteína J (cadeia alfa e beta), imunoglobulinas (Igs) de cadeias leve e pesada, prostaglandina-HS D-sintetase, transferrina e transtirretina (BRENN et al., 2009)..

Posteriormente, usando 2-DE e espectrometria de massas, foi realizado um

mapeamento mais detalhado do proteoma de CSF bovino adulto com amostras individuais de três bovinos hígidos coletados por punção lombar (6-7mL), destacando a presença de 66 proteínas distintas, incluindo 58 que não haviam sido descritas anteriormente. Segundo os autores, dos 66 genes relacionados a estas proteínas, 57 mostraram correlação com a presença de proteínas homólogas em CSF humano, e as demais proteínas relatadas remetem a este baixo número de genes total representados devido a existência de múltiplas isoformas de proteínas bovinas e outras espécies, além de modificações pós transcricionais identificadas nas amostras analisadas (BRENN et al., 2009).

Nakamura et al. (2012) realizaram a análise proteômica do CSF de 40 cães adultos de dois grupos fisiologicamente distintos, sendo 15 cães saudáveis da raça Beagle e 25 cães de diferentes raças e diagnosticados MUE, respectivamente, tiveram amostras de CSF coletadas e analisadas usando 2-DE e espectrometria de massas. Foram encontradas 97 proteínas através do estudo e deste total, 36 foram identificadas e classificadas após pesquisa no *software* MASCOT. Nos animais doentes (MUE), foram encontrados um *spot* em 2-DE que estava *up-regulated* em comparação aos saudáveis (controle). Após a excisão do *spot* de interesse e análise em espectrometria de massas, foi identificada como correspondente ao *spot* a proteína enolase neurônio-específica (NSE). Interessantemente, a NSE é conhecida como um biomarcador em humanos de distúrbios do CNS, como isquemias, traumatismo craniano e medular e alguns tumores. Embora os cães com MUE fossem um pouco mais jovens que os cães saudáveis, a NSE estava elevada no CSF e em conjunto com a inflamação devido a MUE. Como a NSE está geralmente presente no interior de células nervosas, é possível que a NSE aumentada seja um indicativo de destruição neuronal ou processo inflamatório.

Broccardo et al. (2014) caracterizou o perfil proteômico do CSF de seis equinos hígidos utilizando a estratégia *shotgun* e MS (2D-LC-MS / MS) na qual foram detectadas 320 proteínas (NCBI - National Center for Biotechnology Information), sendo as mais abundantes a albumina, a apolipoproteína e a serotransferrina. Deste total, 145 proteínas que tiveram termos ontológicos gênicos "válidos" no banco de dados de proteínas em equinos (UniProt - Universal Protein Resource) foram apresentadas segundo categorias de processos biológicos ao qual estão relacionadas, e as categorias de proteínas mais evidentes foram as envolvidas com processos celulares (18%) e de regulação biológica (13%). Quando avaliadas em categorias quanto às funções moleculares, as mais predominantes incluem funções de associação (42%), atividade catalítica (21%) e atividade de regulação enzimática (14%).

Modelos experimentais com grandes animais mostram-se uma ótima oportunidade para investigar o CSF através de abordagens proteômicas baseadas em espectrometria de massas, devido a possibilidade de se obter amostras com maior volume e facilidade de se realizar múltiplas coletas.

Esses achados são relevantes e de grande importância pois são o ponto de partida para o estudo de possíveis biomarcadores para diversas enfermidades neurológicas que podem acometer os equinos como a leucoencefalomalácia, SVCM, doença do neurônio motor, EHV-1, entre outras; como a EPM, também abordada no presente estudo.

Ademais, visto que muitas vezes estas enfermidades são fatais ou levam a sequelas, que comumente determinam a eutanásia dos animais, gerando inúmeros prejuízos na indústria equina (RECH; BARROS, 2015). Neste contexto, mais estudos devem ser realizados para a melhor caracterização do perfil proteômico do CSF de equinos hígidos e com doenças neurológicas, assim como a resposta aos diferentes tipos de tratamentos e a terapia celular, foco deste estudo.

3 HIPÓTESES

Múltiplos transplantes intratecal de células estromais mesenquimais multipotentes (MSCs) altera o perfil proteômico do líquido cefalorraquidiano (CSF) de equinos.

4 OBJETIVOS

4.1 Objetivo geral

Avaliar o perfil proteômico do líquido cefalorraquidiano (CSF) antes e após múltiplos transplantes intratecal de células estromais mesenquimais multipotentes em equinos saudáveis e o perfil proteômico do CSF de equinos cronicamente afetados pela Mieloencefalite Protozoária Equina (EPM).

4.2 Objetivos específicos

- Comparar as linhagens celulares de MSCs provenientes do AD e da BM quanto aos efeitos sobre o perfil proteômico do CSF após três transplantes intratecal em equinos saudáveis.
- Avaliar o perfil proteômico do CSF de equinos cronicamente afetados pela EPM e compará-lo com o perfil proteômico do CSF de equinos clinicamente saudáveis.

CAPÍTULO 2

TRABALHO CIENTÍFICO

Trabalho a ser enviado para a revista *Stem Cell Research & Therapy.*

PROTEOMIC PROFILING OF CEREBROSPINAL FLUID AFTER INTRATHECAL TRANSPLANTATION OF MULTIPOTENT MESENCHYMAL STROMAL CELLS IN HORSES

ABSTRACT

Background: Studies with multipotent mesenchymal stromal cells (MSCs) are in increasing progress due to their immunomodulatory, anti-inflammatory, anti-apoptotic and tissue regeneration properties, making this modality of cell therapy promising in the treatment of several diseases. Due the limited regenerative capacity of the central nervous system (CNS), causing functional sequelae, MSCs are being investigated as a therapeutic alternative for inflammatory, vascular, traumatic and degenerative neurological conditions in several animal species. The application of proteomics with its range of tools in equine clinics can contribute significantly to the understanding of pathological processes and facilitate the discovery of new therapeutic targets or diagnostic markers. In this context, the objective of this study was to evaluate the proteomic profile of cerebrospinal fluid (CSF) before and after multiple intrathecal transplants of CSFs in healthy horses.

Methods: Twelve clinically healthy adult horses were randomly divided into three experimental groups: control group (Control; n=4) where intrathecal Dulbecco's phosphate-buffered saline solution (DPBS) was administered; adipose tissue group (AD-G; n=4), in which intrathecal transplants with allogeneic MSCs from adipose tissue (AD-MSCs) were performed; and bone marrow group (BM-G; n=4), in which intrathecal transplants with allogeneic MSCs from bone marrow (BM-MSCs) were performed. All the experimental groups received three successive treatments (DPBS or MSCs) with an interval of 30 days between them. CSF samples were collected from the experimental groups immediately before the first treatment (named M0 or Before) and 30 days after the third treatment (named M90 or After).

Results: Considering proteomics platform used, the six pools compared: Control After vs. Control Before, AD-G After vs. AD-G Before and BM-G After vs. Before (After:Before paired modes), obtained 208, 211 and 131 identified access numbers corresponding to proteins and verified on UniProt database. Among the proteins identified, the presence of the three types of enolases interacting (ENO 1, ENO 2 and ENO 3) was also observed in the comparison of the group Control and BM-G 30 days after transplantation.

Conclusion: This study allowed us to initially verify the presence of 3 types of enolases interacting exclusively 30 days after transplantation in both the Control and BM-G groups. In this context, a deeper analysis of these data should be carried out, since the NSE (ENO-2) may play an important role in the pathogenesis of neural injury, as described in others species.

Keywords: Equine, cerebrospinal fluid, multipotent mesenchymal stromal cells, proteomics, neural lesion biomarkers, enolases.

Background

Nowadays multipotent mesenchymal stromal cells (MSCs) have been presented as a promising alternative in the treatment of inflammatory, traumatic, vascular and degenerative diseases in various organ systems and in particular in the central nervous system (CNS) due to its anti-inflammatory, immunomodulatory and neuroregenerative properties. The cells are also easily isolated from a variety of sources and are easily cultured (BURK et al., 2013; CARRADE et al., 2012; CHAMBERLAIN et al., 2007; GUTIERREZ-NIBEYRO, 2011; NÖTH et al., 2010). Studies in animal models and clinical trials have demonstrated the therapeutic potential of MSCs in treating nervous system diseases such as Alzheimer's, multiple sclerosis, stroke, traumatic spinal cord injuries, among others, showing beneficial effects on the neuroregeneration process (CHENG et al., 2015; COHEN, 2013; HYATT et al., 2014; KARUSSIS; PETROU; KASSIS, 2013; LINDVALL; KOKAIA, 2006; MAZZINI et al., 2010; PENHA et al., 2014; UCCELLI et al., 2011). In the context of veterinary medicine, there are several neurological diseases that affect horses whose recommended treatments are not fully efficient and often lead to important side effects, such as equine protozoal myeloencephalitis (EPM) and cervical vertebral stenotic myelopathy (CVSM) (DIRIKOLU; FOREMAN; TOBIN, 2013; REED; GRANT; NOUT, 2008). The use of MSCs for these neurological diseases in horses appears to be a promising alternative.

However, the efficacy results of cell therapy with MSCs in neurological lesions presented in preclinical and clinical studies are heterogeneous, postponing the use of this biotechnology in the clinical routine, which demonstrates the need for more studies in the area and in particular, studies focusing on safety and standardization of cell transplantation techniques.

The transplantation pathway is one of the factors that may influence the efficacy of cell therapy since the therapeutic properties of MSCs are fundamentally related to the ability of these cells to produce and secrete justacrine and paracrine factors that promote immunomodulation and neuroregeneration. In order to do so, MSCs need to migrate to the site of the lesion, a process known as cell homing (CHENG et al., 2015; COHEN, 2013; HYATT et al., 2014; KARUSSIS; PETROU; KASSIS, 2013; LINDVALL; KOKAIA, 2006; MAZZINI et al., 2010; PENHA et al., 2014; UCCELLI et al., 2011); Several MSC transplantation routes have been used to reach the CNS, including the intravenous (IV), intra-arterial (IA), intrathecal (IT), intralesional and intranasal routes. Although there is no consensus in the literature, transplantation routes that deliver MSCs as close as possible to the lesion (e.g., intralesional and IT), seem to have advantages over the systemic pathways (IV, IA), since they facilitate cellular homing. The low number of cells delivered to target tissues may result in ineffective treatment (ZHANG et al., 2015).

Cerebrospinal fluid (CSF) can be considered an information-rich biomarker "compartment" in which the peptides and biomarker proteins of its proteome represent the physiological state of the affected neural tissue and produce valuable information about the pathogenesis and therapeutic response of ongoing disease.

To our knowledge, there are no studies on the proteomic profile of CSF of horses after multiple intrathecal transplantations of MSCs from bone marrow (BM-MSCs) and adipose tissue (AD-MSCs). Proteomic analysis of CSF from healthy horses receiving multiple intrathecal MSCs transplants may reveal which metabolic pathways and/or potential biomarkers expression differs from before and after cells transplantations.

The results obtained with this approach can contribute both to the understanding of the mechanisms of action of MSCs in the process of neuroregeneration CNS of horses, and to the development of a protocol of safe and effective cellular therapy (GYGI et al., 1999; IDEKER et al., 2001).

Materials and methods

All stages of the development of this work followed the Ethical Principles in Animal Experimentation and were approved by the Ethics Committee for the Use of Animals (CEUA) of the School of Veterinary Medicine and Animal Science (FMVZ), Sao Paulo State University "Júlio de Mesquita Filho" (UNESP), Botucatu campus, Brazil (BRA) under the CEUA Protocol 161/2014.

Groups and experimental design

For the development of this study, 12 clinically healthy horses (Equus caballus) of both sexes (9 females and 7 males), mixed-breed, aged between 4 and 15 years and body weight between 300-500 kg, belonging to the School of Veterinary Medicine and Animal Science - Sao Paulo State University (Botucatu, Sao Paulo, Brazil) were used. These animals were considered healthy based on the evaluation of the physical examination, complete blood count and CSFanalysis (data not shown).

The horses were randomly divided into three experimental groups: control group (Control; n=4) where intrathecal Dulbecco's phosphate-buffered saline solution (DPBS) was administered; adipose tissue group (AD-G; n=4), in which intrathecal transplants with allogeneic AD-MSCs were performed; and bone marrow group (BM-G; n=4), in which intrathecal transplants with allogeneic BM-MSCs were performed. All the experimental groups received three successive treatments (DPBS or MSCs) with an interval of 30 days between them. CSF samples were collected from the experimental groups immediately before the first treatment (named M0 or Before) and 30 days after the third treatment (named M90 or After).

MSCs used for transplantation

Allogeneic cryopreserved AD-MSCs and BM-MSCs from young and healthy equine donors were used to perform the cellular transplants. These cell lines were thawed and expanded into culture until 80-90% of cell confluence was obtained.

Immediately before the transplantation procedure, the MSC culture bottles were trypsinized, washed three times with Dulbecco's phosphate buffered saline solution (DPBS: phosphate buffered saline solution (PBS) free of calcium chloride and magnesium chloride) and the cell pellet resuspended in 3 mL of DPBS, containing approximately $50x10^6$ cells with at least 80% cellular viability. Counting and cell viability test with Trypan blue were performed in a Neubauer chamber. The cells were kept refrigerated at 5°C until the moment of transplantation.

Immunophenotyping

Immunophenotypic analysis of AD-MSCs, BM-MSCs and was performed in the third passage (P3) with the flow cytometer FACS Calibur (BD, Franklin Lakes, NJ, USA), using mouse anti-rat antibodies CD90-FITC (clone OX7, Caltag Laboratories, USA), CD34-FITC anti-human mouse (clone 581, BD, USA), CD105-FITC anti-human mouse (clone SN6, Abcam, FL, USA), CD44 anti-horse mouse (clone CVS18, abD Serotec, UK) and MHC class II monomorphic anti-horse mouse (clone CVS20, abD Serotec, UK). The secondary anti-mouse IgG-FITC goat antibody (abD Serotec, UK) was used for non-conjugated primary markers. The protocols used were those described by the manufacturers.

Tri-lineage differetiation - osteogenic, adipogenic and chondrogenic

During P3, samples of BM-MSCs were stored in 6 Wells plates (Sarstedt, USA) in triplicate for osteogenic and adipogenic differentiation, and incubated in an incubator at 37ºC in a humid atmosphere containing 95% air and 5% CO2. After confluence of 80-90%, the maintenance medium was removed and the Stempro® adipogenesis and Stempro® osteogenesis (Invitrogen, USA) differentiation medium was added to the cultures, with one well from each plate maintained as a control and two wells used to evaluate the differentiation. The medium was changed every 3 days, remaining 15 days, and the differentiation into adipose tissue was confirmed by deposition of fat droplets in the cytoplasm by 0.5% Oil Red staining (Sigma-Aldrich Corp, USA). Bone tissue differentiation was confirmed by positive staining of the extracellular calcium matrix by the Alizarin Red dye 2% (Sigma-Aldrich Corp, USA).

For chondrogenic differentiation, a pellet of MSCs was cultured in a Falcon tube and incubated in an incubator at 37ºC in a humid atmosphere containing 95% air and 5% CO2. After two days, the maintenance medium was removed, and the Stempro chondrogenesis differentiation medium (Invitrogen, USA) was added, with exchange every 3 days, remaining for 21 days. To confirm the chondrogenic differentiation, the pellets were stained with Alcian Blue (pH=2.5) and Toluidine Blue (pH=1) to identify proteoglycans.

CSF collection and transplantation of MSCs

The CSF was collected from the subarachnoid space between the cervical vertebrae C1-C2 according to (PEASE; BEHAN; BOHART, 2012) and with modifications made by (BARBERINI, 2017).

For this, the animals were sedated with detomidine (10-20 µg/kg intravenously) and morphine (0.05 mg/kg intravenously). In quadrupedal position, an area of 15 x 15 cm, centered on the space of the cervical vertebrae C1-C2, was tricotomized and aseptically prepared with 2% degerming chlorhexidine and 70% alcohol for sterile puncture. Skin and subcutaneous tissue infiltration was performed with the anesthetic lidocaine hydrochloride 2% without a constrictor vessel, using a local "button" block. With the aid of ultrasound (ultrasound device MyLab™30Gold VET, ESAOTE®, ITA), the subarachnoid space between the cervical C1-C2 vertebrae was located, a 25Gx90mm spinal needle was inserted and the CSF aspirated using a 3 mL syringe.

The CSF was stored in a sterile cryogenic tube and free of DNase and RNase to undergo freezing in liquid nitrogen at a temperature of -160ºC and stored at a temperature of -80ºC for proteomic analysis.

Soon after this procedure, the DPBS (3 mL) or the MSCs (approximately $50x10^6$ cells in 3 mL of DPBS), according to the respective groups already described, were transplanted by the same route.

Evaluation of CSF proteomic profiling

To evaluate the proteomic profile, the CSF samples were pooled according to the experimental groups (Control, AD-G and BM-G) and moments (immediately before the first transplant - M0 or Before; and 30 days after the third M90 transplant or After).

Each representative sample had its proteins quantitatively identified with a confidence interval (>95%) and the interaction between these proteins identified and differentially expressed with a bidirectional p-value (<0.05) for the down-regulated proteins, and (1-p>0.95), for the up-regulated proteins.

Sample preparation

The CSF samples were thawed slowly and kept refrigerated at 4-5°C for use. Then, each selected sample was homogenized with the aid of a vortex and resuspended with movements up and down in the pipette itself, being then the aliquot of the standardized volume packed in LoBind plastic microtubes (Eppendorf®) corresponding to the destination pool.

In each of the CSF samples of the twelve horses randomly divided into the three groups (Control, AD-G and BM-G) and moments (M0/Before and M90/After) 150µL were resuspended and grouped into a pool of four animals (150µL/animal) according to their group (Control, AD-G and BM-G) and moment (M0/Before and M90/After), totaling six pools representing the experimental groups.

These six pools were named according to their group and time. The M0 was named with Before and M90 After.

The six pools were so named: Control Before and After, AD-G Before and After, BM-G Before and After.

Protein Quantification

The proteins present in the CSF samples were quantified in triplicate according to the Bradford method (BioRad®; Protein Assay, cod. 500-0001), using bovine albumin (BSA) as the standard protein to obtain the calibration curve.

Enzyme digestion in solution

Fifty micrograms (50ug) of each sample were solubilized in 50 uL of 50mM ammonium bicarbonate buffer at pH=7.8. Subsequently, the samples were homogenized in the presence of 25 uL of Rapigest surfactant (Waters) and incubated at 37°C for 60 minutes. After this period, the samples were submitted to reduction and alkylation steps, using 10mM ditiotreitol (DTT) and 45mM iodoacetate (IAA), both solubilized in 50mM ammonium bicarbonate solution. The samples were then submitted to proteolytic digestion in the presence of the enzyme trypsin (Promega) at a concentration of 1:100 (enzyme:substrate), solubilized in 50mM ammonium bicarbonate buffer, pH=7.8. The hydrolysis occurred for 18 hours, being interrupted with the addition of formic acid 1% (v/v) in relation to the volume of the samples. These were then desalinated using Sep Pak Vac C18 cartridges (Agilent). The digested and desalinated samples were reduced in SpeedVac™ (Thermo Scientific) and kept at 4°C until the moment of analysis by mass spectrometry.

Mass Spectrometry Analysis and Protein Identification

Triptych peptide analysis was performed in the nanoACQUITY UPLC system (Waters, Milliford, USA) coupled to the Xevo Q-TOF G2 mass spectrometer (Waters, Milliford, USA). For this purpose, the UPLC nanoACQUITY system was equipped with a column type HSS T3 (Acquity UPLC HSS T3 column 75 mm x 150 mm; 1.8

µm, Waters), previously balanced with 7% of mobile phase B (100% ACN + 0.1% formic acid). The peptides were separated by a linear gradient of 7-85% of mobile phase B for 70 minutes with a flow of 0.35µL/min and the temperature of the column maintained at 45°C. The spectrometer was operated in positive ion mode with a data acquisition time of 75 minutes.

The data obtained were processed using the ProteinLynx GlobalServer (PLGS) software version 3.03 (Waters, Milliford, USA). The samples were submitted to a mass spectrometer in triplicates. The identification of the proteins was obtained through the ion counting algorithm incorporated into the software. The data obtained were retrieved from the *Equus caballus* species database downloaded from the UniProt catalogue (Universal Protein Resource) in September 2018, using the following variables: trypsin enzyme taking into account a lost cleavage; fixed carbamidomethylation modification; variable methionine oxidation modification; MS and MSMS tolerance error 0.1 Da.

Analysis of differential expression through the label-free strategy

All proteins identified with a confidence score (>95%) were included in the quantitative analysis. The ionic peak intensities of each protein were normalized, scaled and compared between topographies by the PLGS software version 3.03 (Waters, Milliford, USA) using a Bayesian algorithm (Monte Carlo method), which returns a bidirectional p-value: (<0.05) for down-regulated proteins, and (1-p>0.95) for up-regulated proteins.

