UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

MUTAÇÕES CAUSADORAS DO NANISMO DO TIPO CONDRODISPLÁSICO EM EQUINOS DA RAÇA MINI-HORSE NO BRASIL

DANILO GIORGI ABRANCHES DE ANDRADE

Botucatu, SP
Agosto de 2020

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

Orientador: Prof. Dr. José Paes de Oliveira Filho
Co-orientador: Prof. Dr. Alexandre Secorun Borges

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Aos animais.

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ANDRADE, D. G. A. **Mutações causadoras do nanismo do tipo condrodisplásico em equinos da raça Mini-Horse no Brasil.** Botucatu – SP, 2019. 76p. Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu.

RESUMO

Em equinos da raça Mini-Horse, o nanismo condrodisplásico possui padrão de herança autossômico recessivo e já foram descritas quatro variantes causais (*D1*, *D2*, *D3** e *D4*) no gene *aggrecan* (*ACAN*). Animais homozigotos ou heterozigotos compostos para essas variantes apresentam a doença. Os objetivos gerais desse estudo foram verificar as mutações já descritas anteriormente e investigar outra(s) possível(is) mutação(ões) relacionada(s) com o nanismo do tipo condrodisplásico em equinos da raça Mini-Horse, além de estimar suas frequências alélicas. O RNAm do gene *ACAN* foi sequenciado e, após a comparação entre animais afetados e seus pais, sugeriu-se que o polimorfismo de nucleotídeo único (SNP) c.6465A>T (g.95271115A>T; p.L2155F – RefSeq XM_005602799.2; XP_005602856.2 – EquCab3.0) fosse uma variante potencialmente associada ao nanismo condrodisplásico ainda não descrita na literatura consultada. Teste genético, baseado em reação em cadeia da polimerase (PCR) seguida de sequenciamento Sanger, para as cinco variantes em 347 equinos da raça Mini-Horse fenotipicamente normais, assim como o estudo *in silico* da estrutura proteica potencialmente associaram c.6465A>T com a enfermidade. Estudos em equinos de raças grandes demonstraram a presença do SNP c.6465A>T em heterozigose e dois cavalos Mangalarga Marchador fenotipicamente normais apresentaram-no em homozigose. Na população estudada de equinos da raça Mini-Horse fenotipicamente normais (n=347), a frequência alélica combinada de *D1*, *D2*, *D3**, *D4* e c.6465A>T foi de 0,392, o que sugere uma taxa de heterozigotos de 47,7%. Interações complexas no genoma de Mini-Horses possivelmente contribuem para a associação de c.6465A>T com o nanismo condrodisplásico ou este é um marcador em desequilíbrio ligado com uma variante causal ainda não identificada.

Palavras chave: agrecano; análise de sequência de DNA; condrodisplasia; doenças genéticas; neonatologia equina.

ANDRADE, D. G. A. **Chondrodysplasia-like dwarfism mutations in Miniature horses in Brazil.** Botucatu – SP, 2019. 76p. Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu.

ABSTRACT

In Miniature horses, chondrodysplastic dwarfism has an autosomal recessive inheritance pattern and four causative variants (*D1*, *D2*, *D3** and *D4*) in the *aggrecan* (*ACAN*) gene have been described previously. Homozygous or compound heterozygotes animals for these variants present the disorder. The general aims of this study were to verify the mutations already described and to investigate other possible variant(s) related to chondrodysplastic dwarfism in Miniature horses; in addition, to estimate the allelic frequency of the mutations. The mRNA of the *ACAN* gene was sequenced, and after comparing the affected animals and their parents, it was suggested that the single nucleotide polymorphism (SNP) c.6465A>T (g.95271115A>T; p.L2155F – RefSeq XM_005602799.2; XP_005602856.2 – EquCab3.0) could be potentially associated with dwarfism not yet described in the literature. Genetic test based in polymerase chain reaction (PCR) and Sanger sequencing for the five variants in 347 phenotypically normal Miniature horses, as well as the *in silico* protein structure study potentially associated c.6465A>T with dwarfism. Study in large sized horses showed the presence of the SNP in heterozygosis and two Mangalarga Marchador horses presented it in homozygosis. In the studied population of phenotypically normal Miniature horses (n=347), the combined frequency of *D1*, *D2*, *D3**, *D4* and c.6465A>T was 0.392, suggesting a heterozygous rate of 47.7%. Complex interactions in the Miniature horse genome could contribute to association of c.6465A>T with chondrodysplastic dwarfism or it could be a marker in linkage disequilibrium with a causative variant not yet identified.

Keywords: aggrecan; chondrodysplasia; equine neonatology; genetic diseases; sequence analysis, DNA.

CAPÍTULO I

Introdução

Os conhecimentos das mutações responsáveis pelas enfermidades de origem genética nos animais domésticos, assim como de suas prevalências, são relevantes para determinar o impacto destas doenças nas raças envolvidas (TRYON et al., 2009). Em equinos, há relatos de nanismo desproporcional principalmente na raça Friesian (BACK et al., 2008; LEEGWATER et al., 2016) e em raças pônei, como nos pôneis Shetland (RAFATI et al., 2016; METZGER et al., 2017) ou na raça Mini-Horse (WATANABE et al., 2014; EBERTH et al., 2018; ANDRADE et al., 2020). Nesta última, sabe-se que o padrão de herança é autossômico recessivo e envolve o gene *aggrecan* (*ACAN*) presente no cromossomo 1. Esse padrão de herança contribui para ampliar a disseminação da enfermidade genética de forma silenciosa, uma vez que os animais que carreiam os alelos mutantes (heterozigotos) não apresentam alteração clínica, somente seus descendentes quando na condição de homozigotos.

Já foram descritas quatro mutações (*D1*, *D2*, *D3** e *D4*) no gene *ACAN*, que são causadoras do nanismo do tipo condrodisplásico (EBERTH et al., 2018). Essa forma da doença envolve encurtamento e más-formações dos ossos longos e provoca o crescimento esquelético anormal em virtude de alterações cartilaginosas e subsequente maturação anormal dos ossos (EBERTH, 2013). Além disso, fetos abortados podem ser observados em algumas espécies (CAVANAGH et al., 2007; EBERTH et al., 2018; STRUCK et al., 2018). Devido à elevada possibilidade de complicações futuras e perda da qualidade de vida, muitos equinos clinicamente afetados são submetidos a eutanásia (WATANABE et al., 2014; METZGER et al., 2017). Estima-se que 26,2% do rebanho dos EUA seja heterozigoto para as mutações (*D1*, *D2*, *D3** e *D4*) no gene *ACAN* (EBERTH et al., 2018).

Muitas associações do cavalo Mini-Horse tentam evitar o registro de animais fenotipicamente anões, mas, infelizmente esses animais ainda são utilizados em programas de acasalamento (EBERTH, 2013). O padrão racial admitido pela Associação Brasileira dos Criadores de Mini-Horse (ABCMH) é de um animal equilibrado e proporcional. A *American Miniature Horse Association* (AMHA) sugere o mesmo padrão racial e ainda informa que o animal deve apresentar características de conformação e proporção idênticas a outros animais de raças grandes e, portanto, quando uma pessoa olhar uma fotografia

sem nenhum tipo de referência de tamanho, não saberia dizer se é uma miniatura ou não (EBERTH, 2013).

Além disso, considera-se que a raça Mini-Horse apresenta dupla aptidão, tração e montaria. No entanto, pode-se incluir uma terceira aptidão, a qual leva ao grande crescimento do número de animais criados por diversos tipos de proprietários: animal de estimação (ABCMH). Isso é explicado pelo fato de os animais não necessitarem de grandes espaços para manutenção e o custo de criação é baixo devido ao porte diminuto, elevadas resistência e rusticidade (ABCMH). Todos esses atributos facilitam sua comercialização e torna a raça um modelo experimental importante para a medicina equina e humana.

Pelo exposto acima, fica evidente a importância do nanismo condrodisplásico tanto para o animal quanto para o negócio do cavalo Mini-Horse. Os conhecimentos aprofundados dos achados clínicos e de dados epidemiológicos das mutações causadoras da doença, bem como a investigação de variantes causais ainda não descritas na literatura são importantes para minimizar o nascimento de animais clinicamente afetados, uma vez que a seleção dos acasalamentos poderá ser instituída como medida de controle e prevenção. Além disso, o sofrimento animal e as perdas econômicas seriam atenuados. Desta forma, este estudo foi idealizado visando caracterizar os achados clínicos de animais acometidos com nanismo condrodisplásico; confirmar a presença ou não das mutações já descritas em animais da raça Mini-Horse acometidos com nanismo condrodisplásico no Brasil; prospectar novas mutações no gene *ACAN* que podem estar associadas ao nanismo condrodisplásico em equinos da raça Mini-Horse; estimar a frequência alélica das mutações e padronizar teste genético que permita orientar os acasalamentos dos animais com o intuito de diminuir a prevalência do nanismo condrodisplásico em equinos da raça Mini-Horse no país.

Revisão de literatura

O nanismo, que consiste no desenvolvimento esquelético anormal de um indivíduo, é determinado por genes que além de contribuírem para uma pequena estatura, podem interferir negativamente em outros pontos importantes, como saúde e reprodução (EBERTH et al., 2009). Há duas categorias morfológicas nesta enfermidade: nanismo proporcional e nanismo desproporcional. Dentro de cada uma destas, existem numerosos fenótipos que têm sido descritos em seres humanos e nos animais domésticos, tais como: equinos, bovinos, ovinos, suínos, ratos, galinhas e outras espécies (WATANABE et al., 1994; NIELSEN et al., 2000; THOMPSON et al., 2005; MURGIANO et al., 2014; BOEGHEIM et al., 2017; HAUER et al., 2017; EBERTH et al., 2018).

Causas de nanismo podem envolver genes responsáveis pela produção, reconhecimento e metabolismo hormonal; por alterações na hipófise e/ou na tireoide e pelo desenvolvimento anormal das cartilagens e/ou da placa de crescimento ósseo (EBERTH, 2013). Em equinos, há relatos de nanismo desproporcional na raça Friesian (BACK et al., 2008; LEEGWATER et al., 2016), no pônei Shetland (RAFATI et al., 2016; MEZGER et al., 2017) e, principalmente, na raça Mini-Horse (EBERTH, 2013; WATANABE et al., 2014; EBERTH et al., 2018; ANDRADE et al., 2020).

Na raça Mini-Horse a enfermidade possui caráter autossômico recessivo e envolve o gene *aggrecan* (*ACAN*) presente no cromossomo 1 (OMIA 001271-9796). O agrecano, codificado pelo gene *ACAN*, é um grande proteoglicano de agregação da cartilagem e essencial para esta estrutura (KIANI et al., 2002; EBERTH, 2013). A cartilagem é composta por condrócitos e pela matriz extracelular produzida por estas células. As propriedades bioquímicas da cartilagem e a função física das articulações são dependentes da integridade da matriz extracelular, que é composta por proteoglicanos, ácido hialurônico, colágeno tipo II, glicoproteínas e fibras elásticas. Entre os proteoglicanos, o mais crucial para o bom funcionamento da cartilagem articular é, justamente, o agrecano (KIANI et al., 2002). No tecido cartilaginoso, os proteoglicanos promovem uma grande pressão osmótica, devido aos seus grupos aniônicos nas cadeias de glicosaminoglicanos ligados, principalmente, aos íons Na^+, e atraem água. Esta, fica retida nas articulações por causa desse desequilíbrio osmótico e porque o agrecano é uma molécula muito grande e imóvel para se redistribuir,

o que favoreceria a diminuição da pressão osmótica e a saída da água. Outra característica desse tecido, é a ligação e a formação de agregados entre o agrecano com o colágeno e com os glicosaminoglicanos, o que produz uma rede resistente e rígida. Essa é a base bioquímica das propriedades de viscoelasticidade da cartilagem que permitem a distribuição de carga nas articulações. Portanto, as interações osmótica e estrutural dessas moléculas asseguram as propriedades biomecânicas do tecido cartilaginoso (KIANI et al., 2002; ASPBERG, 2012). Além disso, o agrecano possui valor importante para o desenvolvimento ósseo, uma vez que serve como molde para a ossificação endocondral (ASPBERG, 2012).

A molécula de agrecano é composta por três domínios globulares (G1, G2 e G3), um domínio interglobular (IGD) entre G1 e G2 e uma extensa região entre G2 e G3 para ligação de cadeias de glicosaminoglicanos, na qual os domínios de sulfato de queratano (KS) e de sulfato de condroitina (CS) estão contidos. Funcionalmente, o domínio G1 interage com o ácido hialurônico e a proteína de ligação formando complexos ternários estáveis na matriz extracelular. O IGD é uma região curta e aparentemente está relacionado com a redistribuição fisiológica do agrecano. O domínio G2 não interage com o ácido hialurônico e nem com a proteína de ligação e sua função ainda é incerta, porém há indícios de que desempenhe papel importante na via de secreção do agrecano. A função do domínio KS também ainda não é muito clara, apesar de que as cadeias de sulfato de queratano presentes nessa região auxiliam na manutenção das moléculas de água no tecido. Semelhantemente, o domínio CS pode abrigar cadeias de aproximadamente 100 moléculas de sulfato de condroitina, as quais são carregadas negativamente e são essenciais para a função mais importante do agrecano como um proteoglicano estrutural, que é a capacidade de reter grande quantidade de água na matriz extracelular. Por fim, o domínio G3 liga os agregados entre o agrecano com o colágeno e com os glicosaminoglicanos aos outros componentes da matriz extracelular (KIANI et al., 2002; ASPBERG, 2012).

Mutações no agrecano são responsáveis por várias anormalidades de desenvolvimento ósseo em diferentes espécies. Em humanos, displasia espondiloepifiseal tipo Kimberley, caracterizada por membros e tronco encurtados, tem como causa uma inserção de um único par de base (pb) no

éxon 12 do gene *ACAN* (GLEGHORN et al., 2005). Em ratos, a deficiência de matriz cartilaginosa, caracterizada por fenda palatina e membros, cauda e focinho curtos, é causada por uma deleção de 7 pb no gene *ACAN*, que resulta em um códon de finalização prematuro e um encerramento prematuro da tradução (WATANABE et al., 1994). Já em bovinos da raça Dexter, normalmente os fetos afetados por nanismo, devido a alterações no gene *ACAN*, morrem por volta do sétimo mês de gestação, precipitando um abortamento (CAVANAGH et al., 2007). Essa forma de nanismo desproporcional é caracterizada clinicamente por coluna vertebral curta, micromelia, cabeça grande, focinho curto, fenda palatina e protrusão da língua (COELHO et al., 2013). Além disso, zebuínos também podem apresentar sinais clínicos semelhantes decorrentes de uma inserção de um pb no éxon 11 do gene *ACAN* (STRUCK et al., 2018).