In order to ensure even greater reliability in this analysis, the PLGS software uses 14 parameters to correctly identify each protein and not only the score. After performing the analysis, using these 14 parameters, we have 3 categories of identification (named Flag or OK): red (less than 50% of chance of correct identification), yellow (between 50 and 95% chance of correct identification) and green (more than 95% chance of correct identification). We only accepted the categories yellow and green (category red was excluded). Considering that this was standard when our proteomics platform was used anda we use 15 distinct parameters, there is no need to describe the parameters used for identification as threshold score or coverage of each protein.

Analysis of interactions between differentially expressed proteins

Bioinformatics analysis was done to compare the treated groups with the control group. These included the database Uniprot (https://www.uniprot.org/) to verify all

acesses numbers provided from analyzes, the web-available STRING v10.5 (http://string-db.org/, by choosing the variables "Multiples proteins" and "organism *Equus caballus*" in order to evaluate the interaction between the proteins identified differently expressed between the groups compared.

Results

The six pools compared: Control After vs. Control Before, AD-G After vs. AD-G Before and BM-G After vs. Before (After:Before paired modes), obtained 217, 238 and 156 identified access numbers corresponding to proteins, respectively, considering the 3 categories of identification Flag (red, yellow and green).

Considering proteomics platform used (category red excluded), the six pools compared: Control After vs. Control Before, AD-G After vs. AD-G Before and BM-G After vs. Before (After:Before paired modes), obtained 208, 211 and 131 identified access numbers corresponding to proteins and verified on UniProt database, respectively (Tables 1-3).

In the Control comparison (After:Before), 208 access numbers were found, 49 were exclusives of the After moment, 50 exclusive of the Before moment, 3 up-regulated, 3 down-regulated and 103 showed no difference in expression (Table 1).

AD-G After vs. AD-G Before, obtained 211 access numbers found, 7 were exclusives of the After moment, 106 exclusive of the Before moment, 19 up-regulated, 22 down-regulated and 57 showed no difference in expression (Table 2).

BM-G After vs. BM-G Before, obtained 131 access numbers found, 35 were exclusives of the After moment, 80 exclusives of the Before moment, 5 up-regulated, 2 down-regulated and 9 showed no difference in expression (Table 3).

Table 1. Identified proteins in the compare group Control After vs. Before

Identified Proteins (n=208)	aAccess Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (After:Before)	*p* Value ratio
Clone 7 transferrin (Pop-variant * transferrin) (Pop-variant D, F1, F2, F3, G, H2, O transferrin) (Fragment)	Q9TQV4		681	10	1	Predicted	Unique After	After	After
Hemopexin	F6X1I8	HPX	297	462	51	Inferred from homology	Unique After	After	After
Alpha-1-microglobulin/bikunin precursor	F6UZH0	AMBP	263	352	39	Predicted	Unique After	After	After
Ubiquitin conjugating enzyme E2 B	F6XR18	UBE2B	226	152	17	Inferred from homology	Unique After	After	After
Ubiquitin conjugating enzyme E2 A	F7BNF7	UBE2A	226	154	18	Predicted	Unique After	After	After
RAD6 homolog (Fragment)	Q9N1X6	EHR6A	226	126	14	Inferred from homology	Unique After	After	After
Ubiquitin-conjugating enzyme E2 A-like protein (Fragment)	K9KCC9		226	98	11	Evidence at transcript level	Unique After	After	After
Dickkopf WNT signaling pathway inhibitor 3	F6WE78	DKK3	188	324	35	Predicted	Unique After	After	After
Uncharacterized protein	H9GZT5		159	335	36	Predicted	Unique After	After	After
Uncharacterized protein	F6WCY1		154	422	47	Predicted	Unique After	After	After
Alpha-enolase-like protein (Fragment)	K9K2B9		154	310	34	Evidence at transcript level	Unique After	After	After
Enolase 2 (Fragment)	I3RM61	ENO2	154	424	46	Evidence at transcript level	Unique After	After	After
Enolase 3	F7C959	ENO3	154	434	47	Evidence at transcript level	Unique After	After	After
Enolase 2	F7CIX6	ENO2	154	434	47	Inferred from homology	Unique After	After	After
Enolase 1	F6V7C1	ENO1	154	434	47	Evidence at transcript level	Unique After	After	After
MINDY lysine 48 deubiquitinase 3	F7DRK3	MINDY3	115	445	50	Predicted	Unique After	After	After
Phospholipid transfer protein	F7CR03	PLTP	112	496	55	Predicted	Unique After	After	After
Fibrinogen A-alpha chain (Fragment)	O97641		107	481	50	Predicted	Unique After	After	After
Fibrinogen alpha chain (Fragment)	Q7M2U4		107	270	28	Evidence at protein level	Unique After	After	After
Uncharacterized protein	F7CYP1		105	423	47	Inferred from homology	Unique After	After	After
Uncharacterized protein	F7CSL8		105	421	47	Inferred from homology	Unique After	After	After
Uncharacterized protein	F7DXM5	LOC100065068	105	422	47	Inferred from homology	Unique After	After	After
Alpha-1-antitrypsin	B5BV12	Spi2-13	105	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV11	Spi2-12	105	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV10	Spi2-11	105	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV09	Spi2-10	105	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV08	Spi2-9	105	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV07	Spi2-8	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV06	Spi2-7	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV05	Spi2-6	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV04	Spi2-5	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV03	Spi2-4	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV02	Spi2-3	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV01	Spi2-2	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antitrypsin	B5BV00	Spi2-1	100	421	47	Evidence at transcript level	Unique After	After	After
Alpha-1-antiproteinase 2 (Alpha-1-antitrypsin 2) (Alpha-1-proteinase inhibitor 2) (SPI2)	P38029	-	100	421	47	Evidence at protein level	Unique After	After	After
Uncharacterized protein	F7BF31	SPI2	100	421	47	Inferred from homology	Unique After	After	After
FAM188A-like protein (Fragment)	K9K4K6		99	367	41	Evidence at transcript level	Unique After	After	After
Secreted phosphoprotein 1	F7AYC1	SPP1	94	313	35	Predicted	Unique After	After	After
Uncharacterized protein	F7E0F5		85	403	46	Predicted	Unique After	After	After
Alpha-1-antitrypsin	B5BV13	Spi2-14	85	421	47	Evidence at transcript level	Unique After	After	After
Glutamic-oxaloacetic transaminase 1 like 1	F6VRG6	GOT1L1	80	408	46	Predicted	Unique After	After	After
Transferrin (Fragment)	O97677		72	53	6	Predicted	Unique After	After	After
Gelsolin	F7DHZ1	GSN	66	771	85	Predicted	Unique After	After	After
Gelsolin	F7E2D1	GSN	66	783	86	Predicted	Unique After	After	After
Dihydropyrimidinase like 2	F7B320	DPYSL2	64	572	62	Predicted	Unique After	After	After
Tuftelin 1	F6ZH40	TUFT1	63	388	44	Predicted	Unique After	After	After
Angiotensinogen	F6W4R2	AGT	58	485	53	Inferred from homology	Unique After	After	After
Serpin family F member 1	F7BKE1	SERPINF1	54	417	46	Inferred from homology	Unique After	After	After
Cardiolipin synthetase-like protein (Fragment)	K9KEE4		1134	82	9	Evidence at transcript level	Unique Before	Before	Before
Cardiolipin synthase 1	F6Y9A3	CRLS1	1134	200	22	Predicted	Unique Before	Before	Before
Transferrin (Fragment)	Q9TQW7		894	52	6	Predicted	Unique Before	Before	Before
Serpin I (Fragment)	Q7M389		571	54	6	Evidence at protein level	Unique Before	Before	Before
Serpin II (Fragment)	Q7M388		571	54	6	Evidence at protein level	Unique Before	Before	Before
Serpin III (Fragment)	Q7M387		571	49	6	Evidence at protein level	Unique Before	Before	Before
Beta-2-microglobulin	K9K497		445	118	13	Evidence at transcript level	Unique Before	Before	Before
Beta-2-microglobulin (Fragment)	Q6X9X1	B2M	445	94	11	Evidence at transcript level	Unique Before	Before	Before
Beta-2-microglobulin (Fragment)	Q95MB8		445	30	3	Predicted	Unique Before	Before	Before
Transferrin (Fragment)	Q9TUP2		404	61	7	Predicted	Unique Before	Before	Before
Transferrin (Fragment)	O97974		404	61	7	Predicted	Unique Before	Before	Before
Plasminogen	F6USP9	PLG	280	817	92	Inferred from homology	Unique Before	Before	Before
Plasminogen [Cleaved into: Plasmin heavy chain A; Plasmin light chain B] (Fragment)	P80010	PLG	280	338	37	Evidence at protein level	Unique Before	Before	Before
Plasminogen (Fragment)	Q9TV90	PLG	238	73	8	Predicted	Unique Before	Before	Before
Syntaxin 19	F6UAH4	STX19	237	294	34	Inferred from homology	Unique Before	Before	Before
Lactotransferrin (Lactoferrin) (Fragment)	O77811	LTF	222	695	76	Evidence at protein level	Unique Before	Before	Before
Lactotransferrin	F6XLB1	LTF	222	708	77	Inferred from homology	Unique Before	Before	Before
Uncharacterized protein	F6TJ01	CNTRL	193	2327	269	Predicted	Unique Before	Before	Before
Centriolin-like protein (Fragment)	K9KD84		177	339	40	Evidence at transcript level	Unique Before	Before	Before
Alpha-fetoprotein	F6U4X2	AFP	161	614	69	Predicted	Unique Before	Before	Before
Alpha-fetoprotein (Alpha-1-fetoprotein) (Alpha-fetoglobulin)	P49066	AFP	154	609	68	Evidence at transcript level	Unique Before	Before	Before
GLI family zinc finger 3	F6XL68	GLI3	149	1541	165	Predicted	Unique Before	Before	Before
GLI3 protein (Fragment)	A2TGR9		147	81	9	Predicted	Unique Before	Before	Before
Phosphatidylinositol specific phospholipase C X domain containing 3	F7DF05	PLCXD3	142	196	22	Predicted	Unique Before	Before	Before
Solute carrier family 25 member 28	F6WZG2	SLC25A28	138	310	34	Inferred from homology	Unique Before	Before	Before
Mitoferrin-2-like protein (Fragment)	K9KAI6		138	191	21	Evidence at transcript level	Unique Before	Before	Before
Androgen receptor	A0A166J860	AR	117	882	95	Inferred from homology	Unique Before	Before	Before
Androgen receptor	A0A166J9F4	AR	117	882	95	Inferred from homology	Unique Before	Before	Before
Androgen receptor	A0A166J9E7	AR	117	882	95	Inferred from homology	Unique Before	Before	Before
Uncharacterized protein	F6R5G1		117	400	46	Inferred from homology	Unique Before	Before	Before
Ethanolamine kinase 2	F6XBK6	ETNK2	113	298	34	Predicted	Unique Before	Before	Before
WD repeat and SOCS box containing 2	F7DH72	WSB2	108	403	45	Predicted	Unique Before	Before	Before
WD repeat and SOCS box containing 2	F7DCC9	WSB2	108	406	46	Predicted	Unique Before	Before	Before
PDZ domain-containing protein 8-like protein (Fragment)	K9KAW9		95	260	29	Evidence at transcript level	Unique Before	Before	Before
PDZ domain containing 8	F6USA2	PDZD8	95	1151	128	Predicted	Unique Before	Before	Before
Anti-silencing function 1B histone chaperone	F6ULM8	ASF1B	89	202	22	Predicted	Unique Before	Before	Before
Nipsnap homolog 2	F6SCB6	NIPSNAP2	79	256	31	Predicted	Unique Before	Before	Before
Protein NipSnap-like protein 2-like protein (Fragment)	K9KAD5		79	157	19	Evidence at transcript level	Unique Before	Before	Before
Coronin	F7DEW5	CORO1C	79	476	53	Inferred from homology	Unique Before	Before	Before
Eukaryotic initiation factor 4A-III-like protein (Fragment)	K9KAI8		78	343	39	Evidence at transcript level	Unique Before	Before	Before
PDZ domain-containing 8-like protein (Fragment)	L7MRG7	PDZD8	75	114	13	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	F7E454	LOC100060539	69	1640	184	Predicted	Unique Before	Before	Before
Coiled-coil domain containing 15	F6YK38	CCDC15	66	934	108	Predicted	Unique Before	Before	Before
Uncharacterized protein	F7CJG3	LOC100060539	65	1661	186	Predicted	Unique Before	Before	Before
Uncharacterized protein	F7BTW7	LOC100060539	65	1664	186	Predicted	Unique Before	Before	Before
IQ motif containing GTPase activating protein 3	F6WGS2	IQGAP3	65	1637	185	Predicted	Unique Before	Before	Before
UPF0528 protein FAM172A-like protein (Fragment)	K9KD23		61	344	40	Evidence at transcript level	Unique Before	Before	Before
Family with sequence similarity 172 member A	F7CUH0	FAM172A	61	370	43	Predicted	Unique Before	Before	Before
Nuclear receptor subfamily 4 group A member 3	F6TE84	NR4A3	59	622	70	Inferred from homology	Unique Before	Before	Before
IQ motif containing GTPase activating protein 1	F6YRC5	IQGAP1	49	1639	187	Predicted	Unique Before	Before	Before
Serum albumin	F7BAY6	ALB	12731	607	68	Predicted	Up-regulated	1.14	1
Serum albumin (allergen Equ c 3)	P35747	ALB	5778	607	69	Evidence at protein level	Up-regulated	1.09	0.99
Albumin (Fragment)	Q95MC2		4597	44	5	Predicted	Up-regulated	1.79	0.98
Uncharacterized protein	F6ZI35		233	236	27	Predicted	No difference vs. control	1.84	0.94
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B6	IGL	337	225	24	Evidence at transcript level	No difference vs. control	1.46	0.85
Clusterin [Cleaved into: Clusterin beta chain; Clusterin alpha chain]	Q29482	CLU	402	449	52	Evidence at transcript level	No difference vs. control	1.21	0.85
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Y3	IGL	395	225	23	Evidence at transcript level	No difference vs. control	1.43	0.82
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X7	IGL	395	226	24	Evidence at transcript level	No difference vs. control	1.46	0.82
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E493	IGL	337	224	23	Evidence at transcript level	No difference vs. control	1.39	0.81
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H7	IGL	395	225	23	Evidence at transcript level	No difference vs. control	1.45	0.81
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E933	IGL	395	223	23	Evidence at transcript level	No difference vs. control	1.39	0.8
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4E7	IGL	395	225	24	Evidence at transcript level	No difference vs. control	1.42	0.8
Apolipoprotein A1	F6Z2L5	APOA1	635	266	30	Inferred from homology	No difference vs. control	1.46	0.8
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E944	IGL	337	225	24	Evidence at transcript level	No difference vs. control	1.35	0.79
Uncharacterized protein	F6YYP6		1409	99	11	Inferred from homology	No difference vs. control	1.46	0.79
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E938	IGL	395	226	24	Evidence at transcript level	No difference vs. control	1.39	0.77
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H3	IGL	337	221	23	Evidence at transcript level	No difference vs. control	1.32	0.77
Serpin family C member 1	F7CYR1	SERPINC1	70	463	52	Inferred from homology	No difference vs. control	2.12	0.77
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X3	IGL	337	222	23	Evidence at transcript level	No difference vs. control	1.42	0.76
Uncharacterized protein	F6QAU5		395	107	11	Predicted	No difference vs. control	1.32	0.75
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D7	IGL	395	225	23	Evidence at transcript level	No difference vs. control	1.39	0.75
Uncharacterized protein	F6SQD7		337	107	11	Predicted	No difference vs. control	1.30	0.72
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B2	IGL	395	224	23	Evidence at transcript level	No difference vs. control	1.25	0.71
Prostaglandin-H2 D-isomerase (Glutathione-independent PGD synthase) (Lipocalin-type prostaglandin-D synthase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS) (PGDS2)	O97921	PTGDS	570	194	22	Evidence at protein level	No difference vs. control	1.20	0.71
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4B1	IGL	337	225	24	Evidence at transcript level	No difference vs. control	1.27	0.7
Haptoglobin	F6XWM5		1143	346	38	Inferred from homology	No difference vs. control	1.07	0.67
Uncharacterized protein	H9GZQ9		108	337	37	Predicted	No difference vs. control	1.16	0.66
Transferrin (Fragment)	O97966		263	61	7	Predicted	No difference vs. control	1.30	0.66
Immunoglobulin gamma 1 heavy chain constant region (Fragment)	Q95M34	IGHC1	108	337	37	Evidence at transcript level	No difference vs. control	1.16	0.66
Transferrin (Fragment)	O97973		263	61	7	Predicted	No difference vs. control	1.21	0.64
Transferrin (Fragment)	O97678		263	61	7	Predicted	No difference vs. control	1.22	0.63
GC, vitamin D binding protein	F6T0P6	GC	154	474	53	Predicted	No difference vs. control	1.09	0.55
Transferrin (Fragment)	Q9TQR4		3737	63	7	Predicted	No difference vs. control	1.01	0.51
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4R8	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.99	0.49
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E417	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.96	0.48
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E447	IGL	1668	223	23	Evidence at transcript level	No difference vs. control	0.93	0.47
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E532	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.91	0.46
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4A9	IGL	1668	223	23	Evidence at transcript level	No difference vs. control	0.91	0.46
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E464	IGL	1610	224	24	Evidence at transcript level	No difference vs. control	0.94	0.44
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K4	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.87	0.44
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J8	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.88	0.44
Carboxylic ester hydrolase	F6VXP7		91	565	62	Inferred from homology	No difference vs. control	0.92	0.43
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E971	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.84	0.43
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E966	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.92	0.43
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4S5	IGL	1668	222	23	Evidence at transcript level	No difference vs. control	0.88	0.42
Uncharacterized protein	F6ZEH8		8798	990	109	Predicted	No difference vs. control	0.98	0.42
Uncharacterized protein	F7CAC5		1668	117	12	Predicted	No difference vs. control	0.90	0.42
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E452	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.91	0.42
Transferrin (Fragment)	Q9TQW8		1145	52	6	Predicted	No difference vs. control	0.90	0.42
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E588	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.91	0.42
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J6	IGL	1668	219	23	Evidence at transcript level	No difference vs. control	0.89	0.42
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E567	IGL	1610	224	23	Evidence at transcript level	No difference vs. control	0.93	0.42
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A3	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.86	0.41
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z9	IGL	1668	222	23	Evidence at transcript level	No difference vs. control	0.89	0.41
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N5	IGL	1668	226	24	Evidence at transcript level	No difference vs. control	0.82	0.41
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E961	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.90	0.41
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E406	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.76	0.41
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E8	IGL	1668	223	23	Evidence at transcript level	No difference vs. control	0.84	0.4
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q6	IGL	1668	225	24	Evidence at transcript level	No difference vs. control	0.90	0.4
Carboxylic ester hydrolase	F6PRI5	LOC100051065	124	565	62	Inferred from homology	No difference vs. control	0.90	0.4
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4N8	IGL	1668	222	23	Evidence at transcript level	No difference vs. control	0.89	0.4
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E562	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.89	0.4
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E955	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.93	0.4
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E556	IGL	1668	225	24	Evidence at transcript level	No difference vs. control	0.89	0.39
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H1	IGL	1668	226	24	Evidence at transcript level	No difference vs. control	0.84	0.39
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E2	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.90	0.38
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q9	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.82	0.38
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E483	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.87	0.38
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K2	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.90	0.38
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H5	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.84	0.38
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E538	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.89	0.37
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D2	IGL	1668	225	24	Evidence at transcript level	No difference vs. control	0.86	0.37
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E477	IGL	1668	222	23	Evidence at transcript level	No difference vs. control	0.84	0.37
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E976	IGL	1668	209	22	Evidence at transcript level	No difference vs. control	0.89	0.37
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N9	IGL	1668	220	23	Evidence at transcript level	No difference vs. control	0.84	0.36
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9C4	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.85	0.36
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z2	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.88	0.35
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E470	IGL	1668	226	24	Evidence at transcript level	No difference vs. control	0.88	0.35
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R5	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.89	0.34
Carboxylic ester hydrolase	F6ZMG7		91	567	62	Inferred from homology	No difference vs. control	0.85	0.34
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D7	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.78	0.34
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9E9	IGL	1668	225	24	Evidence at transcript level	No difference vs. control	0.87	0.34
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A8	IGL	1610	224	23	Evidence at transcript level	No difference vs. control	0.84	0.33
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J3	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.76	0.33
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E412	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.77	0.33
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6L0	IGL	1668	222	23	Evidence at transcript level	No difference vs. control	0.79	0.32
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4T2	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.80	0.31
Uncharacterized protein	H9GZR2		279	338	37	Predicted	No difference vs. control	0.90	0.31
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4L2	IGL	1668	224	23	Evidence at transcript level	No difference vs. control	0.79	0.31
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6I9	IGL	1668	223	23	Evidence at transcript level	No difference vs. control	0.84	0.3
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D0	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.83	0.29
Alpha-1B-glycoprotein	F6VJR6	A1BG	169	500	54	Predicted	No difference vs. control	0.72	0.28
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R9	IGL	1668	225	23	Evidence at transcript level	No difference vs. control	0.76	0.28
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K7	IGL	1668	221	23	Evidence at transcript level	No difference vs. control	0.84	0.26
Eukaryotic translation initiation factor 3 subunit A-like protein (Fragment)	K9KBY6		160	214	27	Evidence at transcript level	No difference vs. control	0.51	0.26
Transferrin (Fragment)	Q9TQR3		623	48	5	Predicted	No difference vs. control	0.76	0.24
Eukaryotic translation initiation factor 3 subunit A (eIF3a) (Eukaryotic translation initiation factor 3 subunit 10) (eIF-3-theta)	F6XE64	EIF3A EIF3S10	107	1345	162	Inferred from homology	No difference vs. control	0.53	0.23
Uncharacterized protein	H9GZS6		279	338	37	Predicted	No difference vs. control	0.87	0.22
Uncharacterized protein	H9GZU9		1177	325	36	Predicted	No difference vs. control	0.86	0.17
Transferrin (Fragment)	Q9TQV7		1460	54	6	Predicted	No difference vs. control	0.69	0.17
Transferrin (Fragment)	Q71UB5		2391	53	6	Predicted	No difference vs. control	0.64	0.14
Transferrin (Fragment)	O97971		2391	53	6	Predicted	No difference vs. control	0.69	0.13
Gelsolin (Actin-depolymerizing factor) (ADF) (Brevin)	Q28372	GSN	302	731	81	Evidence at protein level	No difference vs. control	0.64	0.12
Uncharacterized protein	H9GZU8		322	324	36	Predicted	No difference vs. control	0.75	0.1
Transferrin (Fragment)	Q9TQV8		865	189	21	Predicted	No difference vs. control	0.75	0.1
Uncharacterized protein	F6PKE1		838	340	37	Predicted	No difference vs. control	0.42	0.08
Transferrin (Fragment)	Q9TQV1		1459	58	7	Predicted	Down-regulated	0.52	0.01
Alpha 2-HS glycoprotein	F7C450	AHSG	681	358	38	Predicted	Down-regulated	0.52	0
Serotransferrin (Transferrin) (Beta-1 metal-binding globulin) (Siderophilin)	P27425	TF	1098	706	78	Evidence at transcript level	Down-regulated	0.79	0

aAccess Number is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Table 2. Identified proteins in the compare group AD-G After vs. Before