Na raça Mini-Horse, existem, pelo menos, quatro mutações presentes no gene *ACAN* (*D1*, *D2*, *D3** e *D4*), que levam ao nanismo na forma condrodisplásica (EBERTH et al., 2018). Animais homozigotos ou heterozigotos compostos para essas variantes apresentam a doença. A enfermidade possui padrão de herança autossômico recessivo, envolve encurtamento e más-formações de todos os ossos longos e provoca o crescimento esquelético anormal em virtude de deformidades da cartilagem e subsequente maturação anormal dos ossos (EBERTH, 2013; EBERTH et al., 2018).

A deleção de uma base (adenina) no nucleotídeo 245 da sequência codificante (c.245delA) do gene *ACAN* (éxon 2) define a mutação *D1*. Genótipos envolvendo a mutação *D1* em homozigose (*D1*/*D1*) ou em combinação com qualquer outro alelo mutante (*D1*/*D2*, *D1*/*D3** ou *D1*/*D4*) parecem ser letais ainda na fase fetal (EBERTH et al., 2018). Por outro lado, os outros genótipos (*D2*/*D2*, *D2*/*D3**, *D2*/*D4*, *D3**/*D3**, *D3**/*D4* e *D4*/*D4*) estão envolvidos com nascimentos de animais clinicamente afetados (METZGER et al., 2017; EBERTH et al., 2018; ANDRADE et al., 2020). Entretanto, demonstrou-se que um indivíduo fenotipicamente anão chegou a nascer com genótipo *D1*/*D4* (EBERTH et al., 2018).

Uma substituição de uma guanina por uma adenina no nucleotídeo 1.270 da sequência codificante (c.1270G>A) do gene *ACAN* (éxon 6) indica a mutação *D2*. Os animais com os genótipos (*D2*/*D2*, *D2*/*D3** e *D2*/*D4*) são caracterizados clinicamente por membros curtos e tortuosos, pescoço encurtado, cabeça

grande e desproporcional em relação ao corpo, olhos proeminentes, estreitamento dos seios nasais e prognatismo (EBERTH et al., 2018).

Há apenas um relato de animal com genótipo homozigoto para *D3** (*D3**/*D3**). Esse equino era um pônei Shetland miniatura (METZGER et al., 2017), porém nenhum genótipo *D3**/*D3** foi relatado na literatura consultada em animais da raça Mini-Horse. Entretanto, o homozigoto *D3** não é considerado letal nessa raça, já que produtos envolvendo a mutação *D3** em heterozigose com as mutações *D2* (*D2*/*D3**) ou *D4* (*D3**/*D4*), também em heterozigose, chegam a nascer. Animais com genótipos *D2*/*D3** ou *D3**/*D4* apresentam características fenotípicas semelhantes aos equinos com genótipos envolvendo *D2* (*D2*/*D2*, *D2*/*D3** e *D2*/*D4*). A deleção de uma citosina no nucleotídeo 6.700 da sequência codificante (c.6700delC) do gene *ACAN* (éxon 11) definia esta mutação e era denominada *D3* (EBERTH, 2013). Entretanto, este resultado foi considerado um artefato de técnica e, posteriormente, o mesmo grupo de pesquisa modificou a definição desta mutação para uma substituição de uma guanina por uma citosina no éxon 7 do gene *ACAN* (g.95282140C>G) e passou a denominar tal mutação como *D3** (EBERTH et al., 2018), a mesma relatada por Metzger et al. (2017) em um pônei Shetland miniatura.

Animais com genótipos envolvendo *D4* (*D2*/*D4*, *D3**/*D4* e *D4*/*D4*), demonstram os mesmos sinais clínicos descritos anteriormente para os equinos com genótipos envolvendo *D2* e *D3** (EBERTH et al., 2018; ANDRADE et al., 2020). O estudo de Eberth (2013) definia a mutação *D4* pela deleção de 21 pb (CGTGGTGATGATCTGGCACGA) a partir do nucleotídeo 7.299 da sequência codificante (c.7299-7319del) do gene *ACAN* (éxon 15), mas foi alterada posteriormente para os 21 pb anteriores (g.95257458_95257500del) em estudo realizado pelo mesmo grupo de pesquisa (EBERTH et al., 2018).

Além disso, Eberth et al. (2018) sugeriram que existissem outras variantes causais de nanismo condrodisplásico em equinos Mini-Horse, uma vez que animais fenotipicamente anões foram encontrados, porém com genótipos sem a presença das mutações *D1*, *D2*, *D3** ou *D4* (N/N) ou com genótipos heterozigotos apenas para *D2* (N/*D2*), *D3** (N/*D3**) ou *D4* (N/*D4*). Ademais, ocorreram nascimentos de animais clinicamente afetados pelo nanismo condrodisplásico em um rebanho de equinos da raça Mini-Horse no interior paulista e a genotipagem destes animais não evidenciou os genótipos *D2*/*D2*,

D2/*D3**, *D2*/*D4*, *D3**/*D3**, *D3**/*D4* ou *D4*/*D4* descritos anteriormente como responsáveis pela enfermidade (ANDRADE et al., 2017; ANDRADE et al., 2018). Consequentemente, a avaliação de variantes no gene *ACAN*, possivelmente causadoras de nanismo condrodisplásico e ainda não relatados na literatura, torna-se necessária para um melhor entendimento da enfermidade.

O diagnóstico diferencial do nanismo condrodisplásico é o atavismo esquelético, que também é considerado uma forma de nanismo por diminuir o tamanho do indivíduo afetado, porém não possui as características histológicas de condrodisplasia (RAFATI et al., 2016). Por definição, atavismo é o reaparecimento de uma característica que foi vista em toda linha evolutiva anterior de uma espécie, mas não tem sido vista em antepassados recentes (TYSON et al., 2004). Como exemplos na espécie equina, citam-se: ulnas e fíbulas completas que se estendem até as articulações do carpo e tarso, respectivamente. Isso causa alterações na estrutura esquelética, promove o movimento de rotação dos membros e prejudica a movimentação. A maioria dos potros afetados são submetidos a eutanásia (TYSON et al., 2004; RAFATI et al., 2016). O atavismo esquelético também possui padrão de herança autossômico recessivo e deleções no gene *short stature homeobox* (*SHOX*) provocam a enfermidade (RAFATI et al., 2016).

O padrão de herança do nanismo relacionado com o gene *ACAN* (autossômico recessivo) contribui para ampliar a disseminação da enfermidade no plantel de forma silenciosa, uma vez que os animais que carreiam os alelos mutantes não apresentam alteração clínica, somente seus descendentes quando na condição de homozigotos. Além disso, ressalta-se o fato de existirem várias mutações envolvidas com o nanismo condrodisplásico em indivíduos da raça Mini-Horse, o que leva a uma complicação ainda maior em relação à probabilidade de aparecimento de animais afetados. Portanto, a identificação dos animais heterozigotos, tanto por metodologias de biologia molecular como por combinação dessas técnicas com a análise genealógica para minimizar custos e otimizar o processo, é importante para minimizar perdas econômicas significativas, diminuir a probabilidade de nascimentos de animais afetados resultantes de reprodutores e matrizes fenotipicamente normais e preservar o bem-estar animal evitando-se nascimentos de animais que poderiam ter indicação de eutanásia. Muitas associações do cavalo Mini-Horse tentam evitar

o registro de animais com nanismo condrodisplásico, mas, infelizmente, esses animais ainda são utilizados em programas de acasalamento (EBERTH, 2013). O padrão racial admitido pela Associação Brasileira dos Criadores de Mini-Horse (ABCMH) é de um animal equilibrado e proporcional. A *American Miniature Horse Association* (AMHA) sugere o mesmo padrão racial e ainda informa que o animal deve apresentar características de conformação e proporção idênticas a outros animais de raças grandes e, portanto, quando uma pessoa olhar sua fotografia sem nenhum tipo de referência de tamanho, não saberia dizer se é uma miniatura ou não (EBERTH, 2013).

Além disso, o cavalo Mini-Horse possui características únicas quando é abordado como modelo experimental para a medicina equina. Os indivíduos da raça são bons candidatos à cirurgia em decorrência de seu porte, uma vez que permitem aos cirurgiões tentativas de procedimentos que teriam baixas taxas de sucesso nas raças maiores, como por exemplo nas cirurgias ortopédicas (MUURLINK, 2012). Procedimentos cirúrgicos relacionados com fixação dorsal da patela; luxação da patela; deformidades angulares; colapso de traqueia; síndrome cólica, principalmente compactações de intestino delgado e distocia são problemas comuns em algumas linhagens. As técnicas cirúrgicas relatadas nos equinos de raças grandes para estas condições se aplicam a estes animais com favoráveis taxas de sucesso (KELMER e WILSON, 2007; MUURLINK, 2012; EASLEY e FREEMAN, 2013). Ademais, apresentam valores individuais, custos com alimentação, manutenção, treinamento/doma e materiais para montaria menores em relação às outras raças de equinos; ao passo que os custos com sanidade e média de expectativa de vida são os mesmos. Por fim, necessitam de menores áreas de pastejo e, consequentemente, a taxa de lotação pode ser maior comparada com equinos de raças grandes (AMHA).

Provavelmente, a condição do nanismo condrodisplásico muitas vezes não é divulgada amplamente pelos criadores devido ao impacto financeiro associado, no entanto, sabe-se que as más-formações relacionadas à enfermidade não são condições infrequentes no rebanho brasileiro de equinos Mini-Horse (ANDRADE et al., 2019). Portanto, esta enfermidade é um problema para o criador que deseja produzir um animal saudável, com proporções e conformação de acordo com as diretrizes do padrão racial.

CAPÍTULO II

Trabalho científico

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Description of the D4/D4 genotype in Miniature horses with dwarfism

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Running title: D4/D4 dwarfism genotype in Miniature horses

Abstract. Four causative mutations (D1, D2, D3*, and D4) of chondrodysplastic dwarfism have been described in the equine *aggrecan* (*ACAN*) gene. Homozygotes for one of these mutations and heterozygotes for any combination of these mutations exhibit the disproportionate dwarfism phenotype. However, no case description of homozygotes for D4 (D4/D4) has been reported in the literature, to our knowledge. We report 2 Miniature horses with the genotype D4/D4 in the *ACAN* gene. Clinically, the 2 dwarfs had a domed head that was large compared to the rest of the body, mandibular prognathism, and short and bowed limbs, mainly in the proximal region of the metatarsal bones. Radiographic examination revealed contour irregularities of the subchondral bone in the long bones and confirmed mandibular prognathism; histopathology revealed irregular chondrocyte organization. To determine the genotypes of the horses, we performed DNA extraction from white blood cells, PCR, and Sanger sequencing. Genotyping demonstrated that these 2 animals had the D4/D4 genotype in the *ACAN* gene. The D4/D4 dwarfs were clinically similar to animals with the other *ACAN* genotypes reported for this disease. Identification of heterozygous animals makes mating selection possible and is the most important control measure to minimize economic losses and casualties.

Key words: aggrecan; chondrodysplasia; dwarfism; equine.

Dwarfism, proportionate or disproportionate, is a form of abnormal development that is determined by genes contributing to small stature and may negatively interfere with the health and reproduction of the affected individual.[3] Aggrecan, encoded by the *aggrecan* (*ACAN*) gene, is a large proteoglycan that is essential for the proper functioning of articular cartilage.[8] Therefore, mutations in the *ACAN* gene are responsible for abnormalities in skeletal development in different species.[1,2,4,5,7,12] In Miniature horses, dwarfism is autosomal recessive, and 4 independently segregating mutations (D1, D2, D3*, and D4) in the *ACAN* gene have been described as causative of chondrodysplastic dwarfism in Miniature horses.[4,9] Genotypes involving D1 mutations cause fetal death, except in a single case, in which a full-term live dwarf was heterozygous for D1 and D4 (D1/D4).[4] In contrast, the other genotypes (D2/D2, D2/D3*, D2/D4, D3*/D3*, D3*/D4, and D4/D4) are theoretically expected to be involved in the birth of dwarf foals.[4] However, animals with the D4/D4 genotype have not been described in the literature, to our knowledge. We describe herein 2 Miniature horses with the D4/D4 genotype in the *ACAN* gene.

A Miniature horse neonatal foal with clinical signs of dwarfism was admitted alive to the Large Animal Internal Medicine Service of São Paulo State University (Unesp; Brazil) during the 2016/2017 foaling season, and a second foal was admitted during the 2017/2018 foaling season. The female (2016/2017) and the male (2017/2018) were both 1 d old and were from 2 different farms. All of the parents were normal in appearance, and mating between the same parents previously produced no dwarf births. In addition, no inbreeding was found between the parents. Blood samples were collected from the parents and affected animals for molecular analysis.

We performed our study in accordance with the policies of the Institutional Animal Care and Use Committee (0219/2016-CEUA) of Unesp. Epidemiologic,

clinical, radiologic, and pathology investigations and *ACAN* genotyping tests were conducted on the 2 Miniature horses with clinical signs of dwarfism and 1 Miniature 1-d-old foal without clinical signs of this disease (control foal), who died as a result of complications of dystocia. The parents of the affected foals were also subjected to *ACAN* genotyping tests. In addition, sequencing of *ACAN* messenger RNA (mRNA) from these 2 affected animals was performed using primer pairs that amplified the exons of the *ACAN* gene, and sequencing of these regions in the parents was also attempted.

During the physical examination, the following body measurements were recorded for the 2 dwarf foals and the control foal: head length (distance from the base of the ear to the lips); head width (distance between the most lateral part of the eyes); height at the withers (vertical distance from the highest point of the thoracic vertebrae to the ground); height at the croup (vertical distance from the highest point of the croup to the ground); thoracic and abdominal circumferences; forelimb length (distance from the humeroradial joint to the metacarpophalangeal joint); hindlimb length (distance from the femorotibial joint to the metatarsophalangeal joint); and cannon (fore and hind) length. The limbs (tibia/fibula and radius/ulna) and head were radiographed. Upper airway endoscopy was also performed in one affected foal.

Unfortunately, the filly died during the first minutes of medical care because of complications of dwarfism; she could not follow her dam to nurse to maintain glucose concentrations and properly hydrate or maintain body temperature until being admitted to the hospital. The colt was euthanized on the second day of life, and the 2 foals were autopsied. Portions of all organs were collected at autopsy on the first and second day of life of the filly and the colt, respectively, and fixed in 10% neutral-buffered formalin for histopathology or frozen for genetic tests. For histologic examination, fixed tissues were

routinely processed, stained with hematoxylin and eosin, and examined by light microscopy. The control foal was also subjected to histologic evaluation.