Identified Proteins (n = 211)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (After:Before)	*p* Value ratio
Uncharacterized protein	F6QVA4		1518	111	12	Predicted	Unique After	After	After
Calcium binding protein 5	F6Q873	CABP5	654	173	20	Predicted	Unique After	After	After
Splicing regulatory glutamic acid and lysine rich protein 1	F6Q3N2	SREK1	372	512	60	Predicted	Unique After	After	After
Splicing factor, arginine/serine-rich 12-like protein (Fragment)	K9KFX6		372	254	31	Evidence at transcript level	Unique After	After	After
WASH complex subunit 5	F6RQ92	WASHC5	372	1165	135	Predicted	Unique After	After	After
Strumpellin-like protein (Fragment)	K9KB53		363	449	52	Evidence at transcript level	Unique After	After	After
Uncharacterized protein	H9GZV9		336	118	13	Predicted	Unique After	After	After
Albumin (Fragment)	Q95MC2		16418	44	5	Predicted	Unique Before	Before	Before
Serpin I (Fragment)	Q7M389		12040	54	6	Evidence at protein level	Unique Before	Before	Before
Serpin II (Fragment)	Q7M388		12040	54	6	Evidence at protein level	Unique Before	Before	Before
Serpin III (Fragment)	Q7M387		12040	49	6	Evidence at protein level	Unique Before	Before	Before
Uncharacterized protein	F6YYP6		6140	99	11	Inferred from homology	Unique Before	Before	Before
Prostaglandin-H2 D-isomerase (Glutathione-independent PGD synthase) (Lipocalin-type prostaglandin-D synthase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS) (PGDS2)	O97921	PTGDS	2652	194	22	Evidence at protein level	Unique Before	Before	Before
Clone 7 transferrin (Pop-variant * transferrin) (Pop-variant D, F1, F2, F3, G, H2, O transferrin) (Fragment)	Q9TQV4		1997	10	1	Predicted	Unique Before	Before	Before
Fibrinogen alpha chain [Cleaved into: Fibrinopeptide A] (Fragment)	P14452	FGA	1632	14	2	Evidence at protein level	Unique Before	Before	Before
tRNA (Adenine-N(1)-)-methyltransferase non-catalytic subunit TRM6-like protein (Fragment)	K9KBP0		1574	247	28	Evidence at transcript level	Unique Before	Before	Before
tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6	F6Z527	TRMT6	1571	497	56	Inferred from homology	Unique Before	Before	Before
Transthyretin	F6UL68	TTR	1106	147	16	Inferred from homology	Unique Before	Before	Before
Alpha-1B-glycoprotein	F6VJR6	A1BG	969	500	54	Predicted	Unique Before	Before	Before
Apolipoprotein A1	F6Z2L5	APOA1	695	266	30	Inferred from homology	Unique Before	Before	Before
Fibrinogen beta chain	F6PH38	FGB	587	490	56	Predicted	Unique Before	Before	Before
Inter-alpha-trypsin inhibitor heavy chain 2	F7CTJ3	ITIH2	529	947	106	Predicted	Unique Before	Before	Before
Inter-alpha-trypsin inhibitor heavy chain 2	F7DAF6	ITIH2	529	949	107	Predicted	Unique Before	Before	Before
Inter-alpha-trypsin inhibitor heavy chain 2	F7DL17	ITIH2	529	946	106	Predicted	Unique Before	Before	Before
Apolipoprotein D (Apo-D)	F6XM13	APOD	496	213	24	Inferred from homology	Unique Before	Before	Before
Inter-alpha (Globulin) inhibitor, H2 polypeptide (Fragment)	A6P3C5	ITIH2	468	19	2	Predicted	Unique Before	Before	Before
Ceruloplasmin (Fragment)	Q9N1X0	CP	427	40	5	Predicted	Unique Before	Before	Before
Ceruloplasmin	F6PQ46	CP	427	1068	122	Inferred from homology	Unique Before	Before	Before
Globin A1 (Hemoglobin subunit beta)	F6RDD3	HBB GLNA1	413	147	16	Inferred from homology	Unique Before	Before	Before
Alpha-1-microglobulin/bikunin precursor	F6UZH0	AMBP	405	352	39	Predicted	Unique Before	Before	Before
Fibrinogen alpha chain (Fragment)	Q7M2U4		382	270	28	Evidence at protein level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV12	Spi2-13	366	421	47	Evidence at transcript level	Unique Before	Before	Before
Chromogranin-A	F7BS54	CHGA	365	448	50	Predicted	Unique Before	Before	Before
Uncharacterized protein	F6ZI35		361	236	27	Predicted	Unique Before	Before	Before
Uncharacterized protein	F7DXM5	LOC100065068	360	422	47	Inferred from homology	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV11	Spi2-12	360	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV10	Spi2-11	360	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV09	Spi2-10	360	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV08	Spi2-9	360	421	47	Evidence at transcript level	Unique Before	Before	Before
Hemoglobin subunit beta (Beta-globin) (Hemoglobin beta chain)	P02062	HBB	356	146	16	Evidence at protein level	Unique Before	Before	Before
NPC intracellular cholesterol transporter 2	F7CIW5	NPC2	348	149	16	Predicted	Unique Before	Before	Before
Fibrinogen A-alpha chain (Fragment)	O97641		345	481	50	Predicted	Unique Before	Before	Before
Hemopexin	F6X1I8	HPX	344	462	51	Inferred from homology	Unique Before	Before	Before
GC, vitamin D binding protein	F6T0P6	GC	338	474	53	Predicted	Unique Before	Before	Before
Uncharacterized protein	H9GZS9		314	354	39	Predicted	Unique Before	Before	Before
Plasminogen	F6USP9	PLG	295	817	92	Inferred from homology	Unique Before	Before	Before
Fibrinogen alpha chain	F6RUZ6	FGA	287	795	88	Predicted	Unique Before	Before	Before
Uncharacterized protein	F7CYP1		278	423	47	Inferred from homology	Unique Before	Before	Before
Uncharacterized protein	F7CSL8		278	421	47	Inferred from homology	Unique Before	Before	Before
FRY like transcription coactivator	F6SV16	FRYL	276	3014	339	Predicted	Unique Before	Before	Before
Protein furry-like protein-like-like protein (Fragment)	K9KAG8		274	142	16	Evidence at transcript level	Unique Before	Before	Before
Globin B1	F6RF57	.OC100068926 GLNB	256	147	16	Inferred from homology	Unique Before	Before	Before
Uncharacterized protein	H9GZT5		255	335	36	Predicted	Unique Before	Before	Before
Uncharacterized protein	Q28383		242	142	15	Inferred from homology	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV07	Spi2-8	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV06	Spi2-7	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV05	Spi2-6	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV04	Spi2-5	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV03	Spi2-4	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV02	Spi2-3	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV01	Spi2-2	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV00	Spi2-1	235	421	47	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	F7BF31	SPI2	235	421	47	Inferred from homology	Unique Before	Before	Before
Hemoglobin-alpha 1 (Fragment)	Q95MA1		229	90	10	Inferred from homology	Unique Before	Before	Before
Serpin family C member 1	F7CYR1	SERPINC1	222	463	52	Inferred from homology	Unique Before	Before	Before
Uncharacterized protein	F6PX34	LOC100629770	218	102	12	Predicted	Unique Before	Before	Before
Alpha-1-antitrypsin	B5BV13	Spi2-14	215	421	47	Evidence at transcript level	Unique Before	Before	Before
Serpin family F member 1	F7BKE1	SERPINF1	205	417	46	Inferred from homology	Unique Before	Before	Before
Uncharacterized protein	F7BLE3		200	1215	135	Predicted	Unique Before	Before	Before
Uncharacterized protein	F6UU86		192	384	42	Inferred from homology	Unique Before	Before	Before
Carboxylic ester hydrolase	F6VXP7		191	565	62	Inferred from homology	Unique Before	Before	Before
Carboxylic ester hydrolase	F6ZMG7		191	567	62	Inferred from homology	Unique Before	Before	Before
Carboxylic ester hydrolase	F6PRI5	LOC100051065	191	565	62	Inferred from homology	Unique Before	Before	Before
Pigment epithelium-derived factor	B8K250		189	417	46	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	F7CJG3	LOC100060539	188	1661	186	Predicted	Unique Before	Before	Before
Uncharacterized protein	F7E454	LOC100060539	188	1640	184	Predicted	Unique Before	Before	Before
Uncharacterized protein	F7BTW7	LOC100060539	188	1664	186	Predicted	Unique Before	Before	Before
Prothrombin (Coagulation factor II)	F7BFJ1	F2	156	623	70	Inferred from homology	Unique Before	Before	Before
Complement factor B-like protein (Fragment)	K9KBR1		145	227	26	Evidence at transcript level	Unique Before	Before	Before
Transferrin (Fragment)	Q9TQR3		139	48	5	Predicted	Unique Before	Before	Before
Fibrinogen gamma chain	F6W2Y1	FGG	135	437	50	Predicted	Unique Before	Before	Before
Alpha-2-macroglobulin	F6RI47	A2M	124	1477	164	Predicted	Unique Before	Before	Before
Angiotensinogen	F6W4R2	AGT	121	485	53	Inferred from homology	Unique Before	Before	Before
Secreted phosphoprotein 1	F7AYC1	SPP1	121	313	35	Predicted	Unique Before	Before	Before
Atlastin GTPase 3	F7BTZ8	ATL3	118	541	60	Predicted	Unique Before	Before	Before
TSC22 domain family member 4	F6TZA6	TSC22D4	115	395	41	Predicted	Unique Before	Before	Before
PR/SET domain 2	F6RX96	PRDM2	115	1709	187	Predicted	Unique Before	Before	Before
Ankyrin repeat domain 13D	F6VQ75	ANKRD13D	114	604	68	Predicted	Unique Before	Before	Before
PR domain zinc finger protein 2-like protein (Fragment)	K9KCY3		111	343	39	Evidence at transcript level	Unique Before	Before	Before
Atlastin-3-like protein (Fragment)	K9KFN6		105	354	40	Evidence at transcript level	Unique Before	Before	Before
Phospholipid transfer protein	F7CR03	PLTP	105	496	55	Predicted	Unique Before	Before	Before
Alpha-2-macroglobulin	F6R942	A2M	103	1462	162	Predicted	Unique Before	Before	Before
Plasminogen (Fragment)	Q9TV90	PLG	103	73	8	Predicted	Unique Before	Before	Before
Neuronal cell adhesion molecule	F6YWR0	NRCAM	101	1216	134	Predicted	Unique Before	Before	Before
Amyloid beta A4 protein-like protein (Fragment)	K9KAU3		100	252	29	Evidence at transcript level	Unique Before	Before	Before
Neuronal cell adhesion molecule	F6YXY0	NRCAM	96	1286	142	Predicted	Unique Before	Before	Before
Neuronal cell adhesion molecule	F6WJG4	NRCAM	96	1301	143	Predicted	Unique Before	Before	Before
Neuronal cell adhesion molecule	F6YWP6	NRCAM	96	1199	132	Predicted	Unique Before	Before	Before
Neuronal cell adhesion molecule	F6XBU8	NRCAM	96	1306	144	Predicted	Unique Before	Before	Before
Procollagen C-endopeptidase enhancer	F6WZ69	PCOLCE	85	459	49	Predicted	Unique Before	Before	Before
Uncharacterized protein	F7E0F5		83	403	46	Predicted	Unique Before	Before	Before
ADP ribosylation factor related protein 1	F7DJ61	ARFRP1	82	201	23	Inferred from homology	Unique Before	Before	Before
Uncharacterized protein	F6XSF7	LOC100059239	78	1750	193	Predicted	Unique Before	Before	Before
Serine palmitoyltransferase long chain base subunit 2	F6T8N8	SPTLC2	76	518	59	Inferred from homology	Unique Before	Before	Before
Amyloid beta precursor protein	F6XKR3	APP	68	732	83	Predicted	Unique Before	Before	Before
Amyloid beta precursor protein	F6XL78	APP	68	751	85	Predicted	Unique Before	Before	Before
APC down-regulated 1 like	F6UT81	APCDD1L	66	411	46	Predicted	Unique Before	Before	Before
Sulfotransferase	F6UTD8		60	278	32	Inferred from homology	Unique Before	Before	Before
Lactotransferrin (Lactoferrin) (Fragment)	O77811	LTF	59	695	76	Evidence at protein level	Unique Before	Before	Before
Lactotransferrin	F6XLB1	LTF	59	708	77	Inferred from homology	Unique Before	Before	Before
Antithrombin III protein (Fragment)	Q9N1X2	AT3	53	60	7	Predicted	Unique Before	Before	Before
Apolipoprotein H	F6Z041	APOH	52	345	39	Predicted	Unique Before	Before	Before
Uncharacterized protein	F6RMD0	CFB	50	768	86	Inferred from homology	Unique Before	Before	Before
Transferrin (Fragment)	O97678		290	61	7	Predicted	Up-regulated	8.67	1
Transferrin (Fragment)	Q71UB5		3109	53	6	Predicted	Up-regulated	10.38	1
Alpha 2-HS glycoprotein	F7C450	AHSG	1023	358	38	Predicted	Up-regulated	2.41	1
Uncharacterized protein	F6ZEH8		7667	990	109	Predicted	Up-regulated	10.59	1
Haptoglobin	F6XWM5		779	346	38	Inferred from homology	Up-regulated	5.81	1
Gelsolin	F7E2D1	GSN	111	783	86	Predicted	Up-regulated	9.12	1
Transferrin (Fragment)	Q9TQW7		1047	52	6	Predicted	Up-regulated	4.66	1
Transferrin (Fragment)	O97973		290	61	7	Predicted	Up-regulated	9.21	1
Transferrin (Fragment)	O97971		3109	53	6	Predicted	Up-regulated	12.06	1
Transferrin (Fragment)	Q9TQV7		423	54	6	Predicted	Up-regulated	10.07	1
Transferrin (Fragment)	Q9TQV1		1508	58	7	Predicted	Up-regulated	9.21	1
Transferrin (Fragment)	Q9TQR4		3710	63	7	Predicted	Up-regulated	8.85	1
Dickkopf WNT signaling pathway inhibitor 3	F6WE78	DKK3	602	324	35	Predicted	Up-regulated	5.75	1
Serotransferrin (Transferrin) (Beta-1 metal-binding globulin) (Siderophilin)	P27425	TF	836	706	78	Evidence at transcript level	Up-regulated	11.70	1
Clusterin [Cleaved into: Clusterin beta chain; Clusterin alpha chain]	Q29482	CLU	901	449	52	Evidence at transcript level	Up-regulated	3.22	1
Uncharacterized protein	F6PKE1		80	340	37	Predicted	Up-regulated	11.59	0.99
Transferrin (Fragment)	Q9TQW8		1992	52	6	Predicted	Up-regulated	4.01	0.99
Kallikrein related peptidase 6	F6SIF7	KLK6	319	244	27	Inferred from homology	Up-regulated	5.81	0.99
Transferrin (Fragment)	O97966		119	61	7	Predicted	Up-regulated	10.07	0.96
Immunoglobulin gamma 1 heavy chain constant region (Fragment)	Q95M34	IGHC1	1239	337	37	Evidence at transcript level	No difference vs. control	1.62	0.72
Uncharacterized protein	H9GZQ9		1236	337	37	Predicted	No difference vs. control	1.73	0.66
Uncharacterized protein	H9GZU8		1283	324	36	Predicted	No difference vs. control	0.90	0.36
Uncharacterized protein	H9GZR2		859	338	37	Predicted	No difference vs. control	0.91	0.32
Uncharacterized protein	H9GZS6		859	338	37	Predicted	No difference vs. control	0.90	0.3
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E464	IGL	665	224	24	Evidence at transcript level	No difference vs. control	0.86	0.23
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R9	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.86	0.21
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E976	IGL	665	209	22	Evidence at transcript level	No difference vs. control	0.84	0.21
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E412	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.89	0.21
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H5	IGL	665	222	23	Evidence at transcript level	No difference vs. control	0.82	0.19
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H1	IGL	665	226	23	Evidence at transcript level	No difference vs. control	0.83	0.18
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4T2	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.85	0.17
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4R8	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.81	0.17
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N5	IGL	665	226	24	Evidence at transcript level	No difference vs. control	0.79	0.16
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K4	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.82	0.16
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z9	IGL	665	222	23	Evidence at transcript level	No difference vs. control	0.86	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E8	IGL	665	223	23	Evidence at transcript level	No difference vs. control	0.77	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D2	IGL	665	225	24	Evidence at transcript level	No difference vs. control	0.85	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E470	IGL	665	226	24	Evidence at transcript level	No difference vs. control	0.75	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6L0	IGL	665	222	23	Evidence at transcript level	No difference vs. control	0.84	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9C4	IGL	665	224	24	Evidence at transcript level	No difference vs. control	0.82	0.14
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A3	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.78	0.13
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q6	IGL	665	225	24	Evidence at transcript level	No difference vs. control	0.76	0.13
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E532	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.80	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q9	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.77	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J6	IGL	665	219	23	Evidence at transcript level	No difference vs. control	0.83	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E971	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.77	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E2	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.78	0.11
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E556	IGL	665	225	24	Evidence at transcript level	No difference vs. control	0.77	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E483	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.78	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4N8	IGL	665	222	23	Evidence at transcript level	No difference vs. control	0.80	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D0	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.76	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E567	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.75	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E538	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.77	0.09
Uncharacterized protein	F7CAC5		665	117	12	Predicted	No difference vs. control	0.76	0.09
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E447	IGL	665	223	23	Evidence at transcript level	No difference vs. control	0.76	0.09
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K2	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.77	0.09
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E588	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.77	0.09
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6I9	IGL	665	223	23	Evidence at transcript level	No difference vs. control	0.79	0.09
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A8	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.79	0.08
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N9	IGL	665	220	23	Evidence at transcript level	No difference vs. control	0.77	0.08
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9E9	IGL	665	225	24	Evidence at transcript level	No difference vs. control	0.76	0.08
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K7	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.76	0.08
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E417	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.76	0.08
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E966	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.75	0.08
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z2	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.77	0.07
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R5	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.78	0.07
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E452	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.76	0.07
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E961	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.78	0.07
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E562	IGL	665	221	23	Evidence at transcript level	No difference vs. control	0.76	0.07
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4S5	IGL	665	222	23	Evidence at transcript level	No difference vs. control	0.76	0.06
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D7	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.76	0.06
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J3	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.76	0.06
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E406	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.76	0.06
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E955	IGL	665	225	23	Evidence at transcript level	No difference vs. control	0.74	0.06
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4L2	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.77	0.05
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J8	IGL	665	224	23	Evidence at transcript level	No difference vs. control	0.75	0.05
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Y3	IGL	665	225	23	Evidence at transcript level	Down-regulated	0.66	0.04
Uncharacterized protein	F6QAU5		665	107	11	Predicted	Down-regulated	0.66	0.04
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E477	IGL	665	222	23	Evidence at transcript level	Down-regulated	0.77	0.04
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4A9	IGL	665	223	23	Evidence at transcript level	Down-regulated	0.75	0.04
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D7	IGL	665	225	23	Evidence at transcript level	Down-regulated	0.65	0.03
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H3	IGL	665	221	23	Evidence at transcript level	Down-regulated	0.64	0.03
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E938	IGL	665	226	24	Evidence at transcript level	Down-regulated	0.66	0.02
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X3	IGL	665	222	23	Evidence at transcript level	Down-regulated	0.66	0.02
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E493	IGL	665	224	23	Evidence at transcript level	Down-regulated	0.67	0.02
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B2	IGL	665	224	23	Evidence at transcript level	Down-regulated	0.65	0.02
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4B1	IGL	665	225	24	Evidence at transcript level	Down-regulated	0.64	0.02
Transferrin (Fragment)	Q9TQV8		773	189	21	Predicted	Down-regulated	0.44	0.02
Uncharacterized protein	F6SQD7		665	107	11	Predicted	Down-regulated	0.64	0.02
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H7	IGL	665	225	23	Evidence at transcript level	Down-regulated	0.66	0.02
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E944	IGL	665	225	24	Evidence at transcript level	Down-regulated	0.66	0.01
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E933	IGL	665	223	23	Evidence at transcript level	Down-regulated	0.64	0.01
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X7	IGL	665	226	24	Evidence at transcript level	Down-regulated	0.66	0.01
Uncharacterized protein	H9GZU9		2501	325	36	Predicted	Down-regulated	0.67	0
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4E7	IGL	665	225	24	Evidence at transcript level	Down-regulated	0.68	0
Serum albumin (allergen Equ c 3)	P35747	ALB	13118	607	69	Evidence at protein level	Down-regulated	0.50	0
Serum albumin	F7BAY6	ALB	33074	607	68	Predicted	Down-regulated	0.52	0
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B6	IGL	665	225	24	Evidence at transcript level	Down-regulated	0.63	0

aAccess Number is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Table 3. Identified proteins in the compare group BM-G After vs. Before

Identified Proteins (n = 131)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (After:Before)	*p* Value ratio
Uncharacterized protein	F6YYP6		559	99	11	Inferred from homology	Unique After	After	After
Albumin (Fragment)	Q95MC2		464	44	5	Predicted	Unique After	After	After
Clone 7 transferrin (Pop-variant * transferrin) (Pop-variant D, F1, F2, F3, G, H2, O transferrin) (Fragment)	Q9TQV4		369	10	1	Predicted	Unique After	After	After
Plasminogen	F6USP9	PLG	314	817	92	Inferred from homology	Unique After	After	After
Plasminogen [Cleaved into: Plasmin heavy chain A; Plasmin light chain B] (Fragment)	P80010	PLG	314	338	37	Evidence at protein level	Unique After	After	After
Uncharacterized protein	F6RMD0	CFB	301	768	86	Inferred from homology	Unique After	After	After
Myosin light chain kinase family member 4	F7A949	MYLK4	293	310	35	Inferred from homology	Unique After	After	After
Complement factor B-like protein (Fragment)	K9KBR1		279	227	26	Evidence at transcript level	Unique After	After	After
Prostaglandin-H2 D-isomerase (Glutathione-independent PGD synthase) (Lipocalin-type prostaglandin-D synthase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS) (PGDS2)	O97921	PTGDS	278	194	22	Evidence at protein level	Unique After	After	After
Transferrin (Fragment)	Q9TQW8		275	52	6	Predicted	Unique After	After	After
Uncharacterized protein	H9GZQ9		228	337	37	Predicted	Unique After	After	After
Immunoglobulin gamma 1 heavy chain constant region (Fragment)	Q95M34	IGHC1	228	337	37	Evidence at transcript level	Unique After	After	After
Fem-1 homolog C	F7AJU7	FEM1C	214	617	69	Predicted	Unique After	After	After
Golgi phosphoprotein 3-like protein (Fragment)	K9K4P9		210	199	23	Evidence at transcript level	Unique After	After	After
Golgi phosphoprotein 3	F6ZER1	GOLPH3	210	236	27	Predicted	Unique After	After	After
Prothrombin (Coagulation factor II)	F7BFJ1	F2	170	623	70	Inferred from homology	Unique After	After	After
Carboxylic ester hydrolase	F6ZMG7		144	567	62	Inferred from homology	Unique After	After	After
GC, vitamin D binding protein	F6T0P6	GC	131	474	53	Predicted	Unique After	After	After
Hemopexin	F6X1I8	HPX	129	462	51	Inferred from homology	Unique After	After	After
Carboxylic ester hydrolase	F6VXP7		124	565	62	Inferred from homology	Unique After	After	After
Carboxylic ester hydrolase	F6PRI5	LOC100051065	124	565	62	Inferred from homology	Unique After	After	After
Fibrinogen gamma chain	F6W2Y1	FGG	111	437	50	Predicted	Unique After	After	After
Protein kinase cAMP-activated catalytic subunit beta	F6TVJ6	PRKACB	103	354	41	Inferred from homology	Unique After	After	After
Protein kinase cAMP-activated catalytic subunit alpha	F7AI06	PRKACA	103	343	40	Inferred from homology	Unique After	After	After
Uncharacterized protein	F6PKE1		102	340	37	Predicted	Unique After	After	After
Alpha-1B-glycoprotein	F6VJR6	A1BG	101	500	54	Predicted	Unique After	After	After
Uncharacterized protein	H9GZS6		84	338	37	Predicted	Unique After	After	After
Uncharacterized protein	H9GZR2		84	338	37	Predicted	Unique After	After	After
Uncharacterized protein	H9GZT5		57	335	36	Predicted	Unique After	After	After
Uncharacterized protein	F6WCY1		12	422	47	Predicted	Unique After	After	After
Alpha-enolase-like protein (Fragment)	K9K2B9		12	310	34	Evidence at transcript level	Unique After	After	After
Enolase 2 (Fragment)	I3RM61	ENO2	12	424	46	Evidence at transcript level	Unique After	After	After
Enolase 3	F7C959	ENO3	12	434	47	Evidence at transcript level	Unique After	After	After
Enolase 2	F7CIX6	ENO2	12	434	47	Inferred from homology	Unique After	After	After
Enolase 1	F6V7C1	ENO1	12	434	47	Evidence at transcript level	Unique After	After	After
Antithrombin III protein (Fragment)	Q9N1X2	AT3	452	60	7	Predicted	Unique Before	Before	Before
Alpha-lactalbumin B/C (Lactose synthase B protein)	P08896		302	123	14	Evidence at protein level	Unique Before	Before	Before
Superoxide dismutase [Cu-Zn]	P00443	SOD1	281	154	16	Evidence at protein level	Unique Before	Before	Before
Retinol-binding protein 4 (Plasma retinol-binding protein) (PRBP) (RBP)	Q28369	RBP4	266	201	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A8	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E556	IGL	220	225	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A3	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4T2	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z9	IGL	220	222	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z2	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4S5	IGL	220	222	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R9	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R5	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E538	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4R8	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E532	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E8	IGL	220	223	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E2	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q9	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q6	IGL	220	225	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E483	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D7	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D2	IGL	220	225	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E477	IGL	220	222	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E470	IGL	220	226	24	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	F7CAC5		220	117	12	Predicted	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E464	IGL	220	224	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N9	IGL	220	220	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N5	IGL	220	226	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4N8	IGL	220	222	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E452	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4A9	IGL	220	223	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E447	IGL	220	223	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6L0	IGL	220	222	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4L2	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9E9	IGL	220	225	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K7	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K4	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K2	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E588	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D0	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J8	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J6	IGL	220	219	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J3	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E976	IGL	220	209	22	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E971	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E417	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9C4	IGL	220	224	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E412	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6I9	IGL	220	223	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E966	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E961	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E406	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E567	IGL	220	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E562	IGL	220	221	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H5	IGL	220	222	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H1	IGL	220	226	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E955	IGL	220	225	23	Evidence at transcript level	Unique Before	Before	Before
Apolipoprotein D (Apo-D)	F6W135	RBP4	209	164	19	Inferred from homology	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E944	IGL	176	225	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Y3	IGL	176	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E938	IGL	176	226	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E933	IGL	176	223	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X7	IGL	176	226	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X3	IGL	176	222	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E493	IGL	176	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4E7	IGL	176	225	24	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	F6QAU5		176	107	11	Predicted	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B6	IGL	176	225	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B2	IGL	176	224	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4B1	IGL	176	225	24	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D7	IGL	176	225	23	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	F6SQD7		176	107	11	Predicted	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H7	IGL	176	225	23	Evidence at transcript level	Unique Before	Before	Before
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H3	IGL	176	221	23	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	F6ZI35		174	236	27	Predicted	Unique Before	Before	Before
Secreted phosphoprotein 1	F7AYC1	SPP1	146	313	35	Predicted	Unique Before	Before	Before
DnaJ heat shock protein family (Hsp40) member B14	F6TQ51	DNAJB14	142	335	37	Predicted	Unique Before	Before	Before
Uncharacterized protein	F6VH22		138	220	24	Inferred from homology	Unique Before	Before	Before
Eukaryotic translation initiation factor 3 subunit A-like protein (Fragment)	K9KBY6		125	214	27	Evidence at transcript level	Unique Before	Before	Before
Uncharacterized protein	H9GZU9		172	325	36	Predicted	Up-regulated	0.53	1
Uncharacterized protein	H9GZU8		149	324	36	Predicted	Up-regulated	1.01	1
Clusterin [Cleaved into: Clusterin beta chain; Clusterin alpha chain]	Q29482	CLU	317	449	52	Evidence at transcript level	Up-regulated	0.49	1
Serotransferrin (Transferrin) (Beta-1 metal-binding globulin) (Siderophilin)	P27425	TF	460	706	78	Evidence at transcript level	Up-regulated	0.32	0.99
Serum albumin (allergen Equ c 3)	P35747	ALB	3865	607	69	Evidence at protein level	Up-regulated	0.06	0.97
Haptoglobin	F6XWM5		361	346	38	Inferred from homology	No difference vs. control	1.78	0.91
Transferrin (Fragment)	Q9TQV8		265	189	21	Predicted	No difference vs. control	0.87	0.86
Transferrin (Fragment)	O97973		194	61	7	Predicted	No difference vs. control	2.14	0.73
Transferrin (Fragment)	O97966		194	61	7	Predicted	No difference vs. control	1.83	0.64
Transferrin (Fragment)	O97678		194	61	7	Predicted	No difference vs. control	1.75	0.63
Transferrin (Fragment)	Q9TQV1		190	58	7	Predicted	No difference vs. control	1.97	0.52
Uncharacterized protein	F6ZEH8		4546	990	109	Predicted	No difference vs. control	0.58	0.44
Transferrin (Fragment)	Q9TQR4		3105	63	7	Predicted	No difference vs. control	1.03	0.08
Transferrin (Fragment)	O97971		2087	53	6	Predicted	No difference vs. control	1.46	0.07
Transferrin (Fragment)	Q71UB5		2087	53	6	Predicted	Down-regulated	1.39	0.04
Serum albumin	F7BAY6	ALB	5480	607	68	Predicted	Down-regulated	0.13	0.02

aAccess Number is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

The web-available STRING v10.5 (http://string-db.org/), by choosing the variables "Multiples proteins" and "organism *Equus caballus*", identified 66 access numbers from our total data obtained on Control comparison. Likewise, 69 and 38 access numbers were availabled for the comparisons of AD-G and BM-G, respectively.

Evaluating the interactions between the proteins identified with differential expression and exclusives between the groups compared in the web-available STRING v10.5, in the Control comparison (After:Before), 45 access numbers were found, 22 were exclusives of the After moment, 21 exclusives of the Before moment, 1 up-regulated and 1 down-regulated (Table 4). Among these proteins, it was possible to

identify an interaction between 3 types of enolases (Figure 1), respectively, ENO 1 (Enolase 1), ENO 2 (Enolase 2) and ENO 3 (Enolase 3).

Table 4. Identified proteins exclusives and with diferential expression in the compare group Control After vs. Before from the web-available STRING v10.5

[a]Access Number	Protein names	Gene names	Expression
F6X1I8	Hemopexin	HPX	Unique after
F6XR18	Ubiquitin conjugating enzyme E2 B	UBE2B	Unique after
F7BNF7	Ubiquitin conjugating enzyme E2 A	UBE2A	Unique after
F6WE78	Dickkopf WNT signaling pathway inhibitor 3	DKK3	Unique after
H9GZT5	Uncharacterized protein		Unique after
F6WCY1	Uncharacterized protein		Unique after
F7C959	Enolase 3	ENO3	Unique after
F7CIX6	Enolase 2	ENO2	Unique after
F6V7C1	Enolase 1	ENO1	Unique after
F7DRK3	MINDY lysine 48 deubiquitinase 3	MINDY3	Unique after
F7CR03	Phospholipid transfer protein	PLTP	Unique after
F7CYP1	Uncharacterized protein		Unique after
F7DXM5	Uncharacterized protein	LOC100065068	Unique after
P38029	Alpha-1-antiproteinase 2 (Alpha-1-antitrypsin 2) (Alpha-1-proteinase inhibitor 2) (SPI2)		Unique after
F7AYC1	Secreted phosphoprotein 1	SPP1	Unique after
F7E0F5	Uncharacterized protein		Unique after
F6VRG6	Glutamic-oxaloacetic transaminase 1 like 1	GOT1L1	Unique after
F7E2D1	Gelsolin	GSN	Unique after
F7B320	Dihydropyrimidinase like 2	DPYSL2	Unique after
F6ZH40	Tuftelin 1	TUFT1	Unique after
F6W4R2	Angiotensinogen	AGT	Unique after
F7BKE1	Serpin family F member 1	SERPINF1	Unique after
F6Y9A3	Cardiolipin synthase 1	CRLS1	Unique before
P80010	Plasminogen [Cleaved into: Plasmin heavy chain A; Plasmin light chain B] (Fragment)	PLG	Unique before
F6UAH4	Syntaxin 19	STX19	Unique before
O77811	Lactotransferrin (Lactoferrin) (Fragment)	LTF	Unique before
F6TJ01	Uncharacterized protein	CNTRL	Unique before
P49066	Alpha-fetoprotein (Alpha-1-fetoprotein) (Alpha-fetoglobulin)	AFP	Unique before
F6XL68	GLI family zinc finger 3	GLI3	Unique before
F7DF05	Phosphatidylinositol specific phospholipase C X domain containing 3	PLCXD3	Unique before
F6WZG2	Solute carrier family 25 member 28	SLC25A28	Unique before
F6R5G1	Uncharacterized protein		Unique before
F6XBK6	Ethanolamine kinase 2	ETNK2	Unique before
F7DCC9	WD repeat and SOCS box containing 2	WSB2	Unique before
F6USA2	PDZ domain containing 8	PDZD8	Unique before
F6ULM8	Anti-silencing function 1B histone chaperone	ASF1B	Unique before
F6SCB6	Nipsnap homolog 2	NIPSNAP2	Unique before
F7DEW5	Coronin	CORO1C	Unique before
F7BTW7	Uncharacterized protein	LOC100060539	Unique before
F6WGS2	IQ motif containing GTPase activating protein 3	IQGAP3	Unique before
F7CUH0	Family with sequence similarity 172 member A	FAM172A	Unique before
F6TE84	Nuclear receptor subfamily 4 group A member 3	NR4A3	Unique before
F6YRC5	IQ motif containing GTPase activating protein 1	IQGAP1	Unique before
P35747	Serum albumin (allergen Equ c 3)	ALB	Up-regulated
F7C450	Alpha 2-HS glycoprotein	AHSG	Down-regulated

aAccess Number is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

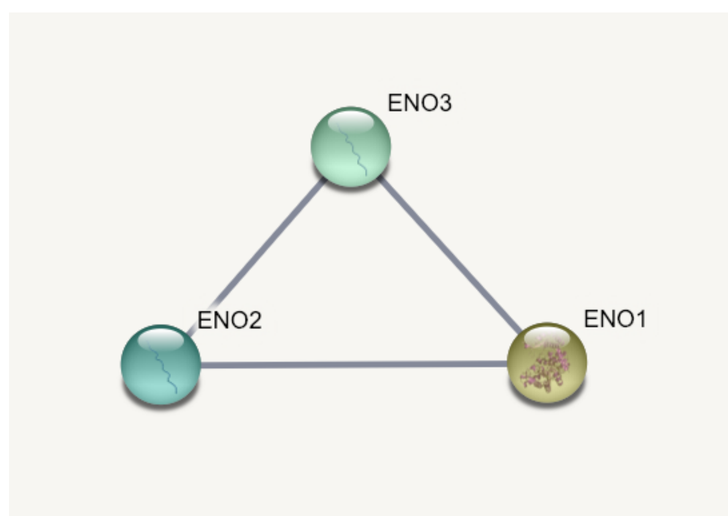

Figure 1. String interaction between 3 types of enolases,
ENO 1 (Enolase 1), ENO 2 (Enolase 2) and ENO 3 (Enolase 3)

The interactions between the proteins identified with differential expression and exclusives between AD-G After vs. AD-G Before in the web-available STRING v10.5, obtained 64 access numbers, 4 were exclusives of the After moment, 49 exclusives of the Before moment, 7 up-regulated and 4 down-regulated (Table 5). In addition, 35 GO terms (Gene Ontology) related to the biological processes associated with these proteins were identified (Table 6).

Table 5. Identified proteins exclusives and with diferential expression in the compare group AD-G After vs. Before from the web-available STRING v10.5

[a]Access Number	Protein names	Gene names	Expression
F6Q873	Calcium binding protein 5	CABP5	Unique after
F6Q3N2	Splicing regulatory glutamic acid and lysine rich protein 1	SREK1	Unique after
F6RQ92	WASH complex subunit 5	WASHC5	Unique after
H9GZV9	Uncharacterized protein		Unique after
O97921	Prostaglandin-H2 D-isomerase (Glutathione-independent PGD synthase) (Lipocalin-type prostaglandin-D synthase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS) (P	PTGDS	Unique before
F6Z527	tRNA (adenine(58)-N(1))-methyltransferase non-catalytic subunit TRM6	TRMT6	Unique before
F6UL68	Transthyretin	TTR	Unique before
F6VJR6	Alpha-1B-glycoprotein	A1BG	Unique before
F6Z2L5	Apolipoprotein A1	APOA1	Unique before
F6PH38	Fibrinogen beta chain	FGB	Unique before
F7DAF6	Inter-alpha-trypsin inhibitor heavy chain 2	ITIH2	Unique before
F6XM13	Apolipoprotein D (Apo-D)	APOD	Unique before
F6PQ46	Ceruloplasmin	CP	Unique before
F6ZI35	Uncharacterized protein		Unique before
F7DXM5	Uncharacterized protein	LOC100065068	Unique before
P02062	Hemoglobin subunit beta (Beta-globin) (Hemoglobin beta chain)	HBB	Unique before
F7CIW5	NPC intracellular cholesterol transporter 2	NPC2	Unique before
F6X1I8	Hemopexin	HPX	Unique before
F6T0P6	GC, vitamin D binding protein	GC	Unique before
H9GZS9	Uncharacterized protein		Unique before
F6RUZ6	Fibrinogen alpha chain	FGA	Unique before
F7CYP1	Uncharacterized protein		Unique before
F6SV16	FRY like transcription coactivator	FRYL	Unique before
F6RF57	Globin B1	OC100068926 GLNE	Unique before
H9GZT5	Uncharacterized protein		Unique before
F7CYR1	Serpin family C member 1	SERPINC1	Unique before
F7BKE1	Serpin family F member 1	SERPINF1	Unique before
F7BLE3	Uncharacterized protein		Unique before
F6UU86	Uncharacterized protein		Unique before
F6VXP7	Carboxylic ester hydrolase		Unique before
F6ZMG7	Carboxylic ester hydrolase		Unique before
F6PRI5	Carboxylic ester hydrolase	LOC100051065	Unique before
F7BTW7	Uncharacterized protein	LOC100060539	Unique before
F7BFJ1	Prothrombin (Coagulation factor II)	F2	Unique before
F6W2Y1	Fibrinogen gamma chain	FGG	Unique before
F6RI47	Alpha-2-macroglobulin	A2M	Unique before
F6W4R2	Angiotensinogen	AGT	Unique before
F7AYC1	Secreted phosphoprotein 1	SPP1	Unique before
F7BTZ8	Atlastin GTPase 3	ATL3	Unique before
F6TZA6	TSC22 domain family member 4	TSC22D4	Unique before
F6RX96	PR/SET domain 2	PRDM2	Unique before
F6VQ75	Ankyrin repeat domain 13D	ANKRD13D	Unique before
F7CR03	Phospholipid transfer protein	PLTP	Unique before
F6XBU8	Neuronal cell adhesion molecule	NRCAM	Unique before
F6WZ69	Procollagen C-endopeptidase enhancer	PCOLCE	Unique before
F7E0F5	Uncharacterized protein		Unique before
F7DJ61	ADP ribosylation factor related protein 1	ARFRP1	Unique before
F6XSF7	Uncharacterized protein	LOC100059239	Unique before
F6T8N8	Serine palmitoyltransferase long chain base subunit 2	SPTLC2	Unique before
F6XL78	Amyloid beta precursor protein	APP	Unique before
O77811	Lactotransferrin (Lactoferrin) (Fragment)	LTF	Unique before
F6Z041	Apolipoprotein H	APOH	Unique before
F6RMD0	Uncharacterized protein	CFB	Unique before
F7C450	Alpha 2-HS glycoprotein	AHSG	Up-regulated
F6ZEH8	Uncharacterized protein		Up-regulated
F6XWM5	Haptoglobin		Up-regulated
F7E2D1	Gelsolin	GSN	Up-regulated
F6WE78	Dickkopf WNT signaling pathway inhibitor 3	DKK3	Up-regulated
Q29482	Clusterin [Cleaved into: Clusterin beta chain; Clusterin alpha chain]	CLU	Up-regulated
F6SIF7	Kallikrein related peptidase 6	KLK6	Up-regulated
F6QAU5	Uncharacterized protein		Down-regulated
F6SQD7	Uncharacterized protein		Down-regulated
H9GZU9	Uncharacterized protein		Down-regulated
P35747	Serum albumin (allergen Equ c 3)	ALB	Down-regulated

aAccess Number is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Table 6. Biological processes linked to the proteins exclusives and with diferential expression in the compare group AD-G After vs. Before from the web-available STRING v10.5