Genomic DNA was extracted from blood samples with a commercial kit (illustra blood genomicPrep mini spin kit; GE Healthcare Life Sciences, Opfikon, Switzerland) according to the manufacturer's instructions. PCR was performed using specific primers designed with an online tool (PrimerQuest tool; Integrated DNA Technologies, Coralville, IA). The primers amplified 4 fragments (Supplementary Table 1) containing the mutations (D1, D2, D3*, and D4) that were previously described[4] in coding exons 2, 6, 7, and 14, respectively, in the *ACAN* gene. To search for new mutations, 42 primer pairs (Supplementary Table 2) were designed to sequence the 16 coding exons of the predicted *ACAN* mRNA. Using dwarf skin samples, total RNA purification, DNase treatment, and complementary DNA (cDNA) synthesis were performed using a previously described methodology.[10] PCR was standardized to a final volume of 25 μL (12.5 μL of PCR master mix–GoTaq Green PCR master mix; Promega, Madison, WI; 300 nM of each primer, 8.5 μL of nuclease-free water, and 2.5 μL of DNA or cDNA template) with the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The products with the correct size were purified (Wizard SV gel and PCR clean-up system; Promega) according to the manufacturer's instructions. To sequence the DNA, 10 μL of purified PCR product and 5 μL of forward primers were used with a sequencing kit (BigDye terminator cycle sequencing kit; Thermo Fisher Scientific, Waltham, MA). The sequences were determined (3500 series genetic analyzer; Thermo Fisher Scientific). The obtained sequences and the electropherograms were analyzed (Geneious v.10.0.9; Biomatters,

Auckland, New Zealand) and compared with the normal equine *ACAN* gene sequence (RefSeq 100033876 and ENSECAG00000007493).

Physical examination revealed that the 2 dwarfs had a domed head that was large compared to the rest of the body, mandibular prognathism, and short and bowed limbs, mainly in the proximal region of the metatarsal bones (Figs. 1–4; Table 1).

In the radiographic examination of the 2 affected animals, irregularities of the subchondral bone in the long bones were observed in the proximal margin of the olecranon, in the distal region of the radius, in the dorsal aspect of the proximal metatarsal metaphysis, and in the femoral condyles. Angular deformities with deviations of the valgus and recurvatum types at the tarsal/metatarsal level and mandibular prognathism in the skull were also observed (Supplementary Figs. 1–4). Airway endoscopy of the affected colt revealed dorsal displacement of the soft palate, thickening of both arytenoid cartilages, and mild tracheal collapse (Supplementary Figs. 1–4).

No alteration was observed in the internal organs during autopsy, but microscopically, the proximal portion of the metatarsus (both in the articular cartilage and in the physis) showed mild and severe disorganization of the layers of chondrocytes in the affected filly and colt, respectively, and reduced extracellular matrix in both animals. The usual columnar alignment of hypertrophic cells was not present, and the demarcation between the epiphyseal plate growth zones was not apparent, mostly in the resting and proliferative zones. The ossifying trabeculae were also irregular. In addition, differentiated chondrocytes were absent from the articular cartilage in the colt, and the physis was thickened in the filly, but the same could not be stated about the colt given the intense disorganization of these cells (Supplementary Figs. 5–16).

The g.95257480_95257500del (D4) in the *ACAN* gene, which is responsible for dwarfism in Miniature horses,[4] was found to be homozygous (D4/D4) in the affected foals in our study. In addition, their parents were heterozygous at the same position (N/D4; Supplementary Figs. 17–20). All parents and the 2 affected foals were wild type (N/N) for the other 3 mutations (D1, D2, and D3*) in the *ACAN* gene that were described previously.[4] Two additional missense polymorphisms were found when sequencing exons of the *ACAN* gene (g.95271452C>T/p.A1875V and g.95270484A>G/p.R2198G, RefSeq 100033876). However, given that the stallions were also demonstrated to be homozygous for the same single-nucleotide polymorphisms and both mares were heterozygous for g.95271452C>T and homozygous for g.95270484A>G, these amino acid changes did not interfere with the functionality of the protein. Unfortunately, as reported in another study,[4] we were also unable to sequence the initial region of coding exon 11 (~1,000 bases).

The pathologic findings revealed chondrodysplasia-like characteristics, and genetic alterations confirmed the etiology of dwarfism in the affected foals. The foals were also evaluated radiographically (before confirmatory genetic test results for dwarfism), and the findings were not consistent with skeletal atavism because the horses did not have a complete fibula and ulna, as described previously.[11] In addition, the endoscopic findings were similar to those previously described in another foal with dwarfism.[6]

Phenotypically, these two D4/D4 dwarfs were similar to the D2/D2, D2/D3*, D2/D4, D3*/D3*, and D3*/D4 genotypes described previously in dwarf foals.[4,9] All of the dwarfs had a disproportionately short stature, a malformed skull, a shortened nasal bone, and mandibular prognathism,[4,9] but the front and hind cannon length appeared to be unique to D4/D4 dwarfs. Compared with a control foal, these animals appeared to

have shortened cannon bones and hindlimb deformity, with a plantarodorsal deviation in the region of the tarsometatarsal joint. The foals described in our study were neonates, and the adult phenotype of D4/D4 animals is still unknown. Possibly, in adult life, these animals could have a misshapen short barrel, similar to the other genotypes with D4 (D2/D4 and D3*/D4) that were described in a study conducted in the USA (Eberth JE. Chondrodysplasia-like dwarfism in the Miniature horse [master's thesis]. Lexington, KY: University of Kentucky, 2013).

The D4 mutation is not an embryonic lethal mutation in heterozygotes given that the combinations D2/D4 and D3*/D4 have produced live dwarf foals previously.[4] However, D4/D4 animals have not been detected previously, possibly because of the small sample sizes of previous studies or because D4 is considered a rare mutation in the populations already studied and was therefore not examined.[4] We suggest that a prevalence study should be performed to better understand the occurrence of this disease, given that 2 D4/D4 Miniature foals were found within a short period of time, and homozygous D4 mutations have not been described previously.

The autosomal recessive inheritance pattern silently contributes to the spread of the disease in the population because the parents (heterozygotes) are normal in appearance. Therefore, the identification of heterozygous animals is the most important control measure to minimize economic losses and casualties of affected animals given that such identification makes mating selection possible.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplementary material

Supplementary material for this article is available online.

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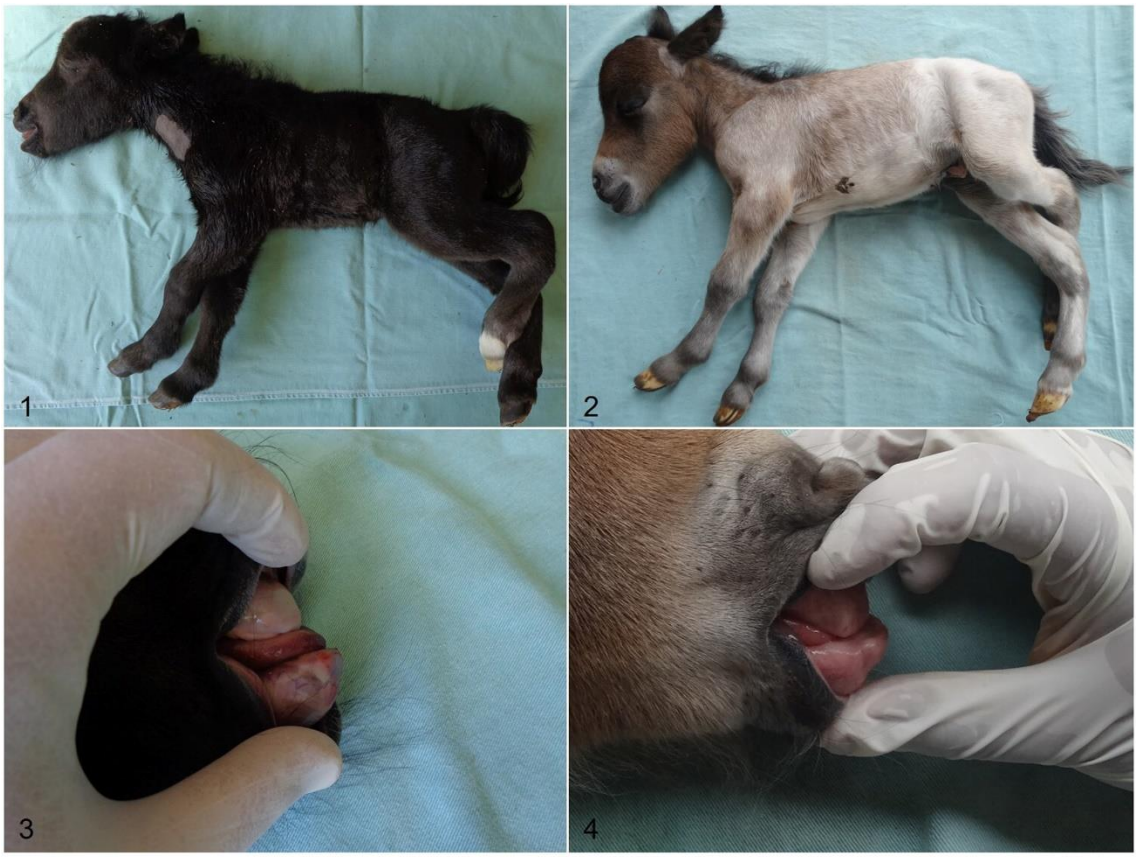
208 **Table 1.** Body measurements obtained from two 1-d-old Miniature horses with
209 disproportionate dwarfism (D4/D4) and a 1-d-old Miniature horse without
210 dwarfism (control).

Body measurement	Filly (D4/D4)	Colt (D4/D4)	Control filly (N/N)
Weight (kg)	9.5	8.0	10.0
Head length	23.0	19.0	19.0
Head width	11.0	12.0	10.0
Wither height (cm)	44.0	41.0	43.0
Croup height (cm)	40.0	38.0	42.0
Thoracic circumference (cm)	43.0	42.0	45.0
Abdominal circumference (cm)	43.0	36.0	38.0
Forelimb length (cm)	24.0	24.0	27.0
Third metacarpal length (cm)	10.0	10.0	12.0
Hindlimb length (cm)	27.0	27.0	31.0
Third metatarsal length (cm)	11.0	11.0	15.0

211



Figures 1–4. Clinical signs of dwarfism in Miniature horses. **Figures 1, 2.** Domed head and bowed limbs. **Figures 3, 4.** Mandibular prognathism. Figures 1 and 3 are of the same animal, as are Figures 2 and 4.

Supplementary Table 1. Primer sequences used for D1, D2, D3* and D4 *ACAN* variants (RefSeq 100033876 and ENSECAG00000007493).

Primer	Sequence (5' - 3')	Amplicon length (bp)	Coding exon
JP_ACAN_D1_Forward JP_ACAN_D1_Reverse	ACCCTGACAACTCGCTGA TCACCTCGCAGCGATAGAT	336	2
JP_ACAN_D2_Forward JP_ACAN_D2_Reverse	AGATGCCACTGCCACAAA GTGGTCACCTGTACCACAAG	268	6
JP_ACAN_D3*_Forward JP_ACAN_D3*_Reverse	GGAAAGAGGGAATGAACAGAGG AGTGACTGAATTAACCCACAGG	462	7
JP_ACAN_D4_Forward JP_ACAN_D4_Reverse	CGGTGAGGCCAGTTCTTT TCAGCTCCAATCTGCTTGTC	461	14

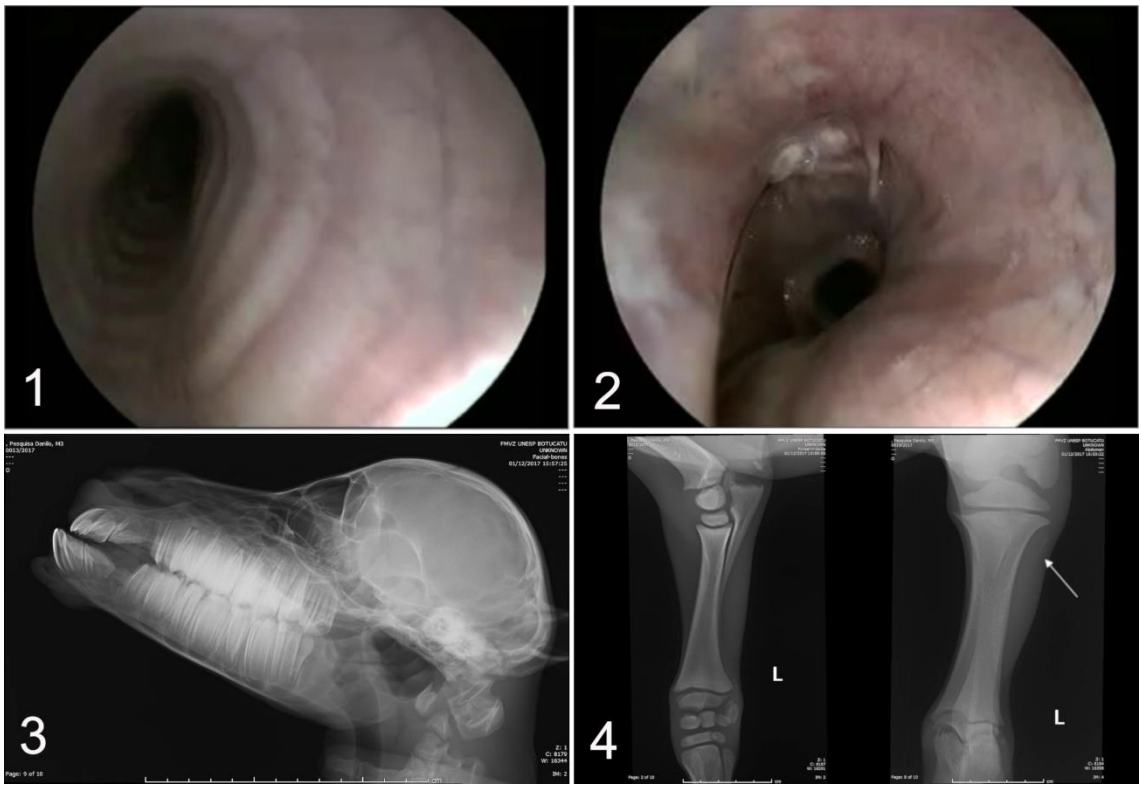
Supplementary Table 2. Primer sequences used for sequencing the exons of the *ACAN* gene (RefSeq 100033876 and ENSECAG00000007493).