Pathway ID	Pathway description	Observed gene count	False discovery rate
GO.0065007	biological regulation	8	7.38e-06
GO.0006810	transport	6	1.89e-05
GO.0048523	negative regulation of cellular process	6	1.89e-05
GO.0050789	regulation of biological process	7	1.96e-05
GO.1902578	single-organism localization	5	0.000114
GO.0065008	regulation of biological quality	5	0.00021
GO.0006950	response to stress	5	0.000491
GO.0051179	localization	5	0.000491
GO.0008150	biological process	7	0.000718
GO.0043066	negative regulation of apoptotic process	3	0.00155
GO.0044765	single-organism transport	4	0.00215
GO.0009653	anatomical structure morphogenesis	3	0.00361
GO.0030162	regulation of proteolysis	3	0.00408
GO.0031333	negative regulation of protein complex assembly	2	0.00445
GO.0051248	negative regulation of protein metabolic process	3	0.00445
GO.0000902	cell morphogenesis	2	0.0163
GO.0031324	negative regulation of cellular metabolic process	3	0.0163
GO.0032680	regulation of tumor necrosis factor production	2	0.0163
GO.0050790	regulation of catalytic activity	3	0.0163
GO.0044699	single-organism process	5	0.0164
GO.0050896	response to stimulus	4	0.0172
GO.0009605	response to external stimulus	3	0.0183
GO.0055072	iron ion homeostasis	2	0.0183
GO.0048583	regulation of response to stimulus	3	0.0196
GO.0051130	positive regulation of cellular component organization	2	0.0218
GO.0051239	regulation of multicellular organismal process	3	0.0239
GO.0051704	multi-organism process	3	0.0239
GO.0006953	acute-phase response	2	0.0248
GO.0044089	positive regulation of cellular component biogenesis	2	0.0248
GO.0006952	defense response	3	0.0268
GO.0048856	anatomical structure development	3	0.0279
GO.0071822	protein complex subunit organization	2	0.0283
GO.0010951	negative regulation of endopeptidase activity	2	0.0339
GO.0009968	negative regulation of signal transduction	2	0.0375
GO.0044767	single-organism developmental process	3	0.0396

BM-G After vs. BM-G Before interactions between the proteins identified with differential expression and exclusives between AD-G After vs. AD-G Before in the web-available STRING v10.5, obtained 36 access numbers found, 24 were exclusives of the After moment, 8 exclusives of the Before moment, 4 up-regulated and no down-regulated found (Table 7).

Table 7. Identified proteins exclusives and with diferential expression in the compare group BM-G After vs. Before from the web-available STRING v10.5

[a]Access Number	Protein names	Gene names	Expression
P80010	Plasminogen [Cleaved into: Plasmin heavy chain A; Plasmin light chain B] (Fragment)	PLG	Unique After
F6RMD0	Uncharacterized protein	CFB	Unique After
F7A949	Myosin light chain kinase family member 4	MYLK4	Unique After
O97921	Prostaglandin-H2 D-isomerase (Glutathione-independent PGD synthase) (Lipocalin-type prostaglandin-D synthase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS) (PGDS2)	PTGDS	Unique After
H9GZQ9	Uncharacterized protein		Unique After
F7AJU7	Fem-1 homolog C	FEM1C	Unique After
F6ZER1	Golgi phosphoprotein 3	GOLPH3	Unique After
F7BFJ1	Prothrombin (Coagulation factor II)	F2	Unique After
F6ZMG7	Carboxylic ester hydrolase		Unique After
F6T0P6	GC, vitamin D binding protein	GC	Unique After
F6X1I8	Hemopexin	HPX	Unique After
F6VXP7	Carboxylic ester hydrolase		Unique After
F6PRI5	Carboxylic ester hydrolase	LOC100051065	Unique After
F6W2Y1	Fibrinogen gamma chain	FGG	Unique After
F6TVJ6	Protein kinase cAMP-activated catalytic subunit beta	PRKACB	Unique After
F7AI06	Protein kinase cAMP-activated catalytic subunit alpha	PRKACA	Unique After
F6VJR6	Alpha-1B-glycoprotein	A1BG	Unique After
H9GZS6	Uncharacterized protein		Unique After
H9GZR2	Uncharacterized protein		Unique After
H9GZT5	Uncharacterized protein		Unique After
F6WCY1	Uncharacterized protein		Unique After
F7C959	Enolase 3	ENO3	Unique After
F7CIX6	Enolase 2	ENO2	Unique After
F6V7C1	Enolase 1	ENO1	Unique After
P00443	Superoxide dismutase [Cu-Zn]	SOD1	Unique Before
Q28369	Retinol-binding protein 4 (Plasma retinol-binding protein) (PRBP) (RBP)	RBP4	Unique Before
F7CAC5	Uncharacterized protein		Unique Before
F6QAU5	Uncharacterized protein		Unique Before
F6SQD7	Uncharacterized protein		Unique Before
F6ZI35	Uncharacterized protein		Unique Before
F7AYC1	Secreted phosphoprotein 1	SPP1	Unique Before
F6TQ51	DnaJ heat shock protein family (Hsp40) member B14	DNAJB14	Unique Before
H9GZU9	Uncharacterized protein		Up-regulated
H9GZU8	Uncharacterized protein		Up-regulated
Q29482	Clusterin [Cleaved into: Clusterin beta chain; Clusterin alpha chain]	CLU	Up-regulated
P35747	Serum albumin (allergen Equ c 3)	ALB	Up-regulated

aAccess Number is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Among the proteins identified, the presence of the three types of enolases interacting (ENO 1, ENO 2 and ENO 3) was also observed in the comparison of this group BM-G.

Discussion

The traditional or routine clinical laboratory analysis of the CSF is focused on physical-chemical and cytological examination. One of the challenges in characterizing the CSF proteome is the low abundance of proteins, peptides and proteolytic fragments compared to blood serum. The introduction of microscale/molecular analysis has led to immunoassay methods (e.g., Western Blot, WB), isoelectric focusing, two-dimensional electrophoresis (2-DE) and MS for CSF analysis.

Broccardo et al. (2014) characterized the protein profile of the CSF of six healthy horses and 320 proteins were detected, the most abundant were albumin, apoliprotein and serotransferrin. Of this total, 145 proteins that had "valid" access numbers in the UniProt database related with *Equus* proteins were presented according to categories of biological processes to which they are related, and the most evident categories of proteins were those involved with cellular processes (18%) and biological regulation (13%). When evaluated in categories regarding molecular functions, the most predominant include association functions (42%), catalytic activity (21%) and enzymatic regulation activity (14%).

To our knowledge, there are no studies on the proteomic profile of CSF of horses after multiple intrathecal transplantations of MSCs. According to our results, the numbers of proteins identified exceed the values found in this unique proteomic study addressing horses. In addition, we have taken a step further by trying to evaluate transplantation of different types of MSCs.

Enolase (2-phospho-D glycerate hydrolyase or phosphopyruvate hydratase, EC 4.2.1.11) is a glycolytic enzyme that converts 2-phospho-D glycerate to phospho-enolpyruvate, being responsible for catalyzing the only dehydration step in the glycolytic pathway (LIMA et al., 2004; WILSON et al., 2004). It is a protein which is functionally active as a heterodimer assembled from a combination of three subunits: α, ß and γ (LIMA et al., 2004). Enolase is found from archaebacteria to mammals, and its sequence is highly conserved (GILBERT et al., 2005). In vertebrates, the enzyme occurs as three isoforms: α-enolase (Eno1) is found in almost all human tissues, whereas β-enolase (Eno3) is predominantly found in muscle tissues, and γ-enolase (Eno2) is only found in neuron and neuroendocrine tissues (MARANGOS; PARMA; GOODWIN, 1978). The γγ and αγ isoenzymes are referred to as neuron-specific enolase (NSE) (MARANGOS et al., 1979; MARANGOS; SCHMECHEL, 1987), although it was found in neuroendocrine cells and several non-neuronal and non-neuroendocrine cells, as in platelets and red blood cells (MARANGOS et al., 1980).

However, neurons express the γγ isoenzyme, while non-neuronal cells contain predominantly the αγ isoenzyme (MARANGOS; SCHMECHEL, 1987). In rat, monkey and human`s adult brains, higher concentrations of NSE are found in the gray matter (e.g., neocortex) and lower levels in the white matter (e.g., pyramidal tract and corpus callosum) (MARANGOS et al., 1979). NSE has a high stability in biological fluids and, as a free soluble cytoplasmic protein, can easily diffuse to the extracellular medium and CSF when neuronal membranes are injured. Our findings have shown the presence of all three isofroms of enolase in the Control and BM groups, in contrast with the AD-G.

Several studies in humans have shown that CSF-NSE yields a reliable estimate of the severity of neuronal injury, as well as, clinical outcome of patients with serious clinical manifestations such as in cases of stroke (HAY et al., 1984), head injury (PERSSON et al., 1987), anoxic encephalopathy (ROINE et al., 1989), encephalitis (STUDAHL et al., 2000), brain metastasis (ROYDS; TIMPERLEY; TAYLOR, 1981), and status epilepticus (CORREALE et al., 1998). In dogs CSF-NSE concentrations are increased in cases of GM1 gangliosidosis (SATOH et al., 2007) and meningoencephalitis (NAKAMURA et al., 2012).

Besides measurements of CSF-NSE have been described as an important marker of neuronal damage, the nature, location and extension of the lesion; CSF turnover and time elapsed between neuronal injury and CSF sample collection have to be considered to avoid misinterpretation (ROYDS; TIMPERLEY; TAYLOR, 1981; HARDEMARK et al., 1989). For example, CSF-NSE may be increased secundarily due CSF blood cells contamination during spinal tap. However, there were no relevant blood cells contaminations during CSF collection in all three experimental groups (data not shown).

It has been suggested that there may be an increase of CSF-NSE at earlier stages of neurodegenerative disorders, followed by a gradual decrease in chronic stages due neuronal degeneration. In contrast to other human demential disorders, high CSF-NSE and 14-3-3 protein levels have been described in Creutzfeldt-Jacob disease as a relevant information to establish the diagnosis (KROPP et al., 1999). The presence of CSF-NSE in the control and BM groups, suggest that this brain-specific protein may be a potential neuronal injury biomarker for horses.

Conclusion

This study allowed us to initially verify the presence of 3 types of enolases interacting exclusively 30 days after transplantation in both the Control and BM-G

groups. In this context, a deeper analysis of these data should be carried out, since the NSE (ENO-2) may play an important role in the pathogenesis of neural injury, as described in others species.

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CAPÍTULO 3

TRABALHO CIENTÍFICO

Trabalho a ser enviado para a revista *Brazilian Journal of Veterinary Pathology.*

PROTEOMIC PROFILING OF CEREBROSPINAL FLUID FROM HORSES WITH EQUINE PROTOZOAL MYELOENCEPHALITIS

Abstract

Equine protozoal myeloencephalitis (EPM) caused by both apicomplexa protozoans, *Sarcocystis neurona* and *Neospora hughesi*, remains as an important neurological disease of horses in the Americas, although the majority of cases are due *S. neurona* infection. Several studies have reported the seroprevalence of *S. neurona* in horses from North and South America ranging from as low as 15% to a high of 89%, depending on geographic location. The aim of this study was to evaluate the proteomic profiling of cerebrospinal fluid (CSF) of chronically EPM-affected horses and to compare it with the proteomic profiling of CSF of clinically healthy horses. Results of this work allow to know the profiling of the CSF of equines in a pathological state and provide new insights driven to identify CSF markers of equine neural injury, as well as for EPM, applying modern proteomic tools. The CSF was collected from the subarachnoid space between the cervical vertebrae C1-C2 from 16 clinically healthy horses named control group (Control; n=16) and from 9 chronically EPM-affected horses (EPM group; n=9). Each one of the CSF samples of Control group and EPM group were resuspended and grouped totaling 2 pools representing each one of the experimental groups to evaluate the proteomic profiling of them. Of the total 247 proteins founded in CSF groups from EPM vs. Control, 201 proteins were confidently

identified in UniProt database and amount 33 proteins exclusively observed in the CSF from EPM group, the enolases (ENO), may play an important role in the pathogenesis of equine neurological disease. In conclusion, the results obtained in this study support the hypothesis-driven for CSF enolases as an exploitable protein marker for neural injury and *S. neurona* infection in horses.

Keywords: Equine, cerebrospinal fluid, enolase, proteomics, neural lesion markers, Equine protozoal myeloencephalitis.

Introduction

Equine protozoal myeloencephalitis (EPM) caused by both apicomplexa protozoans, Sarcocystis neurona and Neospora hughesi, remains as an important neurological disease of horses in the Americas, although the majority of cases are due *S. neurona* infection [1], [2], [3]. Several studies have reported the seroprevalence of S. neurona in horses from North and South America ranging from as low as 15% to a high of 89%, depending on geographic location [4], [5], [6], [7], [8], [9]. There are no pathognomonic clinical signs for the disease and the neurological signs depending of anatomical localization of the lesion within central nervous system (CNS) [3]. Moreover, affected horses can have focal or multifocal CNS disease involving both white and gray matter in the brain, brainstem, or spinal cord. [1], [2], [3]. Signs of gray matter involvement include focal muscle atrophy and severe muscle weakness, whereas damage to white matter frequently results in ataxia and weakness in limb. Usually, the signs of brain/brainstem disease include abnormal consciousness, head tilt, facial nerve paralysis, dysphagia or even seizures, although others neurologic signs can be present. Severely affected horses might have difficulty standing or walking and the disease can

progress very rapidly, resulting in recumbency [3]. EPM can be difficult to diagnose antemortem and several serological tests, as Western blot (WB) [10], *S. neurona* SAT [11], IFAT [12] and ELISAs based on the SnSAG2, SnSAG3, and SnSAG4 surface antigens [13], have been developed to help overcome the negative impact of antigenic diversity in the *S. neurona* population and the varied immune responses that occur in different horses. Fusion of SnSAG3 and SnSAG4 into a single chimeric protein (rSnSAG4/3) and concurrent analysis with two ELISAs (rSnSAG2 ELISA and rSnSAG4/3 ELISA) have been employed for commercial testing of equine samples (Equine Diagnostic Solutions, LLC, Lexington, KY, USA). Furthermore, extensive validation studies have shown that the SnSAG ELISAs are specific and do not crossreact with serum from horses infected with other species of *Sarcocystis* [14], [15]. SnSAGs are valuable biomarkers that accurately detect infection with *S. neurona* [2]. However, since seroprevalence of S. *neurona* in horses vary widely, the simple detection of serum antibodies against this protozoan has limited diagnostic value. Detection of antibodies in cerebrospinal fluid (CSF) is more clinically relevant for EPM diagnosis.

CSF can be considered to be the best fluid for the analysis of protein and peptide biomarkers to study neurological disorders and to support CNS drug development [16], [17]. In addition to containing proteins that are secreted from brain regions and components of blood, CSF also plays a more active role as a transport medium for signaling proteins and peptides. CSF proteome is expected to constitute a rich biomarker compartment in which protein represent the physiological state of the CNS and reflect valuable information about the disease process [17]. One of the goals of proteomics is to characterize cellular proteins, secreted proteins and peptides and proteolytic fragments as potential biomarkers in light of specific pathologic processes. Serial evaluation of CSF for monitoring disease progression or treatment efficacy

demonstrates the clinical utility of CSF proteomic analysis [16]. Otherwise, studies of CSF are challenging because of the invasiveness of sample collection and the difficulty in obtaining sufficient sample from humans and small animal models. In this context, large animals models present a unique opportunity to investigate CSF due to the ease of obtaining large sample volumes at distinct moments of the disease [18].

The aim of this study was to evaluate the proteomic profiling of CSF of chronically EPM-affected horses and to compare it with the proteomic profiling of CSF of clinically healthy horses. Results of this work allow to know the profiling of the CSF of equines in a pathological state and provide new insights driven to identify CSF markers of equine neural injury, as well as for EPM, applying modern proteomic tools.

Materials and Methods

Experimental groups

Clinically healthy horses

For the development of this study were used 16 clinically healthy horses (*Equus caballus*) of both sexes (9 females and 7 males), mixed-race, aged between 4 and 15 years and body weight between 300-500 kg, belonging to the School of Veterinary Medicine and Animal Science (FMVZ), Sao Paulo State University "Júlio de Mesquita Filho" (UNESP), Botucatu campus, Brazil. These animals were considered healthy based on the evaluation of the physical examination, complete blood count and CSF (data not shown), and named control group (Control; n=16).

Equines with EPM

Nine horses were selected using the following minimum criteria: positive laboratory diagnosis for EPM (SAG ELISA), having already been treated with

antiprotozoal drugs for at least 30 days, and showing neurological signs of myeloencephalopathy, such as motor incoordination resulting from EPM sequelae. These animals were from owners who applied to participate in the present study after referral performed by referenced veterinarians. The owners signed a consent term (in accordance with the Ethical Principles on Animal Experimentation) and committed not to initiate any other treatment during the study.

After this previous selection, this group (EPM group; n=9) consisted of horses of both sexes (5 females and 4 males), distinct races (Quarter of Mile (QM; n=4), Brazilian Equestrian (BH; n=3) and mestizo (SRD; n=2)), with ages between 2 and 23 years and body weight between 350-560 kg; and minimum criteria mentioned above. EPM group were kept in pickets and/or stalls in the properties in which they were found and appropriate feeding regime, but varied according to the location.

CSF collection

The CSF was collected from the subarachnoid space between the cervical vertebrae C1-C2 according to Pease [43] and with modifications made by Barberini [42].

For this, the animals were sedated with detomidine (10-20 µg/kg intravenously) and morphine (0.05 mg/kg intravenously). In quadrupedal position, an area of 15 x 15 cm, centered on the space of the cervical vertebrae C1-C2, was tricotomized and aseptically prepared with 2% degerming chlorhexidine and 70% alcohol for sterile puncture. Skin and subcutaneous tissue infiltration was performed with the anesthetic lidocaine hydrochloride 2% without a constrictor vessel, using a local "button" block. With the aid of ultrasound (ultrasound device MyLab™30Gold VET, ESAOTE®, ITA), the subarachnoid space between the cervical C1-C2 vertebrae was located, a 25Gx90mm spinal needle was inserted and the CSF aspirated using a 3 mL syringe.

The CSF was stored in a sterile cryogenic tube and free of DNase and RNase to undergo freezing in liquid nitrogen at a temperature of -160ºC and stored at a temperature of -80ºC for proteomic analysis.

Soon after this procedure, 3 mL of DPBS were administrated by the same route according already described.

Evaluation of the proteomic profile of the CSF

To evaluate the proteomic profile, the CSF samples were pooled according to the experimental groups (Control and EPM).

Each representative sample had its proteins quantitatively identified with a confidence interval (>95%) and the interaction between these proteins identified and differentially expressed with a bidirectional p-value (<0.05) for the down-regulated proteins, and (1-p>0.95), for the up-regulated proteins.

Sample preparation

The CSF samples were thawed slowly and kept refrigerated at 4-5°C for use. Then, each selected sample was homogenized with the aid of a vortex and resuspended with movements up and down in the pipette itself, being then the aliquot of the standardized volume packed in LoBind plastic microtubes (Eppendorf®) corresponding to the destination pool.

In each one of the CSF samples of the 16 healthy horses (Control group) and 9 EPM affected horses (EPM group), 100µL were resuspended and grouped into a pool of the 16 healthy horses (100µL/animal) (representing the experimental group Control) and the 9 EPM affected horses (100µL/animal) (representing the experimental group EPM), totaling 2 pools representing the each one experimental groups.