Primer	Template	Sequence (5' – 3')	Amplicon length (bp)	Coding exon
DG_ACAN_Eq_F47 DG_ACAN_Eq_R47	DNA	CACGTACAGCCTCCTTATCTTG CTTCAGCGCCTGTGGATAAA	259	1
JP_ACAN_D1_Forward JP_ACAN_D1_Reverse	DNA	ACCCTGACAACTCGCTGA TCACCTCGCAGCGATAGAT	336	2
DG_ACAN_Eq_F34 DG_ACAN_Eq_R34	DNA	CTCCAATGACTCTGGCATCTATC CAGGGTCCAGAAAGAACGATAC	283	2
DG_ACAN_Eq_F35 DG_ACAN_Eq_R35	DNA	CCTCTGACTATCGCCGTAATTC AAGGATTTGGGACAGGTCATC	421	3
DG_ACAN_Eq_F36 DG_ACAN_Eq_R36	DNA	CCACAAGGGAGTCAGATACAAG TGTGATCCCGAGTGGTAGT	268	4
DG_ACAN_Eq_F37 DG_ACAN_Eq_R37	DNA	ACCCGGGCTTTGAGAATTTA CTGACATGAGGTTCAGGTATCC	554	5
DG_ACAN_Eq_F38 DG_ACAN_Eq_R38	DNA	GACGTCTTGTGCTATGATGACT CTTCACCCTCGGTGATGTTT	336	6
JP_ACAN_D2_Forward JP_ACAN_D2_Reverse	DNA	AGATGCCACTGCCACAAA GTGGTCACCTGTACCACAAG	268	6
DG_ACAN_Eq_F48 DG_ACAN_Eq_R48	DNA	AGGCGGAGAACGAGACT GGCCAAGTTCCTTCCACTT	216	6
JP_ACAN_D3*_Forward JP_ACAN_D3*_Reverse	DNA	GGAAAGAGGGAATGAACAGAGG AGTGACTGAATTAACCCACAGG	462	7
DG_ACAN_Eq_F50 DG_ACAN_Eq_R50	DNA	AGAACAGGCCCTCATTCTG GGTGAAGGTAATGCCCTCT	255	8
DG_ACAN_Eq_F51 DG_ACAN_Eq_R51	DNA	GTCGTTTCCGTGCAGAGT CCGGCGTAGCACTTGTC	261	9
DG_ACAN_Eq_F39 DG_ACAN_Eq_R39	DNA	CACGGGCCAGCTCTATG CTCCTTTCTCTGATGGATGGG	314	9
DG_ACAN_Eq_F40	DNA	CTGGTAAGGAGGAACCTTCAC	355	

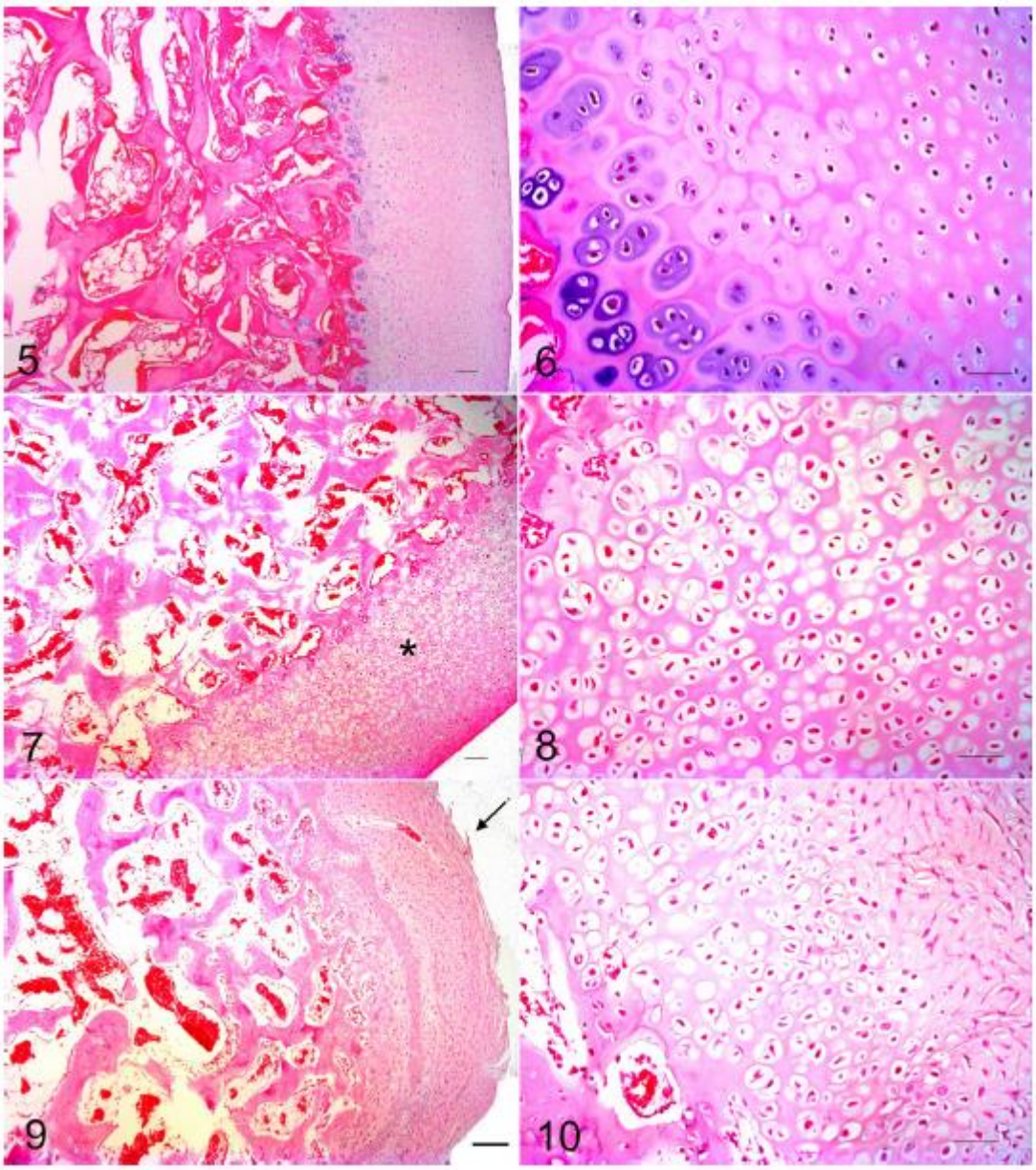
DG_ACAN_Eq_R40		GGGAAGCACAGCTAGGG		10
DG_ACAN_Eq_F41 DG_ACAN_Eq_R41	DNA	TTTCGCTTTGGAAGGAGTAGAG GAGCCCACTGCAGAGATAAG	416	11
DG_ACAN_Eq_F6 DG_ACAN_Eq_R6	DNA	ACCTCAGTGGACTTCCTTCT GGCTCAACATGAATCTCTCCTC	547	11
DG_ACAN_Eq_F7 DG_ACAN_Eq_R7	DNA	CCCTGGAATAGAGGATCTCAGT GAGGGAGTAGTCTCCAGATGAA	318	11
DG_ACAN_Eq_F12 DG_ACAN_Eq_R12	DNA	CTTCTGGAGCCTTGGACTTT ACGGATCCCAAACCTTCTTC	802	11
DG_ACAN_Eq_F13 DG_ACAN_Eq_R13	DNA	CAGTGGGACAACATCTGGAA CTTCTCCACTGGACTCAACAA	803	11
DG_ACAN_Eq_F29 DG_ACAN_Eq_R29	DNA	GCCTCAGGACTTCCAGAAATTA GTAGGTGGTGGTTGACACTC	450	11
DG_ACAN_Eq_F42 DG_ACAN_Eq_R42	DNA	CTTGTTGAGTCCAGTGGAGAAG TGTGCTCAGAACCTTCAATCTC	734	11
DG_ACAN_Eq_F43 DG_ACAN_Eq_R43	DNA	CTCTTGAGGGCATGACATTGA TGCTGACTTCTGGCAAGTG	359	12
DG_ACAN_Eq_F44 DG_ACAN_Eq_R44	DNA	GCCTCTATGAGCTCAACTTTCT CATGCCGTCCGAATGTATCT	245	13
JP_ACAN_D4_Forward JP_ACAN_D4_Reverse	DNA	CGGTGAGGCCAGTTCTTT TCAGCTCCAATCTGCTTGTC	461	14
DG_ACAN_Eq_F45 DG_ACAN_Eq_R45	DNA	CATCACAGGAGCCCTTTCT CGGTCTGTGCAGGTGAT	223	15
DG_ACAN_Eq_F52 DG_ACAN_Eq_R52	DNA	ACGATGAGGAGGGCTCA TGGAATTCCTTAGAGGACAGAAAG	261	16
DG_ACAN_Eq_F1 DG_ACAN_Eq_R1	cDNA	GCCGCTCCAGGTGTATG GGCTCTGTAATGGAACACGA	540	1, 2, 3
DG_ACAN_Eq_F18 DG_ACAN_Eq_R18	cDNA	GAACCTGCGCTCCAATGA CCGGAGACGTTGCGTAAA	422	2, 3, 4
DG_ACAN_Eq_F19 DG_ACAN_Eq_R19	cDNA	TACGACGTGTACTGCTTTGC TTTGTGGCAGTGGCATCT	436	4, 5, 6
DG_ACAN_Eq_F3 DG_ACAN_Eq_R3	cDNA	AACTTCTTCGCTGTGAGTGG CCGGCGTAGCACTTGTC	815	6, 7, 8, 9
DG_ACAN_Eq_F4 DG_ACAN_Eq_R4	cDNA	AAACCTATGATGTCTACTGCTACG CTTCTGTGCTCTCCTCTGTTG	603	8, 9, 10
DG_ACAN_Eq_F6 DG_ACAN_Eq_R6	cDNA	ACCTCAGTGGACTTCCTTCT GGCTCAACATGAATCTCTCCTC	547	11
DG_ACAN_Eq_F7 DG_ACAN_Eq_R7	cDNA	CCCTGGAATAGAGGATCTCAGT GAGGGAGTAGTCTCCAGATGAA	318	11
DG_ACAN_Eq_F11 DG_ACAN_Eq_R11	cDNA	ACTGCCTCTGGAGTAGAAGA CCAGACGGAAGTCCACTAAAG	659	11
DG_ACAN_Eq_F12 DG_ACAN_Eq_R12	cDNA	CTTCTGGAGCCTTGGACTTT ACGGATCCCAAACCTTCTTC	802	11
DG_ACAN_Eq_F13 DG_ACAN_Eq_R13	cDNA	CAGTGGGACAACATCTGGAA CTTCTCCACTGGACTCAACAA	803	11
DG_ACAN_Eq_F29 DG_ACAN_Eq_R29	cDNA	GCCTCAGGACTTCCAGAAATTA GTAGGTGGTGGTTGACACTC	450	11
DG_ACAN_Eq_F30 DG_ACAN_Eq_R30	cDNA	TCTGAGTGCAGCCACCT CTGTCAGTCCTGTCTCTGGAA	216	11
DG_ACAN_Eq_F31 DG_ACAN_Eq_R31	cDNA	CTACCACGTTGGCACAGAG TGACGGCTGTCTCCGATA	308	11
DG_ACAN_Eq_F32	cDNA	GCTTCCATCCCAGCTTCTC	234	

DG_ACAN_Eq_R32		CTGGTAGTCTTGGGCATTGT		11, 12
DG_ACAN_Eq_F33 DG_ACAN_Eq_R33	cDNA	GCCACTGTTACCGCTACTTT CTTCTGTAGTCTGCGCTTGTAG	560	12, 13, 14, 15, 16
DG_ACAN_Eq_F16 DG_ACAN_Eq_R16	cDNA	CACCGAGGGCTTTGTCC GAGGACAGAAAGCGACAAGAA	265	15, 16

221

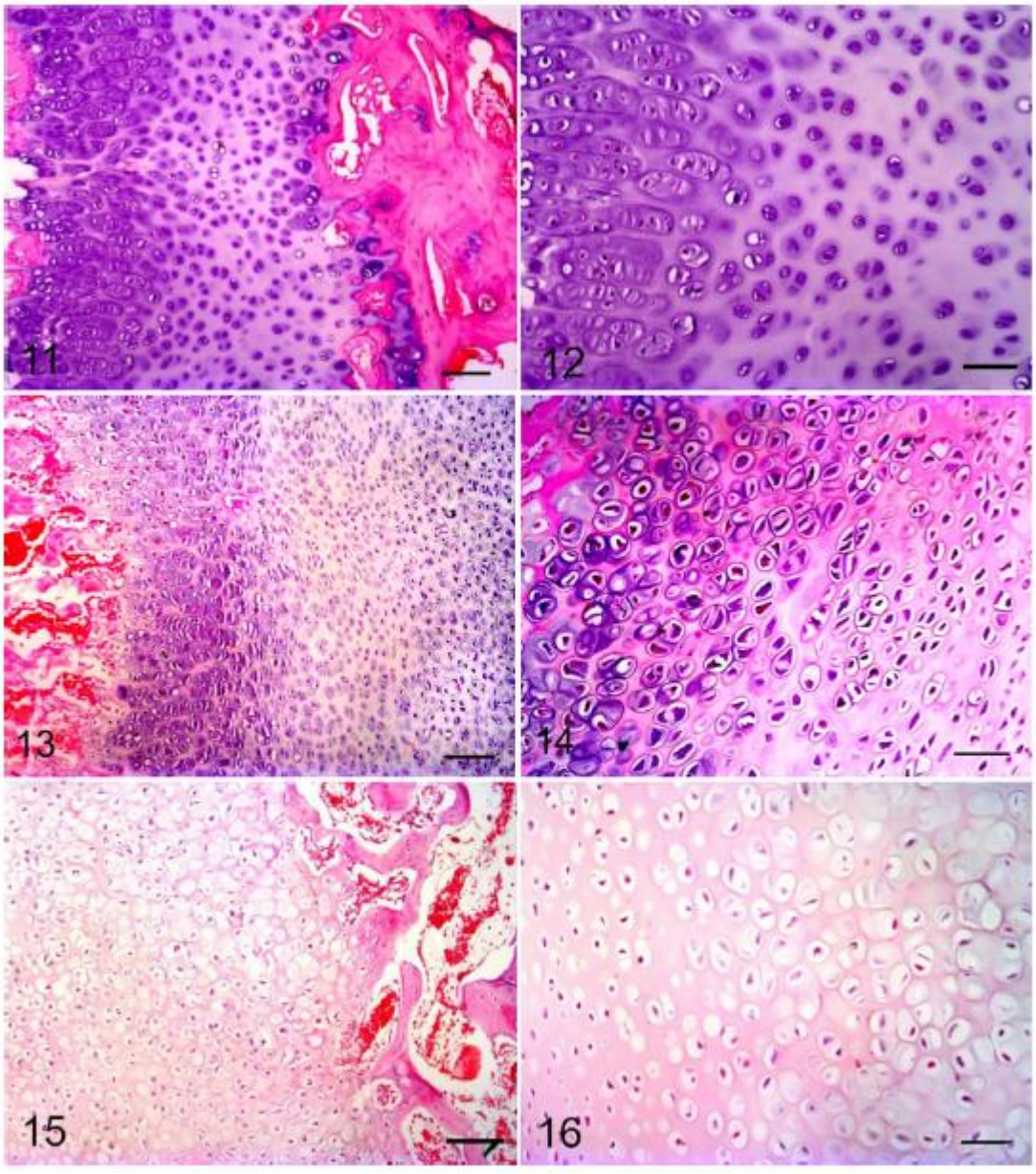


Supplementary Figures 1–4. Endoscopic and radiographic findings in Miniature horses with dwarfism. **Figure 1.** Mild tracheal collapse. **Figure 2.** Dorsal displacement of the soft palate. **Figure 3.** Mandibular prognathism. **Figure 4.** Incomplete ulna with irregular and thick proximal physis. Enlargement of the proximal and distal epiphyses of the radius secondary to chondrodysplasia. Incomplete and filiform fibula (arrow) with incomplete bone mineralization (secondary to age) and reaching the middle third of the tibia.



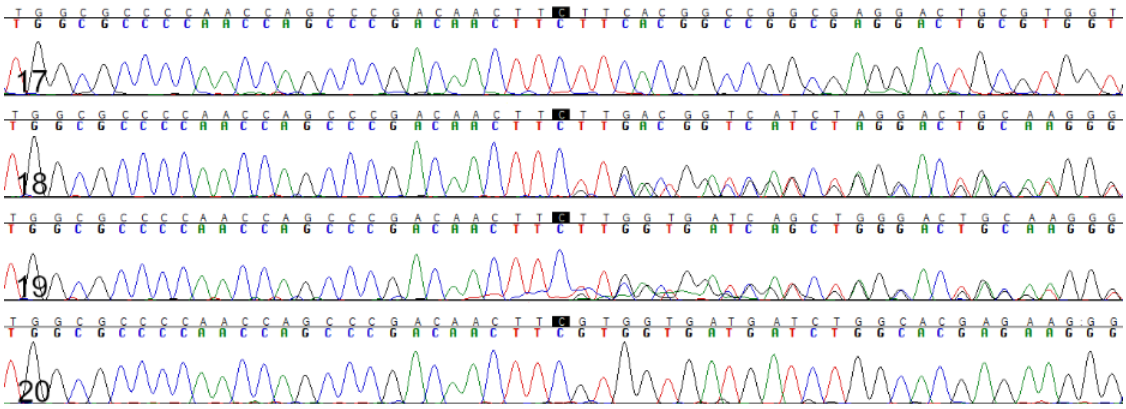
Supplementary Figures 5–10. Histologic images of the articular cartilage. H&E. **Figure 5.** One-day-old Miniature horse (control animal) without dwarfism: with normal cartilage structure. Bar = 100 μm. **Figure 6.** Higher magnification of Fig. 5. Bar = 50 μm. **Figure 7.** One-day-old Miniature horse (filly) with dwarfism: articular cartilage with mild lesion, note the disorganization of the layers of chondrocytes (*) and reduced extracellular matrix. Bar = 100 μm. **Figure 8.** Higher magnification of Fig. 7. Bar = 50 μm. **Figure 9.** One-day-old Miniature horse (colt) with dwarfism: articular cartilage with severe lesion, note the disorganization of the tissue, absence of differentiated

chondrocytes, and clefts in the surface (arrow). Bar = 100 μm. **Figure 10.** Higher magnification of Fig. 9, with the absence of differentiated chondrocytes. Bar = 50 μm.



Supplementary Figures 11–16. Histologic images of the physis cartilage. H&E. **Figure 11.** One-day-old Miniature horse (control animal) without dwarfism: with normal structure. Bar = 100 μm. **Figure 12.** Higher magnification of Fig. 11. Bar = 50 μm. **Figure 13.** One-day-old Miniature horse (filly) with dwarfism: physis cartilage with mild lesion. Bar = 100 μm. **Figure 14.** Higher magnification of Fig. 13. Bar = 50

μm. **Figure 15.** One-day-old Miniature horse (colt) with dwarfism: articular cartilage with severe lesion. Bar = 100 μm. **Figure 16.** Higher magnification of Fig. 15, with severe swelling of chondrocytes and reduced extracellular matrix production. Bar = 50 μm.



Supplementary Figures 17–20. Partial chromatogram obtained from the assembly of *ACAN* gene sequences from a control foal (**Fig. 17**), stallion (**Fig. 18**), mare (**Fig. 19**), and affected foal (**Fig. 20**). A double peak from the selected cytosine ("C" in black) was observed in the heterozygote sequences (**Figs. 18, 19**), and a deletion of 21 base pairs (CTTCACGGCCGGCGAGGACTG) from the selected base was observed in the homozygote sequence (**Fig. 20**).

CAPÍTULO III

Trabalho científico

Trabalho aceito para publicação na revista "*Scientific Reports*". Nesta tese, o artigo está formatado de acordo com as normas para a submissão, que podem ser encontradas em: https://www.nature.com/srep/author-instructions/submission-guidelines.

Evaluation of a new variant in the *aggrecan* gene potentially associated with chondrodysplastic dwarfism in Miniature horses

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ABSTRACT

Chondrodysplastic dwarfism in Miniature horses is an autosomal recessive disorder previously associated with four mutations (*D1, D2, D3*,* and *D4*) in the *aggrecan* (*ACAN*) gene. The aim of this study was to identify additional variants in the candidate *ACAN* gene associated with chondrodysplastic dwarfism in Miniature horses. Fifteen dwarf Miniature horses were found to possess only one of the dwarfism-causing variants, and two possessed none of the variants. The *ACAN* exons (EquCab3.0) of seven dwarf Miniature horses were sequenced. A missense SNP in coding exon 11 (g.95271115A>T, c.6465A>T – RefSeq XM_005602799.2), which resulted in the amino acid substitution p.Leu2155Phe (RefSeq XP_005602856.2), was initially associated with the dwarf phenotype. The variant was tested and found present in 14 dwarf foals as well as one parent of each, and both parents of a dwarf possessing two copies. Genetic testing of 347 phenotypically normal Miniature horses demonstrated that none had more than one of the dwarf alleles or c.6465A>T. However, a study of large breeds revealed the presence of c.6465A>T, which was present in homozygosis in two Mangalarga Marchador horses. We suggest that c.6465A>T as a marker of disequilibrium or complex interactions in the Miniature horse genome could contribute to the associated dwarfism.

Introduction

Chondrodysplastic dwarfism is a genetic disorder that leads to a disproportionate reduction in body size and may negatively interfere with the development and reproduction of the affected individual [1]. In addition, aborted foetuses can be observed in some species as a clinical sign of this disease [2,3]. In horses, reports of dwarfism are mainly related to Friesian horses, Shetland ponies, and Miniature horses

[2,4-6]. In all these breeds, the disorder presents an autosomal recessive inheritance pattern, but the causative mutations are in different genes. In Friesian horses, the *beta-1, 4 galactosyltransferase 7* (*B4GALT7*) gene is involved; in Miniature horses and Miniature Shetland ponies, the causative mutations are in the *aggrecan* (*ACAN*) gene [2,4,5], or in the *short stature homeobox* (*SHOX*) gene [6].

The *ACAN* gene encodes the large proteoglycan aggrecan, which provides a hydrated gel structure for the proper functioning of the articular cartilage, since the joints are dependent on the integrity of the extracellular matrix, which is composed of proteoglycans, hyaluronic acid, collagen type II, glycoproteins and elastic fibres. In addition, among the proteoglycans, aggrecan is the most crucial for the proper functioning of articular cartilage and is essential in chondroskeletal morphogenesis [7].

Mutations in the *ACAN* gene also cause chondrodysplasia-like dwarfism in other species [3,8-10]. In humans, at least 25 pathological *ACAN* mutations have been identified as the cause of short stature [8]. Spondyloepiphyseal dysplasia type Kimberley, characterized by shortened limbs and trunk, is one of these diseases and is caused by a single base pair (bp) insertion in exon 12 of the *ACAN* gene [9]. In rats, cartilaginous matrix deficiency, characterized by cleft palate and short limbs, tail and muzzle, is caused by a seven bp deletion in the *ACAN* gene, resulting in a premature stop codon [10]. In turn, Dexter cattle foetuses affected by dwarfism related to alterations in the *ACAN* gene die around the seventh month of gestation [3].

In Miniature horses, four *ACAN* gene variants (*D1*, *D2*, *D3** and *D4*) have already been described as causative of chondrodysplastic dwarfism [2,5]. Most *D1* genotypes (*D1*/*D1*, *D1*/*D2*, *D1*/*D3** and *D1*/*D4*) cause foetal death [2], whereas the other genotypes (*D2*/*D2*, *D2*/*D3**, *D2*/*D4*, *D3**/*D3**, *D3**/*D4* and *D4*/*D4*) are involved in the birth of dwarf foals [2,5,11]. Clinical diagnosis of chondrodysplastic dwarfism in

Miniature horses has already been described in Brazil [12], and the *D4*/*D4* genotype was recently characterized in the same country [11]. However, it is still not possible to associate all dwarf Miniature horses with the previously described *ACAN* variants [2]. The aims of the present study were to locate and identify other variants in the candidate *ACAN* gene associated with chondrodysplastic dwarfism in Miniature horses and to verify the allele frequencies of the causative variants in a phenotypically normal population of Miniature horses in Brazil.

Results

ACAN variants detection

The genotyping for the four previously described causative variants (*D1, D2, D3** and *D4*) of dwarfism [2] in the 18 dwarf Miniature horses used in this study revealed that 13 animals were heterozygous for only *ACAN-D4* (N/*D4*), two animals were heterozygous for only *ACAN-D2* (N/*D2*), one animal had the *D2*/*D3** genotype, and two animals did not possess any of the known dwarfism alleles (*D1, D2, D3**, or *D4*). *ACAN* exons from seven dwarf Miniature horses (animals 1-7), which were admitted to the Large Animal Internal Medicine Section of São Paulo State University (Unesp), were sequenced except for two fragments (102 bp and 1,252 bp) in coding exon 11, which is located in a highly repetitive region. No alterations in the exon-intron junctions were found, and some genetic alterations were detected after comparison with the equine *ACAN* mRNA sequence (RefSeq XM_005602799.2 – EquCab3.0). A total of six synonymous SNPs and four missense SNPs were found in the *ACAN* gene of dwarf Miniature horses (Table 1).

To check the relationship of these missense SNPs with the dwarf phenotype, the parents of the affected Miniature horses were genotyped according to these SNPs and

the variants *D1*, *D2*, *D3**, and *D4*. The SNPs c.3277A>C and c.7096A>G (RefSeq XM_005602799.2 – EquCab3.0) were also found to be homozygosis in 30.8% and 100% of the parents, respectively. Since the parents were phenotypically normal and these two SNPs were found to be homozygosis, c.3277A>C and c.7096A>G were discarded as responsible for dwarfism in the Miniature horses with inconclusive genotypes in the present study. SNP c.6128C>T (RefSeq XM_005602799.2 – EquCab3.0) was not found to be homozygosis in any of the parents of the affected animals; however, 87.5% of the parents were heterozygous for both this SNP and the *D4* mutation. Since these parents were phenotypically normal, c.6128C>T was also discarded. Conversely, all the parents were homozygous for the reference allele (c.6465A - RefSeq XM_005602799.2 – EquCab3.0) or heterozygous for the SNP c.6465A>T. The parents that were heterozygous for the c.6465A>T variant did not possess any of the *ACAN* causative variants (*D1*, *D2*, *D3**, and *D4*). In addition, six of seven dwarf offspring of the c.6465A>T heterozygous parents were also heterozygous for c.6465A>T and for the *ACAN-D4* causative variant previously described [2], and the other dwarf foal was homozygous for SNP c.6465A>T. Therefore, the analysis of the sequencing results between the affected animals and their parents revealed that c.6465A>T (g.95271115A>T; p.Leu2155Phe - RefSeq XM_005602799.2; RefSeq XP_005602856.2 – EquCab3.0) would be a novel variant potentially associated with chondrodysplastic dwarfism in Miniature horses (Figure 1, Supplementary Figure S1).

The two dwarf Miniature horses assessed in the present study, which did not possess the *D1*, *D2*, *D3**, and *D4* variants (animals 2 and 15), were found to be homozygous for c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0). In addition, the 13 animals that were heterozygous for only *ACAN-D4* (N/*D4*) and the two animals that

were heterozygous for only *ACAN-D2* (N/*D2*) were also heterozygous for c.6465A>T (Table 2).

All the missense SNPs were analysed by PolyPhen-2, SIFT and PROVEAN. The amino acid residue substitution p.Leu2155Phe (RefSeq XP_005602856.2; c.6465A>T, RefSeq XM_005602799.2 – EquCab3.0) was predicted to be "probably damaging" (0.994), and it was noted that this substitution "affects protein function" (0.00) according to PolyPhen-2 and SIFT, respectively. A PROVEAN analysis suggested that p.Leu2155Phe was "neutral" using the default cutoff (score -2.222, cutoff = -2.5). However, the prediction for p.Leu2155Phe would be "deleterious" if the cutoff were higher (cutoff = -1.3), similar to the sensitivity of the detection. In addition, the aggrecan amino acid sequence from an affected Miniature horse homozygous for c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0) was aligned with those of 21 other species. Comparison of the amino acid sequences in this section for 21 species (Supplementary Figure S2) showed a conservation of leucine at this position in 13 but substitution with threonine or glutamine in 8 others.

Finally, 93 horses from different large breeds (23 Mangalarga Marchador, 23 Warmblood, 23 Thoroughbred and 24 Quarter horses) were tested for SNP c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0). Two Mangalarga Marchador horses were homozygous, and heterozygous animals were found in all tested breeds (Table 3). We also sequenced 26 horses from the same breeds for *D1*, *D2*, *D3**, and *D4* (9 Mangalarga Marchador, 5 Warmblood, 6 Thoroughbred, and 6 Quarter horses). All of them were N/N except one Mangalarga Marchador horse that was N/*D2* (Table 4).

Dwarf Miniature horses

Eighteen Miniature horses with dwarfism were used in this study, belonging to five different farms (Table 2), and were tested for existing *ACAN* alleles [2] (Table 1). Fifteen dwarf Miniature horses (animals 1-15) were admitted at the Large Animal Internal Medicine Section of São Paulo State University (Unesp). Of these, 14 animals were one day old, of which two died during medical care and 12 were euthanized on the second day of life due to future dwarfism complications. One horse (animal 15; homozygous for *ACAN*:c.6465A>T) was one year old and was discharged after improvement in respiratory clinical signs; we have no knowledge of his history after hospital discharge. During sample collection for the *ACAN* variants prevalence estimation, three Miniature horses were also phenotypically identified as dwarfs (animals 16-18).

Physical examination of animals 1-16 revealed a disproportionate and short body, shortened limbs relative to overall body size, a disproportionately large cranium, bilateral enlarged eye sockets with prominent eyes, a shortened nasal bone, mandibular prognathism, a shortened neck, and bowed limbs, mainly the hind limbs (Figure 2). The two affected mares that were evaluated at a farm both were heterozygous for *ACAN-D2* and for *ACAN*:c.6465A>T (animals 17 and 18). It was determined that their phenotypic characteristics were different from each other, despite having the same genotype, and they were mildly affected compared with the other affected animals in this study. One had a disproportionate and short body, shortened limbs relative to overall body size and a shortened neck, while the other had shortened limbs relative to overall body size and bilateral enlarged eye sockets with prominent eyes. As their phenotypic characteristics were only mildly observable, their owner did not notice that they were dwarfs, so they had both been used for breeding.