Protein Quantification

The proteins present in the CSF samples were quantified in triplicate according to the Bradford method (BioRad®; Protein Assay, cod. 500-0001), using bovine albumin (BSA) as the standard protein to obtain the calibration curve.

Enzyme digestion in solution

Fifty micrograms (50ug) of each sample were solubilized in 50 uL of 50mM ammonium bicarbonate buffer at pH=7.8. Subsequently, the samples were homogenized in the presence of 25 uL of Rapigest surfactant (Waters) and incubated at 37°C for 60 minutes. After this period, the samples were submitted to reduction and alkylation steps, using 10mM ditiotreitol (DTT) and 45mM iodoacetate (IAA), both solubilized in 50mM ammonium bicarbonate solution. The samples were then submitted to proteolytic digestion in the presence of the enzyme trypsin (Promega) at a concentration of 1:100 (enzyme:substrate), solubilized in 50mM ammonium bicarbonate buffer, pH=7.8. The hydrolysis occurred for 18 hours, being interrupted with the addition of formic acid 1% (v/v) in relation to the volume of the samples. These were then desalinated using Sep Pak Vac C18 cartridges (Agilent). The digested and desalinated samples were reduced in SpeedVac™ (Thermo Scientific) and kept at 4°C until the moment of analysis by mass spectrometry.

Mass Spectrometry Analysis and Protein Identification

Triptych peptide analysis was performed in the nanoACQUITY UPLC system (Waters, Milliford, USA) coupled to the Xevo Q-TOF G2 mass spectrometer (Waters, Milliford, USA). For this purpose, the UPLC nanoACQUITY system was equipped with a column type HSS T3 (Acquity UPLC HSS T3 column 75 mm x 150 mm; 1.8 μm, Waters), previously balanced with 7% of mobile phase B (100% ACN + 0.1%

formic acid). The peptides were separated by a linear gradient of 7-85% of mobile phase B for 70 minutes with a flow of 0.35µL/min and the temperature of the column maintained at 45°C. The spectrometer was operated in positive ion mode with a data acquisition time of 75 minutes.

The data obtained were processed using the ProteinLynx GlobalServer (PLGS) software version 3.03 (Waters, Milliford, USA). The samples were submitted to a mass spectrometer in triplicates. The identification of the proteins was obtained through the ion counting algorithm incorporated into the software. The data obtained were retrieved from the Equus caballus species database downloaded from the UniProt catalogue (Universal Protein Resource) in September 2018, using the following variables: trypsin enzyme taking into account a lost cleavage; fixed carbamidomethylation modification; variable methionine oxidation modification; MS and MSMS tolerance error 0.1 Da.

Analysis of differential expression through the label-free strategy

All proteins identified with a confidence score (>95%) were included in the quantitative analysis. The ionic peak intensities of each protein were normalized, scaled and compared between topographies by the PLGS software version 3.03 (Waters, Milliford, USA) using a Bayesian algorithm (Monte Carlo method), which returns a bidirectional p-value: (<0.05) for down-regulated proteins, and (1-p>0.95) for up-regulated proteins.

In order to ensure even greater reliability in this analysis, the PLGS software uses 14 parameters to correctly identify each protein and not only the score. After performing the analysis, using these 14 parameters, we have 3 categories of identification (named Flag or OK): red (less than 50% of chance of correct identification), yellow (between 50 and 95% chance of correct identification) and green (more than 95% chance of correct identification). We only accepted the categories

yellow and green (category red was excluded). Considering that this was standard when our proteomics platform was used and we use 15 distinct parameters, there is no need to describe the parameters used for identification as threshold score or coverage of esch protein.

Analysis of interactions between differentially expressed proteins

Bioinformatics analysis was done to compare the EPM group with the control group. These included the database Uniprot (https://www.uniprot.org/) to verify all acesses numbers provided from analyzes, the web-available STRING v10.5 (http://string-db.org/, by choosing the variables "Multiples proteins" and "organism *Equus caballus*" in order to evaluate the interaction between the proteins identified differently expressed between the groups compared, as well as the software applications Cytoscape (http://www.cytoscape.org/) and its plug- in ClusterMarker and String.

Results

The 2 pools compared: EPM vs. Control (EPM:Control paired modes), obtained 247 identified access numbers corresponding to proteins considering the 3 categories of identification Flag (red, yellow and green).

Considering proteomics platform used (category red excluded), the 2 pools compared (EPM vs. Control) obtained 201 identified access numbers corresponding to proteins and verified on UniProt database (Tabela 8 - Additional file 1).

In the comparison from 201 access numbers found, 33 were exclusives of the EPM group, 33 exclusives of the Control group, 7 up-regulated, 25 down-regulated and 133 showed no difference in expression (Tabela 8 - Additional file 1).

The web-available STRING v10.5 (http://string-db.org/, by choosing the variables "Multiples proteins" and "organism Equus caballus" in order to evaluate the

interaction between the proteins identified exclusives differently expressed between the groups compared, as well as the software applications Cytoscape (http://www.cytoscape.org/) and its plug- in ClusterMarker and String, allowed to evaluate the interaction of the 35 proteins recognized in this database from 98 access numbers (Tabela 8 - Additional file 1), and build a protein-protein interaction network based on the ratio value of our results (EPM:Control), of which 35 proteins, 5 exclusive of the EPM group, 19 exclusive to the Control group, 6 up-regulated and 5 down-regulated (Figure 2).

Correlating the data obtained in Tabela 8 - Additional file 1 and Figure2, it was possible to associate them to the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and to show the crossing of the gluconeogenesis and HIF-1 pathways to the interactions between the 3 types of enolases.

Statistical analysis

Each representative sample had its proteins quantitatively identified with a confidence interval (>95%), normalized, scaled and compared between topographies by the PLGS software version 3.03 (Waters, Milliford, USA) using a Bayesian algorithm (Monte Carlo method), and the interaction between these proteins identified and differentially expressed with a bidirectional p-value (<0.05) for the down-regulated proteins, and (1-p>0.95), for the up-regulated proteins.

Discussion

Of the total 247 proteins founded in CSF groups from EPM vs. Control, 201 proteins were confidentily identified and converted from protein accession numbers after verifing all of them on UniProt database (https://www.uniprot.org/). Of these 201

proteins, 33 were identified exclusively in the EPM group, 33 exclusively in the control group and 135 in both. From these 135, 7 were up-regulated and 25 were down-regulated in the p value ratio EPM:Control. While 113 there were no differences between the experimental groups.

Biomarker research in equine CSF, may be of particular interest for studying equine neurological disorders such as equine herpesvirus myelitis, EPM, equine degenerative myelopathy, equine epilepsy/narcolepsy, and cervical vertebral stenotic myelopathies (CVSM) [18]. The CSF proteins identified in our study provide new data about healthy equine comprehensive global proteomic analysis and their associated Gene Ontology (GO) terms, as well as, provide a specific disease states in the EPM affected horses, driven to search potential targets for markers related with diagnosis and neural lesions.

In this context, amount 33 proteins exclusively observed in the CSF from EPM group, the enolases (ENO) may play an important role in the pathogenesis of equine neurological disease, as described in humans and rodents [19], [20]. Enolase (2-phospho-D glycerate hydrolyase or phosphopyruvate hydratase, EC 4.2.1.11) is a glycolytic enzyme that converts 2-phospho-D glycerate to phospho-enolpyruvate, being responsible for catalyzing the only dehydration step in the glycolytic pathway [19], [21]. It is a protein which is functionally active as a heterodimer assembled from a combination of three subunits: α, ß and γ [19]. Enolase is found from archaebacteria to mammals, and its sequence is highly conserved [22], thought to be evolutionarily derived from a photosynthetic lineage by a secondary endosymbiosis event between green algae and apicomplexans [23], [24]. In vertebrates, the enzyme occurs as three isoforms: α-enolase (Eno1) is found in almost all human tissues, whereas β-enolase (Eno3) is predominantly found in muscle tissues, and γ-enolase (Eno2) is only found in neuron and neuroendocrine tissues [25]. The γγ and αγ isoenzymes are referred to as

neuron-specific enolase (NSE) [26]; [27], although it was found in neuroendocrine cells and several non-neuronal and non-neuroendocrine cells, as in platelets and red blood cells [28]. However, neurons express the γγ isoenzyme, while non-neuronal cells contain predominantly the αγ isoenzyme [27]. In rat, monkey and human`s adult brains, higher concentrations of NSE are found in the gray matter (e.g., neocortex) and lower levels in the white matter (e.g., pyramidal tract and corpus callosum) [26]. NSE has a high stability in biological fluids and, as a free soluble cytoplasmic protein, can easily diffuse to the extracellular medium and CSF when neuronal membranes are injured. Our results have shown the presence of all three isoforms of enolase exclusively into CSF of the EPM group.

Several studies in humans have shown that CSF-NSE yields a reliable estimate of the severity of neuronal injury, as well as, clinical outcome of patients with serious clinical manifestations such as in cases of stroke [31], head injury [32], anoxic encephalopathy [33], encephalitis [34], brain metastasis [29], and status epilepticus [35]. In dogs CSF-NSE concentrations are increased in cases of GM1 gangliosidosis [36] and meningoencephalitis [37].

Besides measurements of CSF-NSE have been described as an important marker of neuronal damage, the nature, location and extension of the lesion; CSF turnover and time elapsed between neuronal injury and CSF sample collection have to be considered to avoid misinterpretation [29], [30]. For example, CSF-NSE assessment may be inconsistent in mild or chronic neurological disorders [38]. It has been suggested that there may be an increase of CSF-NSE at earlier stages of neurodegenerative disorders, followed by a gradual decrease in chronic stages due neuronal degeneration. In contrast to other human demential disorders, high CSF-NSE and 14-3-3 protein levels have been described in Creutzfeldt-Jacob disease as a relevant information to establish the diagnosis [39]. The presence of CSF-NSE in the EPM group in our study, suggest that

even in chronic stage, this brain-specific protein may be a potential neuronal injury marker for horses. Furthers studies should be performed to state if CSF-NSE is non specific neuronal injury or may also represent an specific EPM marker.

On the other hands, studies have established two distinct isoforms of enolase, ENO1 and ENO2, in *T. gondii* ENO1 primarily exists in the bradyzoite stage of *T. gondii* whereas ENO2 is more highly expressed in the tachyzoite stage [40]. When an unknown event triggers a stage conversion in apicomplexan parasites from tachyzoite to bradyzoite, enolase genes are either overexpressed or exclusively expressed at both the transcriptional and protein levels within the bradyzoite stage [23] as compared to levels detected in the tachyzoite. This is significant considering that related parasites like *T. gondii* rely on anaerobic glycolysis during their bradyzoite encystment period, due to non-functioning mitochondria [21].

However, enolase shown potentially other roles in different protozoa, as in *Leishmania Mexicana*, it can bind host plasminogen or in *Plasmodium* spp., whereas enolase is immunostimulatory, and anti-Plasmodium enolase antibodies can be protective [21]. These studies further support enolase as an exploitable target molecule for immunoprophylaxis of parasite stages. Cross-reactivity of individual enolase isoforms between related parasites has been described. For example, anti-T. gondii enolase 2 antibody demonstrated cross-reactivity to *Neospora* spp. tachyzoites and *S. neurona* merozoites by immunoblot analysis, but the anti-T. gondii enolase 1 antibody did not react with *S. neurona* merozites by immunoblot analysis [21]. Based on the sequence homology of known DNA sequences of enolase 1 and 2 between Sarcocystidae parasites, it would be expected that the proteins may be very similar and may show similar expression profiles [21]. *Sarcocystis neurona* merozoites and schizonts reacted with both anti-enolase 1 and 2 antibodies. The staining for anti-enolase 1 was much weaker as compared to the very strong staining of anti-enolase 2 to

the *S. neurona* merozoites and schizonts, suggesting the existence of at least two putative enolase proteins in *S. neurona* [41]. Thus, the CSF-ENO1 and CSF-ENO2 findings in the horses infected by *S. neurona* in our study may represent a potential EPM marker to be investigate in future studies.

In conclusion, the results obtained in this study support the hypothesis-driven for CSF enolases as an exploitable protein marker for neural injury and *S. neurona* infection in horses.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval and consent for participation

All stages of the development of this work followed the Ethical Principles in Animal Experimentation and were approved by the Ethics Committee for the Use of

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Figures

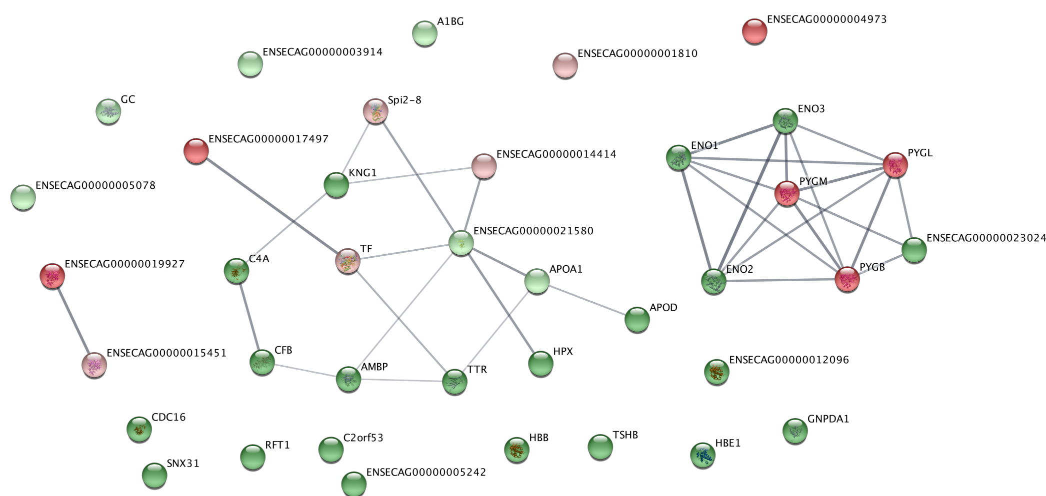

Figure 2. Protein-protein interaction network between the proteins identified exclusives and differently expressed in the compare groupe EPM:Control. The color of nodes indicated proteins exclusives and the differential expression correlated with Additional file 1. The dark red and dark green nodes indicate proteins unique to the control and EPM group, respectively. The light red and light green nodes indicate downregulation and upregulation, respectively.

Additional files

Tabela 8 - Additional file 1 – Identified proteins in the compare group EPM *vs*. Control.

Additional file 1. Identified proteins in the compare group EPM *vs*. Control

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Hemoglobin-alpha 1 (Fragment)	Q95MA1		4617	90	10	Inferred from homology	Unique EPM	EPM	EPM
Transferrin (Fragment)	Q9TQV1		1978	58	7	Predicted	Unique EPM	EPM	EPM
Transthyretin	F6UL68	TTR	805	147	16	Inferred from homology	Unique EPM	EPM	EPM
Globin A1 (Hemoglobin subunit beta)	F6RDD3	HBB GLNA1	654	147	16	Inferred from homology	Unique EPM	EPM	EPM
Hemoglobin subunit alpha (Alpha-globin) (Hemoglobin alpha chain)	P01958	HBA	652	142	15	Evidence at protein level	Unique EPM	EPM	EPM
Hemoglobin subunit beta (Beta-globin) (Hemoglobin beta chain)	P02062	HBB	629	146	16	Evidence at protein level	Unique EPM	EPM	EPM
Uncharacterized protein	Q28383		594	142	15	Inferred from homology	Unique EPM	EPM	EPM
Globin B1	F6RF57	LOC100068926 GLNB1	454	147	16	Inferred from homology	Unique EPM	EPM	EPM
Protein RFT1 homolog	F6TKD2		210	541	60	Inferred from homology	Unique EPM	EPM	EPM
Protein RFT1 homolog (Fragment)	K9KD00		210	223	24	Evidence at transcript level	Unique EPM	EPM	EPM
Cell division cycle 16	F6TY83	CDC16	195	605	70	Predicted	Unique EPM	EPM	EPM
Cell division cycle protein 16-like protein-like protein (Fragment)	K9KA79		180	255	29	Evidence at transcript level	Unique EPM	EPM	EPM
Uncharacterized protein	F6RMD0	CFB	161	768	86	Inferred from homology	Unique EPM	EPM	EPM
Inter-alpha-trypsin inhibitor (ITI) (EI-14) (HI-14) (Inhibitory fragment of ITI) (Fragment)	P04365		161	125	14	Evidence at protein level	Unique EPM	EPM	EPM
Complement factor B-like protein (Fragment)	K9KBR1		148	227	26	Evidence at transcript level	Unique EPM	EPM	EPM
Apolipoprotein D (Apo-D)	F6XM13	APOD	137	213	24	Inferred from homology	Unique EPM	EPM	EPM
Uncharacterized protein	F6S7H7		136	236	27	Predicted	Unique EPM	EPM	EPM
Fibrinogen alpha chain (Fragment)	Q7M2U4		133	270	28	Evidence at protein level	Unique EPM	EPM	EPM
Thyrotropin subunit beta (Thyroid-stimulating hormone subunit beta) (TSH-B) (TSH-beta) (Thyrotropin beta chain)	Q28376	TSHB	131	138	16	Evidence at transcript level	Unique EPM	EPM	EPM
Uncharacterized protein	F6XSF7	LOC100059239	117	1750	193	Predicted	Unique EPM	EPM	EPM
Proline rich 30	F6RGD6	PRR30	114	392	42	Predicted	Unique EPM	EPM	EPM
Glucosamine-6-phosphate isomerase (Glucosamine-6-phosphate deaminase)	F6T471	GNPDA1	100	289	33	Inferred from homology	Unique EPM	EPM	EPM
Uncharacterized protein	F6WCY1		77	422	47	Predicted	Unique EPM	EPM	EPM
Alpha-enolase-like protein (Fragment)	K9K2B9		77	310	34	Evidence at transcript level	Unique EPM	EPM	EPM
Enolase 2 (Fragment)	I3RM61	ENO2	77	424	46	Evidence at transcript level	Unique EPM	EPM	EPM

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Additional file 1. (*continued*)

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6C6	IGL	293	222	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6C0	IGL	293	225	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6L5	IGL	293	225	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6I7	IGL	293	221	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6I2	IGL	293	225	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4I0	IGL	293	219	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4U0	IGL	293	223	23	Evidence at transcript level	Unique Control	Control	Control
Alpha-1,4 glucan phosphorylase	F6ZD04	PYGB	286	764	87	Inferred from homology	Unique Control	Control	Control
Antithrombin III protein (Fragment)	Q9N1X2	AT3	174	60	7	Predicted	Unique Control	Control	Control
Uncharacterized protein	F6PKE1		159	340	37	Predicted	Unique Control	Control	Control
Carboxylic ester hydrolase	F6UN85	LOC100050992	77	565	62	Inferred from homology	Unique Control	Control	Control
Transferrin (Fragment)	Q71UC7		50	31	4	Predicted	Unique Control	Control	Control
Transferrin (Fragment)	Q9N1F7		50	47	5	Predicted	Unique Control	Control	Control
Alpha-1,4 glucan phosphorylase	F6RV94	PYGL	29	830	95	Inferred from homology	Unique Control	Control	Control
Alpha-1,4 glucan phosphorylase	B9A9Y7	PYGL	29	851	97	Evidence at transcript level	Unique Control	Control	Control
Uncharacterized protein	H9GZS6		1046	338	37	Predicted	Up-regulated	1.36	1
Haptoglobin	F6XWM5		1360	346	38	Inferred from homology	Up-regulated	1.49	1
Uncharacterized protein	H9GZR2		1046	338	37	Predicted	Up-regulated	1.36	1
Apolipoprotein A1	F6Z2L5	APOA1	193	266	30	Inferred from homology	Up-regulated	2.51	1
GC, vitamin D binding protein	F6T0P6	GC	163	474	53	Predicted	Up-regulated	1.67	0.99
Alpha-1B-glycoprotein	F6VJR6	A1BG	186	500	54	Predicted	Up-regulated	2.27	0.98
Albumin (Fragment)	Q95MC2		909	44	5	Predicted	Up-regulated	1.34	0.96
Clusterin [Cleaved into: Clusterin beta chain; Clusterin alpha chain]	Q29482	CLU	703	449	52	Evidence at transcript level	No difference vs. control	1.19	0.92
Uncharacterized protein	H9GZU9		5231	325	36	Predicted	No difference vs. control	1.08	0.87
Uncharacterized protein	F6ZI35		263	236	27	Predicted	No difference vs. control	1.52	0.85
Prothrombin (Coagulation factor II)	F7BFJ1	F2	75	623	70	Inferred from homology	No difference vs. control	1.40	0.83
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H3	IGL	387	221	23	Evidence at transcript level	No difference vs. control	1.23	0.82
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X3	IGL	387	222	23	Evidence at transcript level	No difference vs. control	1.21	0.8

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Additional file 1. (*continued*)