In the radiographic examinations of the 15 affected Miniature horses that were referred to the veterinary hospital (animals 1-15), irregularities in the limbs and in the head (e.g., subchondral bone irregularities in the long bones and mandibular prognathism) were observed. In addition, tracheal collapse (Supplementary Figure S3) was radiographically observed in the one-year-old Miniature horse with dwarfism (animal 15; homozygous for *ACAN*:c.6465A>T). This animal presented with respiratory distress, tachypnoea, inspiratory honking noises and dysphagia. The other dwarfs had no clinical signs of tracheal collapse, but airway endoscopy revealed mild tracheal collapse in eight of ten dwarf Miniature horses (animals 5-14). Moreover, other abnormalities were observed during airway endoscopy examination, such as dorsal displacement of the soft palate (5/10) and thickening of both arytenoid cartilages (4/10). Histological evaluation of the proximal portion of the metatarsus revealed lesions consistent with chondrodysplasia. Disorganization of the cartilage structure and a decrease in the extracellular matrix were observed in all of the affected Miniature horses (Supplementary Figure S4) that were examined (n=14).

Population screening

Of the 347 phenotypically normal Miniature horses assigned to determine the allele frequencies of the *ACAN* causative variants, 34.6% (120/347) were males and 65.4% (227/347) were females. These animals belonged to nine farms; all but one had a history of births of affected animals (Table 5).

Among the phenotypically normal Miniature horses, none was identified with more than one of the *D1*, *D2*, *D3**, *D4* or c.6465A>T variants. The cumulative frequency of the four causative alleles of dwarfism (*D1*, *D2*, *D3**, and *D4*) was 0.280 (q), which suggests a heterozygous (2pq) rate of 40.3% and a predicted rate of dwarf

Miniature horses (q^2) of 7.8%. The frequencies of the four *ACAN* gene variants associated with dwarfism are shown in Table 6. The allele frequency of c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0) was 0.112, and if this variant could be associated with dwarfism, the cumulative frequency would be 0.392 (q), which could suggest a heterozygous (2pq) rate of 47.7% and a predicted rate of dwarf Miniature horses (q^2) of 15.4%.

Discussion

The initial genotyping results of the 18 dwarf Miniature horses assessed in this study, similar to the findings of a previous study [2], reinforced the suspicion of the existence of additional mutations responsible for dwarfism in Miniature horses. Although other genes have already been associated with dwarfism in horses [4,6], we decided to investigate novel variants in the candidate *ACAN* gene, since mutations in this gene are related to the birth of dwarf Miniature horses and a dwarf Miniature Shetland pony [2,5]. In addition, several genetic skeletal diseases in humans have been identified as a result of *ACAN* mutations leading to a broad phenotypic spectrum, such as spondyloepiphyseal dysplasia type Kimberley [9], spondyloepimetaphyseal dysplasia [13], and familial osteochondritis dissecans [14].

As in a previous study [2], we were not able to sequence the initial region of coding exon 11 (RefSeq XM_005602799.2 – EquCab3.0), and with our methodology, it was not possible to cover two fragments (102 bp and 1,252 bp) with an interval of 318 bp. However, a comparison between the mRNA sequencing results of seven dwarf Miniature horses and the equine *ACAN* mRNA sequence (RefSeq XM_005602799.2 - EquCab3.0) revealed four missense SNPs, which were compared with the DNA sequence of the affected Miniature horse parents. Three SNPs were discarded due to

198 genetic conditions, and the SNP c.6465A>T (g.95271115A>T; p.Leu2155Phe - RefSeq
199 XM_005602799.2; RefSeq XP_005602856.2 - EquCab3.0) was primarily considered a
200 novel variant potentially associated with chondrodysplastic dwarfism in Miniature
201 horses, although a causative variant could reside in the unsequenced region of coding
202 exon 11, and c.6465A>T could simply be a marker of linkage disequilibrium with other
203 functionally important and presumably causative variant.

204 *ACAN-D1* consists of a single nucleotide deletion in coding exon 2
205 (g.95291270del), which results in a stop codon and a truncated protein (p.Lys82X) [2].
206 On the other hand, *ACAN-D2* and *ACAN-D3** are characterized by missense SNP
207 substitutions, the first in coding exon 6 (g.95284530C>T), which causes an amino acid
208 change (p.Val424Met) in aggrecan globular domain 1 (G1) [2], and the second in
209 coding exon 7 (g.95282140C>G), which results in an amino acid change (p.Ala505Pro)
210 in the interglobular domain (IGD) of aggrecan [2,5]. This last SNP was also described
211 in a Miniature Shetland pony with dwarfism in Germany [5]. Previously, this variant
212 was designated *ACAN-D3* and consisted of a deletion in coding exon 11. However, this
213 result was found to be a technical artefact, and the novel *D3** designation was assigned
214 to differentiate the correct causative variant [2]. Finally, *ACAN-D4* consists of a 21-bp
215 deletion in coding exon 14 (g.95257458_95257500del) that affects aggrecan globular
216 domain 3 (G3) (p.Phe2017-Asp2023del) [2].

217 Aggrecan is a chondroitin sulfate proteoglycan of the lectican family composed of
218 different domains [7], and many causes of dwarfism in humans and animals are
219 associated with mutations in this extracellular matrix protein [13-16]. However, in
220 contrast to the aggrecan alterations already described in Miniature horses [2], the
221 p.Leu2155Phe (*ACAN*:c.6465A>T - RefSeq XP_005602856.2 – EquCab3.0)
222 substitution is located in the chondroitin sulphate (CS) domain. This domain is the

largest aggrecan domain and may harbour chains of approximately 100 CS molecules [7]. These CS molecules are negatively charged and are essential to the most important function of aggrecan as a structural proteoglycan, which is the capacity to retain a large amount of water in the extracellular matrix. Notably, dwarfism in Miniature Zebu calves can be caused by a single base pair insertion in the CS domain, which leads to a truncated protein [17].

Although the p.Leu2155Phe (*ACAN*:c.6465A>T - RefSeq XP_005602856.2 – EquCab3.0) substitution does not produce a truncated aggrecan, an analysis based on computational tools [18-20] indicated the influence of this substitution on proteoglycan function, which corroborated the genetic findings. The PROVEAN score for p.Leu2155Phe (-2.222) was higher than the default cutoff (-2.5), which could indicate that this amino acid substitution would not interfere with protein function. However, a similar PROVEAN finding was observed for *D2* protein function, which was primarily estimated to be neutral based on the physical and chemical properties of the amino acid change (score -0.874) [2]. Unfortunately, a nonhomologous region to the Miniature horse aggrecan CS domain was found in the structural databases. Thus, it was impossible to model the aggrecan region containing the amino acid substitution p.Leu2155Phe and, consequently, analyse the effects of *ACAN*:c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0) on the structure/function of the CS domain. However, the substitution of an aliphatic apolar amino acid residue (Leu) by an aromatic amino acid residue (Phe) has suggestive potential of inducing significant structural/functional modifications in the Miniature horse aggrecan CS domain.

Despite the presence of the *ACAN*:c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0) variant in heterozygosity in horses belonging to the other four tested large breeds and in homozygosity in two Mangalarga Marchador horses, we suggest that

248 further studies are needed to analyse the potential association of this variant with
249 dwarfism in Miniature horses and the role of *D1*, *D2*, *D3**, and *D4* in large-breed
250 horses. Seemingly, Miniature horses are phylogenetically more closely related to
251 Mangalarga Marchador horses compared with the other large breeds (Warmblood,
252 Thoroughbred, and Quarter horses) [21], which could explain the higher frequencies of
253 *ACAN* gene variants in Mangalarga Marchador horses. Since *ACAN-D2* was found in
254 heterozygosity in one Mangalarga Marchador horse, despite the small number of
255 sampled animals, we suggest that the potential association between *ACAN*:c.6465A>T
256 (RefSeq XM_005602799.2 – EquCab3.0) and dwarfism in Miniature horses would
257 continue to be relevant; moreover, *ACAN*:c.6465A>T has provided population, genetic,
258 and informatics prediction evidence that it could be a potential causative variant of
259 dwarfism in this breed [22]. Indeed, a mutation may not always cause the same effects,
260 or it may lead to different phenotypes of the same disorder in different individuals
261 [23,24]. Several genetic modifiers can produce unexpected phenotypes of the primary
262 disease-causing variant [25], and the genetic background of Miniature horses and
263 Shetland ponies, which were selected for diminutive size over the years, differs from
264 horses of large breeds [26-29] and may generate a predisposition to the condition
265 characterized as dwarfism due to *ACAN* variants.

266 In addition, *D1*, *D2*, *D3**, and *D4* can affect height measurements at the withers in
267 Miniature horses; therefore, *ACAN* variant carriers are smaller than non-carriers [30]. In
268 those other large breeds, *ACAN* variants might be involved in height at the withers or
269 with the aetiology of arthropathies [9,14]. As there have been no reports on dwarfism in
270 the tested large breeds (Mangalarga Marchador, Warmblood, Thoroughbred, and
271 Quarter horses) and we do not know the height at the withers of these horses and their

history of articular diseases, more studies are needed to clarify the genetic pathway of aggrecan in Miniature horses and in horses of other breeds.

The clinical signs observed in the Miniature horses with dwarfism described here were similar to those previously reported [2,5,11,12]. As *ACAN-D1* was not observed in the dwarf Miniature horses of the present study, the clinical signs that this variant can cause (cleft palate with protruding tongue, large abdominal hernia and embryonic or late term loss) [2] were not reported in the affected animals of the present study. Therefore, our results also suggest that the genotype of an animal cannot be assumed only from the phenotypic characteristics, except for genotypes associated with *ACAN-D1* [2].

Mutations in genes that encode proteins associated with CS synthesis and sulfation can result in different severities of skeletal dysplasias [31]. This characteristic can explain the phenotypic differences between the two mares that were heterozygous for *ACAN-D2* and *ACAN*:c.6465A>T, since *ACAN*:c.6465A>T is a substitution located in the CS domain. In addition, the clinical signs of *ACAN-D2* are less deleterious than those related to *D1*, *D3** and *D4* [2], and PROVEAN analysis indicated that *D2* [2] and *ACAN*:c.6465A>T were "neutral" using the default cutoff, which may suggest mild phenotypic characteristics.

The radiographic findings of the 15 affected Miniature horses that were referred to the veterinary hospital (animals 1-15) were similar to those of a previous study [11], and they were not consistent with skeletal atavism (e.g., a complete fibula and ulna were not found) [6,32]. The ten animals that were subjected to airway endoscopy did not show inspiratory honking noises at the auscultation of the midcervical tracheal region; however, the airway findings suggested that in young adulthood, they could demonstrate clinical signs of tracheal collapse on auscultation (e.g., mild tracheal collapse in eight of ten dwarf Miniature horses). Chondrodysplasia was reported in all

14 dwarf Miniature horses that had histological evaluation performed. Although a study carried out in Germany [5] reported no apparent histopathological findings of chondrodysplasia in a Miniature Shetland pony with the *D3*/D3** genotype, *ACAN-D3** was classified as causative of chondrodysplasia-like dwarfism in Miniature horses [2].

The data shown in Table 6 suggest that the birth rate of affected animals in Brazil may be mainly due to the *ACAN-D4* variant, since this variant had the highest frequency (0.244) in the studied population. In the USA, a study using 361 randomly selected horses submitted for testing by private Miniature horse owners found that *ACAN-D2* was the most prevalent variant (0.09), followed by *ACAN-D1* (0.03), *ACAN-D4* (0.03) and *ACAN-D3** (0.02). The cumulative frequency of these dwarfism alleles was 0.163, and the predicted rate of affected horses (q^2) was 2.7% [2]. The Brazilian predicted rate of affected horses (q^2) would be 7.8% considering only *D1*, *D2*, *D3**, and *D4* variants, or 15.4% including the *ACAN*:c.6465A>T findings. Therefore, in both scenarios, the predicted rate of affected Miniature horses in Brazil might be more influenced by the other breeds used in the origin and formation of Brazilian Miniature horses than by American Miniature horses, which were also part of the origin of Brazilian Miniature horses and are imported as an option for expanding the gene pool of the Brazilian animals. In addition, the variant prevalence differences might be explained because different foundation horses were used in Miniature horses within the two countries, and the Brazilian Miniature horse population might be more inbred than the American Miniature horse population.

The previously described causative variants of dwarfism (*D1*, *D2*, *D3** and *D4*) were identified in the studied population of Miniature horses. In addition, another potentially causative variant of this disorder was found (c.6465A>T, g.95271115A>T; p.Leu2155Phe - RefSeq XM_005602799.2; RefSeq XP_005602856.2 – EquCab3.0).

This substitution is a novel finding, since it affects the aggrecan CS domain, a protein region never previously associated with dwarf births in Miniature horses. The phenotypic characteristics of heterozygous dwarfs for *ACAN-D4* and for *ACAN*:c.6465A>T and homozygous dwarfs for *ACAN*:c.6465A>T were similar to those of the affected animals with *D2*/*D2*, *D2*/*D3**, *D2*/*D4*, *D3**/*D4*, and *D4*/*D4* genotypes. The cumulative allele frequency of the five variants was 0.392 in Miniature horses in Brazil, and *ACAN-D4* and *ACAN*:c.6465A>T were more prevalent. Based on our findings, we suggest that control measures to identify heterozygous animals based on genetic testing should be adopted to minimize the significant economic losses and casualties related to affected foals.

Methods

The animal study was approved by the Ethics Committee for the Use of Animals in Research (CEUA) of the School of Veterinary Medicine and Animal Science of São Paulo State University – UNESP on February 9th, 2017 (nº 219/2016-CEUA). All methods were carried out in accordance with the guidelines and regulations of CEUA, and samples were collected under a strict confidentiality agreement to ensure the anonymity of establishments, owners, and animals. In addition, all owners allowed the use of their animals in this study through an informed consent statement.

ACAN variant detection

Miniature horse individuals with clinical signs of dwarfism (n=18) were used in this study. These dwarfs were numbered in a sequence from 1 to 18 on the basis of their physical examination date. All of the affected Miniature horses (n=18) were subjected to genetic testing for *D1*, *D2*, *D3** and *D4* according to methodology previously

described [11]. The parents of 14 Miniature horses with dwarfism were also subjected to genetic testing for *D1*, *D2*, *D3** and *D4* with the same methodology [11]. Materials from the parents of four affected animals were not available.

Seventeen out of 18 dwarfs did not present the combined genotypes of previously described mutations (*D1*, *D2*, *D3** or *D4*) associated with this disorder. We then chose the first seven dwarfs admitted to the Large Animal Internal Medicine Section of São Paulo State University (Unesp) for *ACAN* mRNA sequencing. The other nine affected Miniature horses did not have their *ACAN* mRNA sequenced, because genetic testing for the newly found and potentially associated with dwarfism variant *ACAN*:c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0) was already standardized, and their genotypes were consistent with the dwarf phenotype after the results of the *D1*, *D2*, *D3**, *D4* and c.6465A>T genetic tests.