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Enolase 3	F7C959	ENO3	77	434	47	Evidence at transcript level	Unique EPM	EPM	EPM
Enolase 2	F7CIX6	ENO2	77	434	47	Inferred from homology	Unique EPM	EPM	EPM
Enolase 1	F6V7C1	ENO1	77	434	47	Evidence at transcript level	Unique EPM	EPM	EPM
Hemopexin	F6X1I8	HPX	76	462	51	Inferred from homology	Unique EPM	EPM	EPM
Kininogen 1	F7C0D9	KNG1	61	645	71	Predicted	Unique EPM	EPM	EPM
Kininogen 1	F7C0Z0	KNG1	61	653	72	Predicted	Unique EPM	EPM	EPM
Sorting nexin 31	F7BAM6	SNX31	36	440	51	Predicted	Unique EPM	EPM	EPM
Uncharacterized protein	F6VE37	LOC100054161	32	151	17	Inferred from homology	Unique EPM	EPM	EPM
Transferrin (Fragment)	Q9TQW7		1151	52	6	Predicted	Unique Control	Control	Control
Transferrin (Fragment)	Q9TQR3		1049	48	5	Predicted	Unique Control	Control	Control
Alpha-1,4 glucan phosphorylase	B9A9U9	PYGM	791	842	97	Evidence at transcript level	Unique Control	Control	Control
Clone 7 transferrin (Pop-variant * transferrin) (Pop-variant D, F1, F2, F3, G, H2, O transferrin) (Fragment)	Q9TQV4		551	10	1	Predicted	Unique Control	Control	Control
Uncharacterized protein	F6SP11		293	106	11	Predicted	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E949	IGL	293	220	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6G0	IGL	293	219	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Y7	IGL	293	222	24	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6F4	IGL	293	224	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6F3	IGL	293	225	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E696	IGL	293	224	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E691	IGL	293	223	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E921	IGL	293	223	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3W4	IGL	293	224	24	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E482	IGL	293	221	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E916	IGL	293	220	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6P5	IGL	293	225	23	Evidence at transcript level	Unique Control	Control	Control
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3V9	IGL	293	223	23	Evidence at transcript level	Unique Control	Control	Control

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Additional file 1. (*continued*)

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E944	IGL	387	225	24	Evidence at transcript level	No difference vs. control	1.23	0.79
Lactotransferrin (Lactoferrin) (Fragment)	O77811	LTF	31	695	76	Evidence at protein level	No difference vs. control	1.38	0.79
Uncharacterized protein	H9GZT5		112	335	36	Predicted	No difference vs. control	1.54	0.79
Lactotransferrin	F6XLB1	LTF	31	708	77	Inferred from homology	No difference vs. control	1.35	0.78
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E493	IGL	387	224	23	Evidence at transcript level	No difference vs. control	1.19	0.77
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H7	IGL	387	225	23	Evidence at transcript level	No difference vs. control	1.22	0.77
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Y3	IGL	387	225	23	Evidence at transcript level	No difference vs. control	1.19	0.75
Uncharacterized protein	F6QAU5		387	107	11	Predicted	No difference vs. control	1.21	0.75
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B6	IGL	387	225	24	Evidence at transcript level	No difference vs. control	1.23	0.75
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D7	IGL	387	225	23	Evidence at transcript level	No difference vs. control	1.19	0.75
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E933	IGL	387	223	23	Evidence at transcript level	No difference vs. control	1.19	0.74
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4B1	IGL	387	225	24	Evidence at transcript level	No difference vs. control	1.15	0.74
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3X7	IGL	387	226	24	Evidence at transcript level	No difference vs. control	1.16	0.72
Uncharacterized protein	F6SQD7		387	107	11	Predicted	No difference vs. control	1.19	0.72
Serpin family F member 1	F7BKE1	SERPINF1	76	417	46	Inferred from homology	No difference vs. control	1.49	0.72
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4E7	IGL	387	225	24	Evidence at transcript level	No difference vs. control	1.15	0.7
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6B2	IGL	387	224	23	Evidence at transcript level	No difference vs. control	1.15	0.7
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E938	IGL	387	226	24	Evidence at transcript level	No difference vs. control	1.14	0.65
Serpin family C member 1	F7CYR1	SERPINC1	189	463	52	Inferred from homology	No difference vs. control	1.65	0.64
Uncharacterized protein	H9GZS9		118	354	39	Predicted	No difference vs. control	1.08	0.59
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E476	IGL	293	222	23	Evidence at transcript level	No difference vs. control	1.11	0.57
Prostaglandin-H2 D-isomerase (Glutathione-independent PGD synthase) (Lipocalin-type prostaglandin-D synthase) (Prostaglandin-D2 synthase) (PGD2 synthase) (PGDS) (PGDS2)	O97921	PTGDS	2545	194	22	Evidence at protein level	No difference vs. control	1.04	0.51
Immunoglobulin gamma 1 heavy chain constant region (Fragment)	Q95M34	IGHC1	3223	337	37	Evidence at transcript level	No difference vs. control	0.96	0.5
Uncharacterized protein	H9GZQ9		3223	337	37	Predicted	No difference vs. control	0.97	0.44
Uncharacterized protein	F6YYP6		2539	99	11	Inferred from homology	No difference vs. control	0.94	0.38

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Additional file 1. (*continued*)

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Alpha 2-HS glycoprotein	F7C450	AHSG	1028	358	38	Predicted	No difference vs. control	0.90	0.28
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E976	IGL	2009	209	22	Evidence at transcript level	No difference vs. control	0.83	0.24
Uncharacterized protein	H9GZU8		3533	324	36	Predicted	No difference vs. control	0.89	0.22
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E2	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.82	0.22
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E483	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.84	0.22
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4N8	IGL	2009	222	23	Evidence at transcript level	No difference vs. control	0.76	0.22
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K2	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.84	0.22
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4T2	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.79	0.21
Serpin III (Fragment)	Q7M387		1610	49	6	Evidence at protein level	No difference vs. control	0.53	0.21
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z9	IGL	2009	222	23	Evidence at transcript level	No difference vs. control	0.80	0.2
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N5	IGL	2009	226	24	Evidence at transcript level	No difference vs. control	0.83	0.2
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4A9	IGL	2009	223	23	Evidence at transcript level	No difference vs. control	0.76	0.2
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6L0	IGL	2009	222	23	Evidence at transcript level	No difference vs. control	0.70	0.2
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E417	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.79	0.2
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H1	IGL	2009	226	23	Evidence at transcript level	No difference vs. control	0.80	0.2
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R5	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.78	0.19
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K7	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.78	0.19
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E532	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.79	0.18
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E477	IGL	2009	222	23	Evidence at transcript level	No difference vs. control	0.76	0.18
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9C4	IGL	2009	224	24	Evidence at transcript level	No difference vs. control	0.76	0.18
Transferrin (Fragment)	Q9TQR4		3197	63	7	Predicted	No difference vs. control	0.75	0.18
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4R8	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.77	0.17
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J8	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.79	0.17
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E562	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.76	0.17
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E3Z2	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.79	0.16
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4L2	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.77	0.16
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E961	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.76	0.16
Uncharacterized protein	F7CJG3	LOC100060539	79	1661	186	Predicted	No difference vs. control	0.57	0.16

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Additional file 1. (*continued*)

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6E8	IGL	2009	223	23	Evidence at transcript level	No difference vs. control	0.78	0.15
Serpin II (Fragment)	Q7M388		1610	54	6	Evidence at protein level	No difference vs. control	0.55	0.15
Uncharacterized protein	F7CAC5		2009	117	12	Predicted	No difference vs. control	0.75	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E452	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.76	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E447	IGL	2009	223	23	Evidence at transcript level	No difference vs. control	0.75	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6K4	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.75	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E966	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.76	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E406	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.73	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E567	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.70	0.15
Uncharacterized protein	F7BLE3		67	1215	135	Predicted	No difference vs. control	0.68	0.15
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A3	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.73	0.14
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D7	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.76	0.14
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E588	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.78	0.14
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J6	IGL	2009	219	23	Evidence at transcript level	No difference vs. control	0.71	0.14
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6I9	IGL	2009	223	23	Evidence at transcript level	No difference vs. control	0.77	0.14
Uncharacterized protein	F7BTW7	LOC100060539	79	1664	186	Predicted	No difference vs. control	0.56	0.14
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E556	IGL	2009	225	24	Evidence at transcript level	No difference vs. control	0.73	0.13
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q9	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.76	0.13
Serpin I (Fragment)	Q7M389		1610	54	6	Evidence at protein level	No difference vs. control	0.54	0.13
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6J3	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.74	0.13
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6R9	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.75	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6D2	IGL	2009	225	24	Evidence at transcript level	No difference vs. control	0.70	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E464	IGL	2009	224	24	Evidence at transcript level	No difference vs. control	0.79	0.12
Transferrin (Fragment)	O97971		2475	53	6	Predicted	No difference vs. control	0.58	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9D0	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.77	0.12
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E4S5	IGL	2009	222	23	Evidence at transcript level	No difference vs. control	0.70	0.11
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E538	IGL	2009	221	23	Evidence at transcript level	No difference vs. control	0.73	0.11
Transferrin (Fragment)	Q71UB5		2475	53	6	Predicted	No difference vs. control	0.62	0.11

(continued on next page)

Additional file 1. (*continued*)

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6N9	IGL	2009	220	23	Evidence at transcript level	No difference vs. control	0.74	0.11
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6Q6	IGL	2009	225	24	Evidence at transcript level	No difference vs. control	0.70	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E971	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.71	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E955	IGL	2009	225	23	Evidence at transcript level	No difference vs. control	0.73	0.1
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E470	IGL	2009	226	24	Evidence at transcript level	No difference vs. control	0.73	0.09
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E412	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.67	0.09
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9A8	IGL	2009	224	23	Evidence at transcript level	No difference vs. control	0.58	0.08
Uncharacterized protein	F7CSL8		205	421	47	Inferred from homology	No difference vs. control	0.38	0.08
Transferrin (Fragment)	O97966		740	61	7	Predicted	No difference vs. control	0.32	0.08
Transferrin (Fragment)	O97678		1438	61	7	Predicted	No difference vs. control	0.33	0.07
Serum albumin (allergen Equ c 3)	P35747	ALB	12189	607	69	Evidence at protein level	No difference vs. control	0.96	0.07
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E6H5	IGL	2009	222	23	Evidence at transcript level	No difference vs. control	0.67	0.07
Carboxylic ester hydrolase	F6ZMG7		69	567	62	Inferred from homology	No difference vs. control	0.41	0.06
Uncharacterized protein	F7E454	LOC100060539	84	1640	184	Predicted	No difference vs. control	0.48	0.06
Transferrin (Fragment)	O97973		1438	61	7	Predicted	No difference vs. control	0.28	0.05
Alpha-1-antiproteinase 2 (Alpha-1-antitrypsin 2) (Alpha-1-proteinase inhibitor 2) (SPI2)	P38029		289	421	47	Evidence at protein level	No difference vs. control	0.36	0.05
Alpha-1-antitrypsin	B5BV02	Spi2-3	289	421	47	Evidence at transcript level	Down-regulated	0.35	0.04
Uncharacterized protein	F6ZEH8		7624	990	109	Predicted	Down-regulated	0.77	0.03
Alpha-1-antitrypsin	B5BV06	Spi2-7	289	421	47	Evidence at transcript level	Down-regulated	0.38	0.03
Alpha-1-antitrypsin	B5BV01	Spi2-2	289	421	47	Evidence at transcript level	Down-regulated	0.38	0.03
Transferrin (Fragment)	Q9TQV8		2043	189	21	Predicted	Down-regulated	0.60	0.03
Immmunoglobulin lambda light chain variable region (Fragment)	A0A0A1E9E9	IGL	2009	225	24	Evidence at transcript level	Down-regulated	0.64	0.03
Carboxylic ester hydrolase	F6PRI5	LOC100051065	69	565	62	Inferred from homology	Down-regulated	0.44	0.03
Uncharacterized protein	F7CYP1		205	423	47	Inferred from homology	Down-regulated	0.35	0.02
Serum albumin	F7BAY6	ALB	15522	607	68	Predicted	Down-regulated	0.93	0.02
Alpha-1-antitrypsin	B5BV12	Spi2-13	205	421	47	Evidence at transcript level	Down-regulated	0.35	0.02
Alpha-1-antitrypsin	B5BV10	Spi2-11	205	421	47	Evidence at transcript level	Down-regulated	0.39	0.02

(continued on next page)

Additional file 1. (*continued*)

Protein names (n=201)	[a]Access Number	Gene names	Score PLGS	Length (aa)	Mass (kDa)	Protein existence	Expression	Ratio (EPM:Control)	*p* Value ratio
Alpha-1-antitrypsin	B5BV07	Spi2-8	289	421	47	Evidence at transcript level	Down-regulated	0.36	0.02
Alpha-1-antitrypsin	B5BV05	Spi2-6	205	421	47	Evidence at transcript level	Down-regulated	0.29	0.02
Alpha-1-antitrypsin	B5BV04	Spi2-5	289	421	47	Evidence at transcript level	Down-regulated	0.38	0.02
Alpha-1-antitrypsin	B5BV00	Spi2-1	289	421	47	Evidence at transcript level	Down-regulated	0.37	0.02
Uncharacterized protein	F7BF31	SPI2	289	421	47	Inferred from homology	Down-regulated	0.38	0.02
Carboxylic ester hydrolase	F6VXP7		69	565	62	Inferred from homology	Down-regulated	0.44	0.02
Alpha-1-antitrypsin	B5BV13	Spi2-14	289	421	47	Evidence at transcript level	Down-regulated	0.34	0.01
Alpha-1-antitrypsin	B5BV09	Spi2-10	205	421	47	Evidence at transcript level	Down-regulated	0.35	0.01
Transferrin (Fragment)	Q9TQV7		454	54	6	Predicted	Down-regulated	0.41	0.01
Uncharacterized protein	F7DXM5	LOC100065068	205	422	47	Inferred from homology	Down-regulated	0.33	0
Alpha-1-antitrypsin	B5BV11	Spi2-12	205	421	47	Evidence at transcript level	Down-regulated	0.33	0
Alpha-1-antitrypsin	B5BV08	Spi2-9	205	421	47	Evidence at transcript level	Down-regulated	0.33	0
Alpha-1-antitrypsin	B5BV03	Spi2-4	289	421	47	Evidence at transcript level	Down-regulated	0.34	0
Serotransferrin (Transferrin) (Beta-1 metal-binding globulin) (Siderophilin)	P27425	TF	7563	706	78	Evidence at transcript level	Down-regulated	0.66	0

aAccess Number is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

CAPÍTULO 4

1 DISCUSSÃO GERAL

A análise clínica laboratorial tradicional do CSF concentra-se no exame físico-químico e citológico. A introdução de análise de microescala/molecular levou métodos de imunoensaios (e.g., Western Blot, WB), focalização isoelétrica, 2-DE e espectrometria de massas para análise de CSF.

Broccardo et al. (2014) caracterizou o perfil proteômico do CSF de seis equinos hígidos utilizando espectrometria de massas, na qual foram detectadas 320 proteínas, sendo as mais abundantes a albumina, a apoliproteina e a serotransferrina. Deste total, 145 proteínas que tiveram números de acesso encontrados no banco de dados de proteínas em equinos do UniProt e foram apresentadas segundo categorias de processos biológicos ao qual estão relacionadas, e as categorias de proteínas mais evidentes foram as envolvidas com processos celulares (18%) e de regulação biológica (13%). Quando avaliadas em categorias quanto as funções moleculares, as mais predominantes incluem funções de associação (42%), atividade catalítica (21%) e atividade de regulação enzimática (14%).

A análise proteômica do CSF de equinos, sadios ou com EPM, que receberam múltiplos transplantes intratecal de MSCs pode revelar quais vias metabólicas e/ou potenciais marcadores estão diferencialmente expressos antes e após os transplantes celulares. Ao compararmos o perfil proteômico do CSF de equinos saudáveis submetidos a múltiplos transplantes intratecal de BM-MSCs e AD-MSCs, objetivamos, não apenas avaliar as interações proteicas que pudessem elucidar potenciais mecanismos de ação das MSCs, mas também buscar marcadores de lesão/regeneração neural.

Avaliando-se as interações entre as proteínas identificadas como diferentemente expressas e exclusivas dos grupos comparados e seu distintos momentos (Control after *vs*. Control before, AD-G after *vs*. AD-G before e BM-G after *vs*. before) na ferramenta de bioinformática String v10.5 (https://string-db.org/), na comparação controle (After:Before), foram encontrados 45 números de acesso correspondentes a proteínas, sendo 22 exclusivos do momento After, 21 exclusivos do momento Before, 1 *up-regulated* e 1 *down-regulated* (Tabela 4).

As interações entre as proteínas identificadas com diferença de expressão e exclusivas da comparação AD-G (After:Before), obtiveram 64 proteínas reconhecidas, sendo 4 exclusivas do momento After, 49 exclusivas do momento Before, 7 *up-regulated* e 4 *down-regulated* (tabela 5). Além disso, foram identificados 35 termos GO (Gene Ontology) relacionados aos processos biológicos associados a essas proteínas (tabela 6).

BM-G (After:Before), obteve nas interações entre as proteínas identificadas como diferentemente expressas e exclusivas 36 números de proteínas encontradas, 24 eram exclusivas do momento After, 8 exclusivas do momento Before, 4 *up-regulated* e nenhuma *down-regulated* encontrada (tabela 7).

Entre as proteínas identificadas exclusivamentes nos grupos Control e BM-G, no momento After, destacam-se a presença das enolases ENO 1, ENO 2 e ENO 3. Já no AD-G, não foi observado a presença das enolases. Por outro lado, o score PLGS destas enolases no grupo Control foi maior que o do BM-G, sugerindo maior presença das enolases.

A enolase (2-phospho-D glycerate hydrolase or phosphopyruvate hydratase, EC 4.2.1.11) é uma enzima glicolítica que converte o 2-fosfoglicerato em fosfoenolpiruvato, sendo responsável por catalisar a única etapa de desidratação na via glicolítica (LIMA et al., 2004; WILSON et al., 2004). É uma proteína que é funcionalmente ativa como um heterodímero montado a partir de uma combinação de três subunidades: α, ß e γ (LIMA et al., 2004). A enolase é encontrada desde as arqueobactérias até os mamíferos, e sua sequência é altamente conservada (GILBERT et al., 2005). Em vertebrados, a enzima aparece em três isoformas: a α-enolase (Eno1) é encontrada em quase todos os tecidos humanos, enquanto que a β-enolase (Eno3) é encontrada predominantemente nos tecidos musculares, e a γ-enolase (Eno2) é encontrada apenas em neurônios e tecidos neuroendocrinos (MARANGOS; PARMA; GOODWIN, 1978). As isoenzimas γγ e αγ são denominadas enolase neurônio-específica (NSE) (MARANGOS et al., 1979; MARANGOS; SCHMECHEL, 1987), embora tenha sido encontrada em células neuroendócrinas e em várias células não neuronais e não neuroendócrinas, como nas plaquetas e nos eritrócitos (MARANGOS et al., 1980). No entanto, os neurônios expressam a isoenzima γγ, enquanto as células não neuronais contêm predominantemente a isoenzima αγ (MARANGOS; SCHMECHEL, 1987). Nos cérebros adultos do rato, macaco e humano, encontram-se concentrações mais elevadas de NSE na substância cinzenta (por exemplo, neocortex) e níveis mais baixos na substância branca (por exemplo, trato piramidal e corpo caloso)

(MARANGOS et al., 1979). NSE tem uma alta estabilidade em fluidos biológicos e, como uma proteína citoplasmática solúvel livre, pode facilmente se difundir para o meio extracelular e para o CSF quando as membranas neuronais são lesadas. Nossos achados mostraram a presença de todas as três isoformas de enolase nos grupos Control e BM-G, em contraste com o AD-G.

Diversos estudos em humanos mostraram que a CSF-NSE produz uma estimativa confiável da gravidade da lesão neuronal, bem como o resultado clínico de pacientes com manifestações clínicas graves, como em casos de acidente vascular cerebral (HAY et al., 1984), traumatismo craniano (PERSSON et al., 1987), encefalopatia anóxica (ROINE et al., 1989), encefalite (STUDAHL et al., 2000), metástase cerebral (ROYDS et al., 1981) e estado epiléptico (CORREALE et al., 1998). Em cães, as concentrações de CSF-NSE estão aumentadas nos casos de gangliosidose GM1 (SATOH et al., 2007) e meningoencefalite (NAKAMURA et al., 2012).

Além disso, as medições de CSF-NSE foram descritas como um marcador importante de lesão neuronal, da natureza, localização e extensão da lesão; O retorno do CSF e o tempo decorrido entre a lesão neuronal e a coleta da amostra do CSF devem ser considerados para evitar erros de interpretação (ROYDS et al., 1981; HARDEMARK et al., 1989). Por exemplo, o CSF-NSE pode estar aumentado secundariamente devido à contaminação do CSF por células sanguíneas durante a punção. No entanto, não houve contaminação relevante por células sanguíneas durante a coleta do CSF nos três grupos experimentais (dados não mostrados).

Tem-se sugerido que pode haver um aumento do CSF-NSE em estágios iniciais de distúrbios neurodegenerativos, seguido por uma diminuição gradual nos estágios crônicos devido à degeneração neuronal. Em contraste com outros distúrbios de demência em humanos, altos níveis de proteína CSF-NSE e 14-3-3 foram descritos na doença de Creutzfeldt-Jacob como uma informação relevante para estabelecer o diagnóstico (KROPP et al., 1999). A presença de CSF-NSE nos grupos Control e BM-G sugere que essa proteína específica do cérebro pode ser um potencial biomarcador de lesão neuronal para equinos. Já não há nenhuma AD-G mais forte esta fonte de MSC para terapia celular intratecal. Já a ausência no AD-G pode indicar maior segurança desta fonte de MSC para terapia celular intratecal.

A mieloencefalite protozoária equina (EPM) causada por ambos os protozoários apicomplexa, *Sarcocystis neurona* e *Neospora hughesi*, permanece como uma importante doença neurológica dos equinos nas Américas, embora a maioria dos casos seja devida à infecção por *S. neurona* (DUBEY et al., 2001; DUBEY et al., 2015;

REED et al., 2016). Vários estudos relataram a soroprevalência de *S. neurona* em equinos da América do Norte e do Sul variando de 15% a 89%, dependendo da localização geográfica (BENTZ et al., 1997; BENTZ et al., 2003; SAVILLE et al., 1997; TILLOTSON et al., 1999; DUBEY; KERBER; GRANSTROM, 1999; DUBEY et al., 1999). Além disso, os equinos afetados podem ter doença do CSN focal ou multifocal envolvendo tanto a substância branca quanto a cinzenta no cérebro, tronco cerebral ou medula espinhal (DUBEY et al., 2001; DUBEY et al., 2015; REED et al., 2016). Os sinais de envolvimento da substância cinzenta incluem atrofia muscular focal e fraqueza muscular severa, enquanto que os danos à substância branca frequentemente resultam em ataxia e fraqueza no membro. Normalmente, os sinais de doença cerebral / tronco encefálico incluem estado de consciência anormal, inclinação da cabeça, paralisia do nervo facial, disfagia ou mesmo convulsões, embora outros sinais neurológicos possam estar presentes. Cavalos gravemente afetados podem ter dificuldade em ficar em pé ou andar e a doença pode progredir muito rapidamente, resultando em decúbito (REED et al., 2016).

EPM pode ser difícil de diagnosticar antemortem e vários testes sorológicos, como Western blot (WB) (GRANSTROM et al., 1993), *S. neurona* SAT (LINDSAY; DUBEY, 2001), IFAT (DUARTE et al., 2003) e ELISAs baseados nos antígenos de superfície SnSAG2, SnSAG3 e SnSAG4 (YEARGAN; HOWE, 2011), foram desenvolvidos para ajudar a superar o impacto negativo da diversidade antigênica na população de *S. neurona* e as variadas respostas imunes que ocorrem em diferentes cavalos. SnSAGs são valiosos biomarcadores que detectam com precisão a infecção por *S. neurona* (DUBEY et al., 2015). No entanto, como a soroprevalência de *S. neurona* em equinos varia muito, a simples detecção de anticorpos séricos contra esse protozoário tem valor diagnóstico limitado. A detecção de anticorpos no CSF é mais clinicamente relevante para o diagnóstico de EPM.

O CSF pode ser considerado para ser o melhor fluido para a análise de proteínas e peptídeos biomarcadoras para se estudar distúrbios neurológicos e permitir o desenvolvimento de fármacos para o CNS (ROMEO et al., 2005; VAN GOOL; HENDRICKSON, 2012). A pesquisa de biomarcadores no CSF equino, pode ser do interesse particular para se estudar desordens neurológicas de equinos tais como a mielite por Herpesvírus tipo 1 (EHV-1), a EPM, a mielopatia degenerativa equina, a epilepsia equina/narcolepsia e a mielopatia estenótica vertebral cervical (CVSM) (BROCCARDO et al., 2014). O proteoma do CSF constitui-se como um compartimento rico em biomarcadores no qual as proteínas representam o estado fisiológico do CNS e

expoem informações valiosas sobre o processo da doença (VAN GOOL; HENDRICKSON, 2012). Um dos objetivos da proteômica é caracterizar proteínas celulares, proteínas secretadas e peptídeos e fragmentos proteolíticos como potenciais biomarcadores à luz de processos patológicos específicos. A avaliação seriada do CSF para monitorar a progressão da doença ou a eficácia do tratamento demonstra a utilidade clínica da análise proteômica do CSF (ROMEO et al., 2005). Por outro lado, estudos sobre o CSF são desafiadores devido à invasividade do procedimento de coleta de amostras e à dificuldade em obter amostras suficientes de humanos e modelos de experimentação animal de pequeno porte. Neste contexto, modelos experimentais de grandes animais apresentam como uma oportunidade única para investigar o CSF devido à facilidade de se obter grandes volumes de amostra e em momentos distintos da doença (BROCCARDO et al., 2014).

De um total de 247 proteínas encontradas no CSF da comparação EPM *vs*. Control, 201 proteínas foram identificadas após o critério de alta confiança estabelecido na metodologia e conversão de todos os números de acesso verificados no banco de dados UniProt (https://www.uniprot.org/). Destas 201 proteínas, 33 foram identificadas como exclusivas do grupo EPM, 33 exclusivas do grupo Control e 135 presentes em ambos. Das 135 presentes nos dois grupos de comparação, 7 estavam *up-regulated* e 25 *down-regulated* de acordo com o valor *p* da relação EPM:Control. Enquanto 113 não demonstraram diferenças de expressão na comparação. As proteínas do CSF identificadas em nosso estudo forneceram novos dados sobre a análise proteômica global de equinos saudáveis e seus termos GO (Gene Ontology) associados, bem como, fornecem um estado de doença específico nos cavalos afetados pela EPM, conduzindo para pesquisar potenciais alvos para biomarcadores relacionados com o diagnóstico e lesões neurais. Neste contexto, entre as 33 proteínas exclusivas observadas no CSF do grupo EPM, as enolases (Eno) podem desempenhar um papel importante na patogênese da doença neurológica equina, como descrito em humanos e roedores (LIMA et al., 2004; HATFIELD; MCKERNAN, 1992).

Por outro lado, estudos estabeleceram duas isoformas distintas de enolase, ENO1 e ENO2, em *T. gondii* a ENO1 existe principalmente no estágio de bradizoíto de *T. gondii*, enquanto que a ENO2 é mais expressa no estágio de taquizoíto (DZIERSZINSKI et al., 2001). Quando um evento desconhecido desencadeia uma conversão de estágio em parasitas apicomplexa de taquizoítos para bradizoítos, os genes enolase estão sobreexpressos ou exclusivamente expressos tanto no nível transcricional e como de proteína no estágio de bradizoíto (DZIERSZINSKI et al., 1999) quando em

comparação com os níveis detectados no estágio de taquizoíto. Isto é significativo considerando que os parasitas relacionados como *T. gondii* dependem da glicólise anaeróbia durante o período de encistamento de bradizoítos, devido à mitocôndria não funcional (WILSON et al., 2004). Estes estudos sustentam ainda mais a enolase como uma molécula alvo de prospeção para a imunoprofilaxia de estágios do parasita. A reatividade cruzada individual das isoformas de enolase entre os parasitas relacionados foi descrita. Por exemplo, anticorpo anti-*T. gondii* enolase 2 demonstrou a reatividade cruzada com taquizoítos de *Neospora* spp. e merozoítos de *S. Neurona* pela análise de WB, mas o anticorpos anti-T. gondii enolase 1 não reagiu com os merozitas de *S. Neurona* pela análise do WB (WILSON et al., 2004). Com base na sequência de homologia das sequências conhecidas de DNA de enolase 1 e 2 entre os parasitas Sarcocystidae, era de se esperar que as proteínas poderiam ser muito semelhantes e mostrarem perfis de expressão semelhantes (WILSON et al., 2004). Merozoítos de *Sarcocystis neurona* e os esquizontes reagiram com os anticorpos da anti-enolase 1 e 2. A coloração para anti-enolase 1 foi muito mais fraca em comparação com a coloração mais forte de anti-enolase 2 para os merozoítos de *S. Neurona* e esquizontes, sugerindo a existência de pelo menos duas supostas proteínas enolase em *S. Neurona* (BOLTEN et al., 2008). Assim, os achados de CSF-ENO1 e CSF-ENO2 nos cavalos infectados por *S. neurona* em nosso estudo podem representar um potencial marcador de EPM a ser investigado em estudos futuros.

2 CONCLUSÕES GERAIS

Os resultados obtidos com esta abordagem proteômica podem contribuir com o entendimento dos mecanismos de ação das MSCs no processo de neuroregeneração do CNS dos equinos; com o desenvolvimento de um protocolo de terapia celular com MSCs mais seguro e eficiente e com a identificação de potenciais marcadores de lesão neural e/ou de EPM.

Neste contexto, este estudo ao avaliar os perfis proteômico do CSF antes e após três transplantes intratecal de MSCs em equinos hígidos e do CSF de equinos cronicamente afetados pela EPM permitiu identificar as enolases como potenciais marcadores de lesão neural e/ou de EPM. Novos estudos devem ser realizados para confirmar estes achados e avançar no conhecimento dos efeitos da terapia celular com MSCs pela via intratecal no CSF, para o tratamento de lesões neurológicas em equinos.

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4 ANEXOS

4.1 Anexo 1

Normas de publicação da Revista *Stem Cell Research & Therapy.*

Submission Guidelines

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

present a title that includes, if appropriate, the study design e.g.:

"A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"

or for non-clinical or non-research studies a description of what the article reports

list the full names, institutional addresses and email addresses for all authors

if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below

indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

Background: the context and purpose of the study

Methods: how the study was performed and statistical tests used

Results: the main findings

Conclusions: brief summary and potential implications

Trial registration: If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be in stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words 'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

the aim, design and setting of the study

the characteristics of participants or description of materials

a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses

the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

Ethics approval and consent to participate
Consent for publication
Availability of data and material
Competing interests
Funding
Authors' contributions
Acknowledgements
Authors' information (optional)
Please see below for details on the information to be included in these sections.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

include a statement on ethics approval and consent (even where the need for approval was waived)

include the name of the ethics committee that approved the study and the committee's reference number if appropriate

Studies involving animals must include a statement on ethics approval.

See our editorial policies for more information.

If your manuscript does not report on or involve the use of any animal or human data or tissue, please state "Not applicable" in this section.

Consent for publication

If your manuscript contains any individual person's data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent for publication.

You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

See our editorial policies for more information on consent for publication.

If your manuscript does not contain data from any individual person, please state "Not applicable" in this section.

Availability of data and materials

All manuscripts must include an 'Availability of data and materials' statement. Data availability statements should include information on where data supporting the results reported in the article can be found including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. By data we mean the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article. We recognise it is not always possible to share research data publicly, for instance when individual privacy could be compromised, and in such instances data availability should still be stated in the manuscript along with any conditions for access.

Data availability statements can take one of the following forms (or a combination of more than one if required for multiple datasets):

The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

All data generated or analysed during this study are included in this published article [and its supplementary information files].

The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.

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The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].

Not applicable. If your manuscript does not contain any data, please state 'Not applicable' in this section.

More examples of template data availability statements, which include examples of openly available and restricted access datasets, are available here.

BioMed Central also requires that authors cite any publicly available data on which the conclusions of the paper rely in the manuscript. Data citations should include a persistent identifier (such as a DOI) and should ideally be included in the reference list. Citations of datasets, when they appear in the reference list, should include the minimum information recommended by DataCite and follow journal style. Dataset identifiers including DOIs should be expressed as full URLs. For example:

Hao Z, AghaKouchak A, Nakhjiri N, Farahmand A. Global integrated drought monitoring and prediction system (GIDMaPS) data sets. figshare. 2014. http://dx.doi.org/10.6084/m9.figshare.853801

With the corresponding text in the Availability of data and materials statement:

The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS].[Reference number]

Competing interests

All financial and non-financial competing interests must be declared in this section.

See our editorial policies for a full explanation of competing interests. If you are unsure whether you or any of your co-authors have a competing interest please contact the editorial office.

Please use the authors initials to refer to each authors' competing interests in this section.

If you do not have any competing interests, please state "The authors declare that they have no competing interests" in this section.

Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our editorial policies.

Please use initials to refer to each author's contribution in this section, for example: "FC analyzed and interpreted the patient data regarding the hematological disease and the transplant. RH performed the histological examination of the kidney, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript."

Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

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Please note that individual names may not be present in the PubMed record at the time a published article is initially included in PubMed as it takes PubMed additional time to code this information.

Authors' information

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You may choose to use this section to include any relevant information about the author(s) that may aid the reader's interpretation of the article, and understand the standpoint of the author(s). This may include details about the authors' qualifications, current positions they hold at institutions or societies, or any other relevant background information. Please refer to authors using their initials. Note this section should not be used to describe any competing interests.

Endnotes

Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

References

Examples of the Vancouver reference style are shown below.

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Web links and URLs: All web links and URLs, including links to the authors' own websites, should be given a reference number and included in the reference list rather than within the text of the manuscript. They should be provided in full, including both the title of the site and the URL, as well as the date the site was accessed, in the following format: The Mouse Tumor Biology Database. http://tumor.informatics.jax.org/mtbwi/index.do. Accessed 20 May 2013. If an author or group of authors can clearly be associated with a web link, such as for weblogs, then they should be included in the reference.

Example reference style:

Article within a journal

Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

Article within a journal (no page numbers)

Rohrmann S, Overvad K, Bueno-de-Mesquita HB, Jakobsen MU, Egeberg R, Tjønneland A, et al. Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. BMC Medicine. 2013;11:63.

Article within a journal by DOI

Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000; doi:10.1007/s801090000086.

Article within a journal supplement

Frumin AM, Nussbaum J, Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979;59 Suppl 1:26-32.

Book chapter, or an article within a book

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251-306.

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Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

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Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

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ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007.

Dataset with persistent identifier

Zheng L-Y, Guo X-S, He B, Sun L-J, Peng Y, Dong S-S, et al. Genome data from sweet and grain sorghum (Sorghum bicolor). GigaScience Database. 2011. http://dx.doi.org/10.5524/100012.

Figures, tables and additional files

Preparing figures
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Figures should be numbered in the order they are first mentioned in the text, and uploaded in this order.
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Individual figure files should not exceed 10 MB. If a suitable format is chosen, this file size is adequate for extremely high quality figures.

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Figure file types

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PowerPoint (suitable for diagrams and/or images, figures must be a single page)
TIFF (suitable for images)
JPEG (suitable for photographic images, less suitable for graphical images)
PNG (suitable for images)
BMP (suitable for images)
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Figure size and resolution

Figures are resized during publication of the final full text and PDF versions to conform to the BioMed Central standard dimensions, which are detailed below.

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width of 600 pixels (standard), 1200 pixels (high resolution).

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width of 85 mm for half page width figure
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maximum height of 225 mm for figure and legend
image resolution of approximately 300 dpi (dots per inch) at the final size

Figures should be designed such that all information, including text, is legible at these dimensions. All lines should be wider than 0.25 pt when constrained to standard figure widths. All fonts must be embedded.

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Vector figures should if possible be submitted as PDF files, which are usually more compact than EPS files.

TIFF files should be saved with LZW compression, which is lossless (decreases file size without decreasing quality) in order to minimize upload time.

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Conversion of images between file types (especially lossy formats such as JPEG) should be kept to a minimum to avoid degradation of quality.

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Tables larger than one A4 or Letter page in length can be placed at the end of the document text file. Please cite and indicate where the table should appear at the relevant location in the text file so that the table can be added in the correct place during production.

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Tabular data provided as additional files can be uploaded as an Excel spreadsheet (.xls) or comma separated values (.csv). Please use the standard file extensions.

Table titles (max 15 words) should be included above the table, and legends (max 300 words) should be included underneath the table.

Tables should not be embedded as figures or spreadsheet files, but should be formatted using ‘Table object’ function in your word processing program.

Color and shading may not be used. Parts of the table can be highlighted using superscript, numbering, lettering, symbols or bold text, the meaning of which should be explained in a table legend.

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Title of data
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For further guidance on how to use Additional files or recommendations on how to present particular types of data or information, please see How to use additional files.

4.2 Anexo 2

Normas de publicação da *Veterinary Research.*

Submission Guidelines

Preparing your manuscript
Length of article
The length of research articles should not exceed 35 pages. The journal allows a maximum of 70 references for research articles.

Title page
The title page should:

present a title that includes, if appropriate, the study design
list the full names, institutional addresses and email addresses for all authors
if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below
indicate the corresponding author
Abstract
The Abstract should not exceed 250 words

Keywords
Three to ten keywords representing the main content of the article.

Introduction
The Introduction section should be written in a way that is accessible to researchers without specialist knowledge in that area and must clearly state - and, if

helpful, illustrate - the background to the research and its aims. The section should end with a brief statement of what is being reported in the article.

Materials and methods

The Materials and methods section should include:

the aim, design and setting of the study

the characteristics of participants or description of materials

a clear description of all processes, interventions and comparisons. Generic names should generally be used. When proprietary brands are used in research, include the brand names in parentheses

the type of statistical analysis used, including a power calculation if appropriate

Results

This section should describe the outcome of the study. Data should be presented as concisely as possible, if appropriate in the form of tables or figures.

Discussion

The Discussion should be an interpretation of the results and their significance with reference to work by other authors.

Declarations

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

include a statement on ethics approval and consent (even where the need for approval was waived)

include the name of the ethics committee that approved the study and the committee's reference number if appropriate

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Consent for publication

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For information on how to cite your data and format this section, see preparing your manuscript.

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All financial and non-financial competing interests must be declared in this section. See our editorial policies for a full explanation of competing interests. If you are unsure whether you or any of your co-authors have a competing interest please contact the editorial office.

Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our editorial policies.

Acknowledgements

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Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements section.

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Group authorship: if you would like the names of the individual members of a collaboration group to be searchable through their individual PubMed records, please ensure that the title of the collaboration group is included on the title page and in the

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You may choose to use this section to include any relevant information about the author(s) that may aid the reader's interpretation of the article, and understand the standpoint of the author(s). This may include details about the authors' qualifications, current positions they hold at institutions or societies, or any other relevant background information. Please refer to authors using their initials. Note this section should not be used to describe any competing interests.

Endnotes

Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

References

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All references, including URLs, must be numbered consecutively, in square brackets, in the order in which they are cited in the text, followed by any in tables or legends. Each reference must have an individual reference number. Please avoid excessive referencing. The journal allows a maximum of 70 references for Research articles. If automatic numbering systems are used, the reference numbers must be finalized and the bibliography must be fully formatted before submission.

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Examples of the Veterinary Research reference style

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Mihovilovic MD, Leisch HG, Mereiter K (2004) Microwave-mediated intramolecular Diels-Alder cyclization of biodihydroxylated benzoic acid derivatives. Tetrahedron Lett 45:7087-7090

Article within a journal supplement
Fubini B (1997) Surface reactivity in the pathogenic response to particulates. Environ Health Perspect 105(Suppl 5):1013–1020

In press article
Van Damme S, Langenaeker W, Bultinck P (2012) Prediction of Blood-brain partitioning: a model based on ab initio calculated quantum chemical descriptors. Journal of Molecular graphics and modeling, in press

Published abstract
Herres-Pawlis S, Haase R, Bienemann O (2011) Dissecting the role of guanidine copper complexes in atom transfer radical polymerization by density functional theory [abstract] J Cheminf 3(Suppl1):P28

Article within conference proceedings
Schurath U, Wipprecht V (1980) Reactions of peroxiacyl radicals. In: Versino B, Ott H (eds) Proceedings of the 1st European Symposium on the Physico-Chemical Behavior of Atmospheric Pollutants, Ispra, October 1979. Commission of the European Communities, pp 157-166.

Book chapter, or article within a book
Ellis GP (1977) The chemistry of heterocyclic compounds. In: Weissberger A, Taylor ECE (eds) Chromene, Chromanes and Chromone, Volume 2. Wiley, New York

Whole issue of journal
Weckhuysen B (Ed) (2010) In-situ characterisation of heterogeneous catalysts. Chem Soc Rev 39:4541-5072

Whole conference proceedings
Brown S, Clarke I, Williams P (eds) (2001) Proceedings of the 14th International Symposium on Ceramics in Medicine, Palm Springs, California, USA.

Complete book
Görög S (1994) Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis. CRC Press, New York

Monograph or book in a series

Fujiki M (2008) Helix Generation, Amplification, Switching, and Memory of Chromophoric Polymers. In: Soai K (ed) Amplification of Chirality. Topics in Current Chemistry, vol 284. Springer, Heidelberg, pp 119-186

Book with institutional author
IUPAC (1979) Nomenclature of Organic Chemistry. Pergamon Press, Oxford

PhD thesis
Ravi Kishore VVN (2004) Photo-luminescence and electron-luminescence of hydroxy quinoline based organic semiconductors. PhD Thesis, Tata Institute of Fundamental Research, Department of Chemical Sciences

Link / URL
The Chemistry Development Kit. http://sourceforge.net/projects/cdk. Accessed 25 Dec 2012

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Pichon A (2010) Debating cyclobutadiene. http://blogs.nature.com/thescepticalchymist/2010/11/debating_cyclobutadiene.html. Accessed 22 Feb 2011

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