Total RNA was isolated from the skin samples collected at necropsy of seven affected Miniature horses. The RNeasy Fibrous Tissue Mini Kit (QIAGEN) was used for total RNA isolation following the manufacturer's instructions, except for the cell lysis step, which was performed on a Precellys instrument (Bertin Instruments). The relative purity and quality of the isolated RNA was determined by a NanoDrop 2000 Spectrophotometer (Thermo Scientific), and the ratio of A260–A280 nm exceeded 1.8 for all preparations. To ensure the complete removal of traces of genomic DNA, 1 µg of total RNA was incubated with RQ1 RNase-Free DNase (Promega). First-strand cDNA synthesis was performed with 500 ng of total RNA per 45 µl of reaction using random hexamers and the ImProm-II Reverse Transcription System (Promega), following the manufacturer's instructions. The cDNA samples were stored at -20°C.

The primer pairs designed to sequence the 16 coding exons of the predicted *ACAN* mRNA (RefSeq XM_005602799.2 – EquCab3.0) were previously described [11]; these

primers were designed for evaluation of the exons and the exon-intron junctions of the *ACAN* gene. The PCR was standardized to a total volume of 25 μL containing 2 μL of cDNA, 0.2 μM of each primer, 12.5 μL of GoTaq Green Master Mix (Promega), and 9.5 μL of nuclease-free water. In addition, a no-template control reaction was performed to check for the possible presence of contamination in the PCR preparations. The amplification conditions were as follows: initial denaturation at 95°C for 5 minutes; followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 60 seconds, and extension at 72°C for 60 seconds; and a final extension at 72°C for 5 minutes. Amplicons were analysed by 1.5% agarose gel electrophoresis, purified, and subjected to Sanger sequencing.

To sequence the *ACAN* mRNA, 10 μL of purified PCR product and 5 μL of forward or reverse primers and BigDye Terminator Cycle Sequencing Kit were used (Thermo Scientific). The sequences were determined using a genetic analyser (3500 Series Genetic Analyzer, Thermo Scientific). The obtained sequences and electropherograms were analysed using Geneious and Sequencher 5.1 (Gene Codes Corporation), and they were compared to the equine *ACAN* mRNA sequence (RefSeq XM_005602799.2 – EquCab3.0). The comparisons of the sequenced products allowed the interpretation of the results.

Validation of the variant potentially associated with dwarfism (*ACAN*:c.6465A>T)

Variant analysis. The missense SNPs found in the *ACAN* mRNA sequences of the dwarf Miniature horses were compared with the corresponding *ACAN* sequences of the parents of the dwarf Miniature horses. For this, the blood DNA of the parents of the affected Miniature horses was amplified as previously described [11], according to the positions of the missense SNPs. Then, the PCR products were purified, and Sanger

sequencing was performed according to the aforementioned methodology. In addition, this analysis was assigned to estimate the mode of disease inheritance potentially associated with *ACAN*:c.6465A>T.

Protein study. All amino acid substitutions caused by missense SNPs found in the affected Miniature horses were analysed with PolyPhen-2 [18], SIFT [19] and PROVEAN [20] to assess the impact of the amino acid substitutions on the biological function and structure of the *ACAN* protein. In addition, the aggrecan amino acid sequence from a dwarf Miniature horse (homozygous for *ACAN*:c.6465A>T) was aligned with those of 21 other species.

Comparison with other breeds. Ninety-three horses from different large breeds (23 Mangalarga Marchador, 23 Warmblood, 23 Thoroughbred, and 24 Quarter horses) were tested for *ACAN*:c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0) according to the aforementioned methodology. In addition, 26 horses from these breeds (9 Mangalarga Marchador, 5 Warmblood, 6 Thoroughbred, and 6 Quarter horses) were tested for *D1*, *D2*, *D3**, and *D4* as previously described [11]. All the samples were randomly chosen and belonged to the DNA stock of our laboratory. We did not obtain body measurements or articular disease histories of these horses.

Dwarf Miniature horses

Physical examinations were performed on all dwarf Miniature horses (n=18). Radiographs of the limbs (tibia/fibula and radius/ulna) and head were performed on the affected Miniature horses that were referred to the veterinary hospital (n=15). Ten neonatal dwarfs underwent airway endoscopy. Of the 14 animals that were one day old,

two died during medical care, and 12 were euthanized on the second day of life due to future dwarfism complications; all of them underwent necropsy, in which the proximal portion of the metatarsus was collected for histopathology, and skin samples were collected for RNA isolation. Tissue samples of the metatarsus were formalin fixed and decalcified in HNO_3 prior to paraffin embedding. For histopathological examination, the fixed tissues were routinely processed, stained with haematoxylin and eosin, and examined by light microscopy.

Population screening

Genetic testing (*D1*, *D2*, *D3** and *D4*) according to the methodology previously described [11] and using the DG_ACAN_Eq_F13 and DG_ACAN_Eq_R13 primer pair [11] for *ACAN*:c.6465A>T (RefSeq XM_005602799.2 – EquCab3.0) was performed in 347 phenotypically normal Miniature horses randomly sampled from nine farms in Brazil. The parents of the 14 neonatal Miniature horses with dwarfism admitted to the Large Animal Internal Medicine Section of São Paulo State University (Unesp) were included in this sampling. Eight farms were in São Paulo state, and one farm was in Rio Grande do Sul state. The screening was assigned to calculate the prevalence of the *ACAN* variants (*D1*, *D2*, *D3**, *D4* and c.6465A>T) in Brazil.

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Author contributions statement

J.P.O.F. and A.S.B. conceived the experiment. D.G.A.A. and R.M.B. conducted the physical examination, sampling, and molecular analyses. D.G.A.A. conducted the radiographic and endoscopy examinations. R.L.A. and R.M.B. conducted the histological examination. D.G.A.A. and A.J.M. conducted the protein function analysis. D.G.A.A., A.J.M., A.S.B., and J.P.O.F. analysed the results. All authors reviewed the manuscript.

Competing interests

The authors declare that they have no competing interests.

Figure legends

543 **Figure 1**. Partial electropherograms of the *ACAN*:c.6465A>T (RefSeq
544 XM_005602799.2 – EquCab3.0) region obtained after Sanger sequencing. (**a**) Normal
545 horse. (**b**) Carrier (heterozygous). (**c**) Affected foal.
546
547 **Figure 2.** Clinical signs of dwarfism in Miniature horses. (**a**), (**b**) and (**c**) Domed head;
548 enlarged eye sockets with prominent eyes; shortened neck; shortened limbs relative to
549 overall body size; and bowed limbs, mainly the hind limbs. (**d**) Mandibular
550 prognathism. (**a**) and (**b**) represent the same Miniature horse (animal 15) as in (**c**) and
551 (**d**) (animal 2). These two Miniature horses did not possess *D1*, *D2*, *D3**, and *D4*
552 variants and were homozygous for *ACAN*:c.6465A>T.

Tables

Table 1. Synonymous and missense SNPs found in the *ACAN* mRNA sequencing of seven affected Miniature horses.

Animal(s)	Genotype	Coding exon	mRNA (XM_005602799.2)	Protein (XP_005602856.2)	SNP type
1; 3	A/A	11	c.2406G>A	p.Thr802Thr	Synonymous
4; 5; 6; 7	G/A	11	c.2406G>A	p.Thr802Thr	Synonymous
4	T/A	11	c.3453T>A	p.Ser1151Ser	Synonymous
2	A/A	11	c.4950C>A	p.Leu1650Leu	Synonymous
1; 3; 4; 5	C/A	11	c.4950C>A	p.Leu1650Leu	Synonymous
6	C/T	11	c.5145C>T	p.Pro1715Pro	Synonymous
1; 3; 4; 5; 6; 7	C/T	11	c.5883C>T	p.Ser1961Ser	Synonymous
1	T/T	15	c.7836C>T	p.Thr2612Thr	Synonymous
3; 4; 5; 6; 7	C/T	15	c.7836C>T	p.Thr2612Thr	Synonymous
1; 2; 3; 4; 5; 6; 7	C/C	11	c.3277A>C	p.Ile1093Leu	Missense
1	T/T	11	c.6128C>T	p.Ala2043Val	Missense
3; 4; 5; 6; 7	C/T	11	c.6128C>T	p.Ala2043Val	Missense
2	T/T	11	c.6465A>T	p.Leu2155Phe	Missense
1; 3; 4; 5; 6; 7	A/T	11	c.6465A>T	p.Leu2155Phe	Missense
1; 2; 3; 4; 5; 6; 7	G/G	11	c.7096A>G	p.Arg2366Gly	Missense

Table 2. General information about the 18 dwarf Miniature horses, including age, gender, farm, and genetic test results, for *D1*, *D2*, *D3**, *D4*, and c.6465A>T.

Animal	Age	Gender	Farm	*D1*	*D2*	*D3**	*D4*	c.6465 A>T
1	One day	Male	A	N/N	N/N	N/N	N/*D4*	A/T
2	One day	Male	A	N/N	N/N	N/N	N/N	T/T
3	One day	Female	A	N/N	N/N	N/N	N/*D4*	A/T
4	One day	Male	A	N/N	N/N	N/N	N/*D4*	A/T
5	One day	Female	B	N/N	N/N	N/N	N/*D4*	A/T
6	One day	Male	A	N/N	N/N	N/N	N/*D4*	A/T
7	One day	Male	A	N/N	N/N	N/N	N/*D4*	A/T
8	One day	Female	A	N/N	N/N	N/N	N/*D4*	A/T
9	One day	Male	A	N/N	N/N	N/N	N/*D4*	A/T
10	One day	Female	A	N/N	N/N	N/N	N/*D4*	A/T
11	One day	Female	A	N/N	N/N	N/N	N/*D4*	A/T
12	One day	Female	A	N/N	N/N	N/N	N/*D4*	A/T
13	One day	Male	A	N/N	N/N	N/N	N/*D4*	A/T
14	One day	Female	A	N/N	N/N	N/N	N/*D4*	A/T
15	One year	Male	C	N/N	N/N	N/N	N/N	T/T
16	One year	Male	D	N/N	N/*D2*	N/*D3**	N/N	A/A
17	Adult	Female	E	N/N	N/*D2*	N/N	N/N	A/T
18	Adult	Female	E	N/N	N/*D2*	N/N	N/N	A/T

559

560 **Table 3.** Frequencies of the genotypes at position c.6465 (RefSeq XM_005602799.2)
561 after sequencing results from 93 horses of four different large breeds.

Breed	A/A	A/T	T/T
Mangalarga Marchador	30.4% (7/23)	60.8% (14/23)	8.8% (2/23)
Warmblood	91.3% (21/23)	8.7% (2/23)	0.0% (0/23)
Thoroughbred	73.9% (17/23)	26.1% (6/23)	0.0% (0/23)
Quarter horse	79.2% (19/24)	20.8% (5/24)	0.0% (0/23)

562

563 **Table 4.** Frequencies of animals with *D1*, *D2*, *D3**, and *D4* variants after sequencing
564 results from 26 horses of four different large breeds.

Variant	Genotype	Mangalarga Marchador (n=9)	Warmblood (n=5)	Thoroughbred (n=6)	Quarter horse (n=6)
D1	N/N	0.0%	0.0%	0.0%	0.0%
	N/*D1*	0.0%	0.0%	0.0%	0.0%
	D1/*D1*	0.0%	0.0%	0.0%	0.0%
D2	N/N	0.0%	0.0%	0.0%	0.0%
	N/*D2*	11.1% (1/9)	0.0%	0.0%	0.0%
	D2/*D2*	0.0%	0.0%	0.0%	0.0%
*D3**	N/N	0.0%	0.0%	0.0%	0.0%
	N/*D3**	0.0%	0.0%	0.0%	0.0%
	*D3**/*D3**	0.0%	0.0%	0.0%	0.0%
D4	N/N	0.0%	0.0%	0.0%	0.0%
	N/*D4*	0.0%	0.0%	0.0%	0.0%
	D4/*D4*	0.0%	0.0%	0.0%	0.0%

565

566 **Table 5.** Percentages of *D1*, *D2*, *D3**, *D4*, or c.6465A>T heterozygous Miniature
567 horses by farm (n=347).

Farm	Sampling	Heterozygous	Dwarf births in the past
A	79	78.5%	Yes
B	59	91.5%	Yes
D	17	64.7%	Yes
E	82	78.0%	Yes
F	3	100.0%	Yes
G	30	83.3%	Yes
H	18	38.9%	No
I	43	86.0%	Yes
J	16	56.3%	Yes

568

569 **Table 6.** Allele frequencies of *D1*, *D2*, *D3**, and *D4* variants in the *ACAN* gene in

570 Brazil (n=347) and in the USA (n=361).

Brazil (n=347)		**USA (n=361) [2]**	
Variant	**Frequency**	**Variant**	**Frequency**
Normal (N)	0.720	Normal (N)	0.84
g.95291270del (*D1*)	0.003	g.95291270del (*D1*)	0.03
g.95284530C>T (*D2*)	0.017	g.95284530C>T (*D2*)	0.09
g.95282140C>G (*D3**)	0.016	g.95282140C>G (*D3**)	0.02
g.95257458_95257500del (*D4*)	0.244	g.95257458_95257500del (*D4*)	0.03

571

Figure 1.

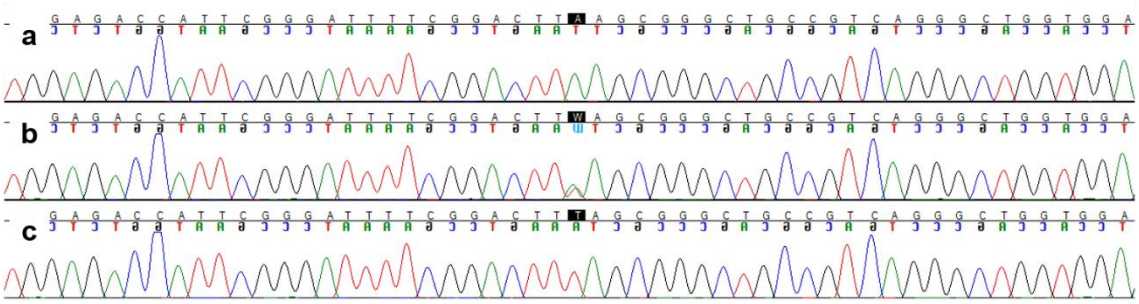

Figure 2.

Supplementary information

Evaluation of a new variant in the *aggrecan* gene potentially associated with chondrodysplastic dwarfism in Miniature horses

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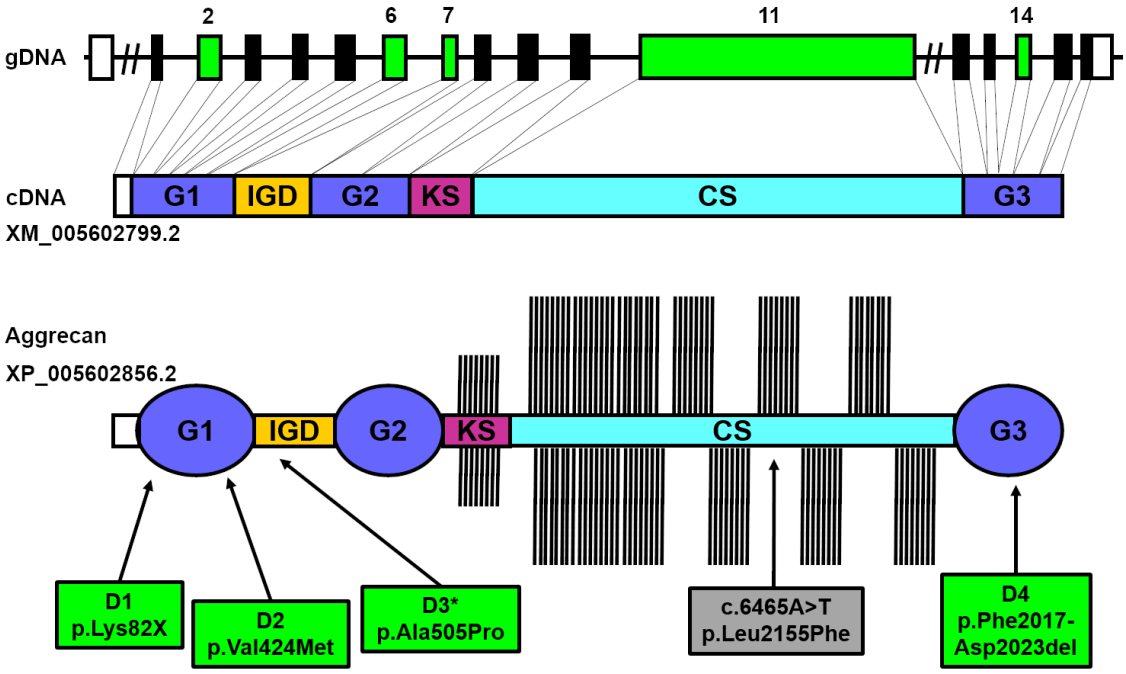

Supplementary Figure S1. Schematic structure of aggrecan and the location of the variants *D1*, *D2*, *D3**, *D4* and c.6465A>T. Genomic DNA (gDNA): the white rectangles indicate the untranslated regions; green rectangles indicate the coding exons where *D1*, *D2*, *D3**, *D4* and c.6465A>T variants are located (2, 6, 7, 11 and 14); black rectangles indicate the coding exons without known variants associated with dwarfism. Coding DNA (cDNA – RefSeq XM_005602799.2) and aggrecan (RefSeq XP_005602856.2): G1, globular domain 1; IGD, interglobular domain; G2, globular domain 2; KS, keratan sulfate domain; CS, chondroitin sulfate domain; G3, globular domain 3. Green boxes with arrows represent the *D1*, *D2*, *D3** and *D4* locations throughout the amino acid chain. Gray box with arrow represents the c.6465A>T (p.Leu2155Phe) location. *D1*, *D2*, *D3** and *D4* first described in Eberth *et al*. (2018). Images generated by Microsoft PowerPoint 2013 and GIMP 2.10.2.

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XP_009679548.1_Struthio_camelus_australis    GMIEISGFPSGDRRISVEGSGAVETSELLSGTGDLSGEPSGIPYFSGDISG--VTDLSGQ    1265
XP_009477624.1_Pelecanus_crispus             GMIEISGFPSGDRGLSGEGSGAVKTSGFPSGTGDFSGEPSGIPYISGDVSG--ATDLSGQ    1297
XP_012867462.1_Dipodomys_ordii               GMWELGGAHS------GEHSGSVDLSRLQPGREASGSDPPSPPDSSGDFSSPAAADLSGD    1717
XP_017523955.1_Manis_javanica                GVLELSGAHSGVPDMSGDHSGILDLSGQQSGMVEPSGEPSSTPYFSGDFPG--TVDVSGE    1950
NP_001348429.1_Mus_musculus                  GILEFSGAHSGTPDISGELSGSLDLSTLQSGQMETSTETPSSPYFSGDFSS--TTDVSGE    1655
Dwarf_p.Leu2155Phe                           GILELSGAHSGAPAVSGDHSGFSDFSGLPSGLVEPSGEPSSTPHFSGDFSG--TIDVSGA    2188
XP_005602856.2_Equus_caballus                GILELSGAHSGAPAVSGDHSGFSDLSGLPSGLVEPSGEPSSTPHFSGDFSG--TIDVSGA    2188
XP_014698037.1_Equus_asinus                  GILELSGAHSGAPAVSGDHSGFSDLSGLPSGLVEPSGEPSSTPHFSGDFSG--TIDVSGA    1516
NP_001106926.2_Canis_lupus_familiaris        GILELSGAHSGAPDVSGDHSGSLDLSGMQSGLVEPSGEPSSTPYFSGDFSG--TMDVTGE    1816
XP_023110702.1_Felis_catus                   GIVELSGAHSGAPDVSGDHSGFLDLSGPRSGLVEPSGEPSSTPYFSGDFSG--TVDVSGE    1787
XP_026366060.1_Ursus_arctos_horribilis       GILELSGAHSGAPDVSGDHSGVLDLSGQQSRLVEPSGEPSSTPYFSGDFSG--TTDVSGE    1758
XP_017922102.1_Capra_hircus                  GILELSGTPSGAPDMSGDHLGSLDQSGLQSGLVEPRGEPASTPYFSGDFSG--ATDVSGE    1891
XP_027812861.1_Ovis_aries                    GILELSGTPSGAPDMSGDHLGSLDQSGLQSGLVEPRGEPASTPYFSGDFSG--ATDVSGE    2013
XP_025126951.1_Bubalus_bubalis               GILELSGAPSGAPDMSGDHLGSLDQSGLQSGLVEPSGEPASTPYFSGDFSG--TTDVSGE    1865
NP_776406.1_Bos_taurus                       GILELSGAPSGAPDMSGDHLGSLDQSGLQSGLVEPSGEPASTPYFSGDFSG--TTDVSGE    1854
XP_010839318.1_Bison_bison_bison             GILELSGAPSGAPDMSGDHLGSLDQSGLQSGLVEPSGEPASTPYFSGDFSG--TTDVSGE    1856
NP_001158124.1_Sus_scrofa                    GTLELSGAHSGVPDMSGDHSGSVDLSGLQSGLAEPSGEPASTPYFSGDFSV--TTDISGD    1773
XP_031296401.1_Camelus_dromedarius           GILELSGAHSGAPDMSGDHSGLLDLSGLQSGLVELSGEPSSTPYFSGDFSG--TTDVSGE    1724
XP_004278291.1_Orcinus_orca                  GILELSGAPSGAPDMSGDHSGLLDLSGLQSGLVEPSGEPSSTPYFSGDFSG--TTDVSGE    1537
XP_004455283.1_Dasypus_novemcinctus          GILDLSGDHSGVPDMSGDHSGVLDTSGLQSGLAEPSGEPQSTPYFSGDFSG--VTDISGE    1576
XP_003952775.3_Pan_troglodytes               GILELSGAHSGAPDMSGEHSGFLDLSGLQSGLVEPSGEPPGTPYFSGDFAS--TTNVSRE    1883
NP_001126.3_Homo_sapiens                     GILELSGAHSGAPDMSGEHSGFLDLSGLQSGLIEPSGEPPGTPYFSGDFAS--TTNVSGE    2017
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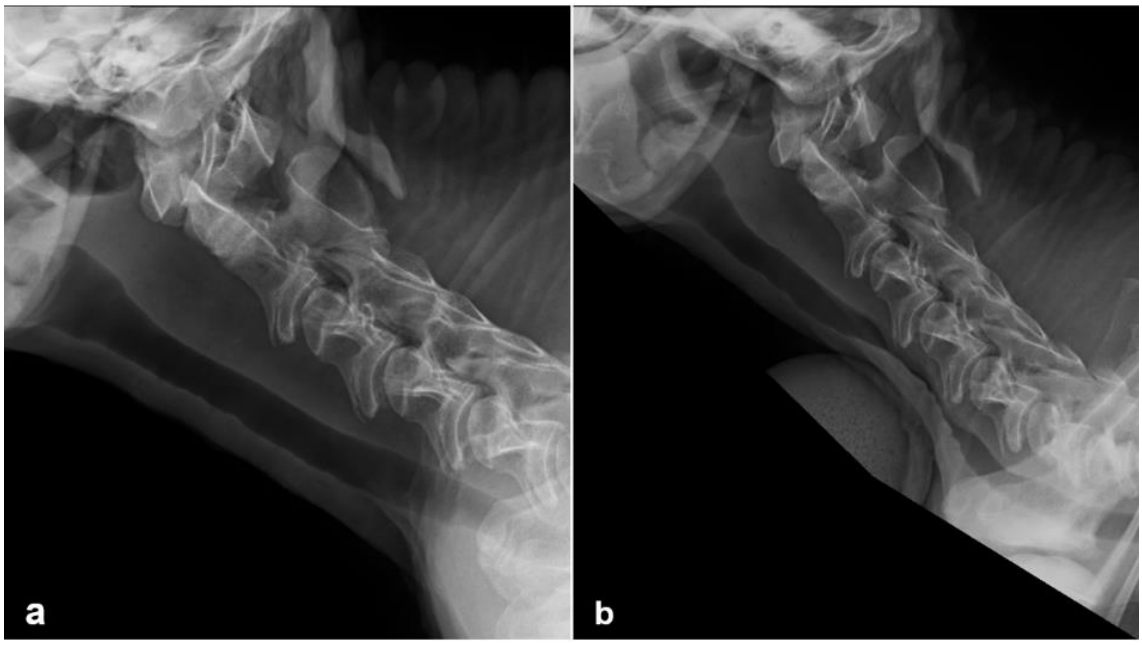
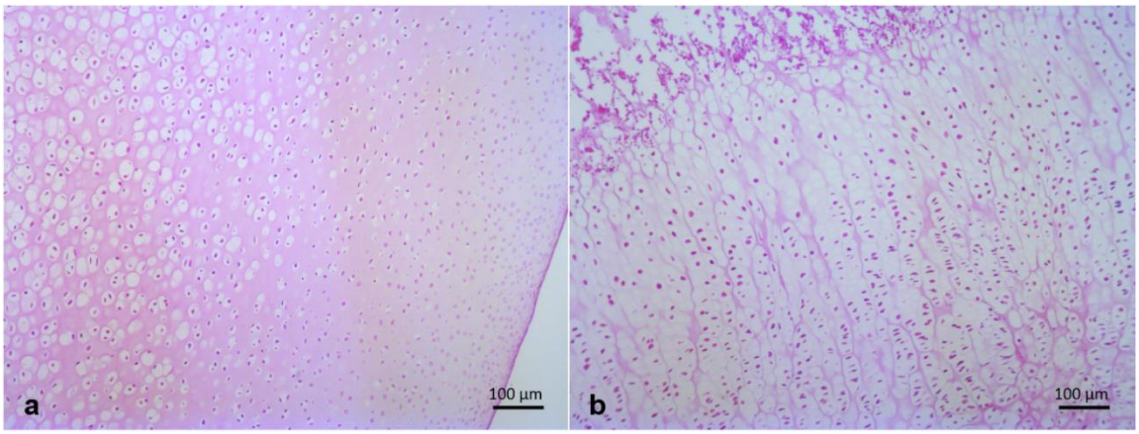
Supplementary Figure S2. Alignment of the aggrecan sequences of 19 mammalian species, two avian species and an affected Miniature horse homozygous for the SNP c.6465A>T between positions 2138–2195 (RefSeq XP_005602856.2). The red box indicates position 2155, and highlighted in blue is p.Leu2155Phe in the Miniature horse homozygous for the SNP c.6465A>T. This alignment was performed with the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).



Supplementary Figure S3. Radiographic findings in a Miniature horse with dwarfism and homozygous for the SNP c.6465A>T. (**a**) Lateral radiograph before compression of

the cervical trachea portion (animal 15). (**b**) Lateral radiograph showing tracheal collapse at the cervical trachea portion (animal 15).



Supplementary Figure S4. Histopathological findings using H&E staining in a Miniature horse with dwarfism and homozygous for the SNP c.6465A>T. Histopathological images of the articular cartilage (**a**) and of the physis cartilage (**b**) of a two-day-old Miniature horse with dwarfism (animal 2). Note the chondrocyte disorganization and the decreased cartilage matrix.

CAPÍTULO IV

Discussão geral

Este estudo descreve os sinais clínicos apresentados por equinos da raça Mini-Horse com nanismo condrodisplásico relacionado com alterações no gene *ACAN*. Dois dos animais fenotipicamente descritos apresentavam genótipo *D4*/*D4* e dois eram homozigotos para c.6465A>T. Assim como em estudos prévios (WATANABE et al., 2014; EBERTH et al., 2018), os animais com nanismo condrodisplásico possuíam membros curtos e tortuosos; pescoço encurtado; cabeça desproporcionalmente grande; olhos pronunciados e prognatismo. Os dois potros neonatos homozigotos para *D4* apresentaram diminuição de comprimento dos ossos metacarpianos/metatarsianos quando comparados com outro potro da raça Mini-Horse de mesma faixa etária e sem os sinais clínicos de nanismo. Entretanto, o fenótipo de animais adultos *D4*/*D4* ainda é desconhecido, uma vez que nenhuma descrição desse genótipo havia sido realizada anteriormente. Possivelmente isso é explicado devido ao pequeno tamanho amostral de estudos anteriores ou porque a variante *D4* é considerada rara nas populações já estudadas (EBERTH, 2013; EBERTH et al., 2018). Além disso, não foi encontrado neste estudo nenhum animal clinicamente afetado com a variante *D1*, o que não nos permitiu analisar os sinais clínicos que essa mutação pode causar: fenda palatina, protrusão de língua, hérnia abdominal e perda embrionária seguida de abortamento (EBERTH et al., 2018).

Os achados radiográficos encontrados em todos os 15 animais avaliados foram similares com estudos prévios (BACK et al., 2008; WATANABE et al., 2014) e nenhum foi compatível com atavismo esquelético (TYSON et al., 2004). Além disso, a histologia das porções proximais dos ossos metatarsianos comprovou condrodisplasia em todos os equinos fenotipicamente anões avaliados (n=14). Tal fato não foi observado anteriormente em um pônei Shetland miniatura com nanismo e apresentando genótipo *D3**/*D3**, pois não foram encontrados sinais de desorganização, deformação ou redução do tamanho dos condrócitos, bem como redução da matriz extracelular (METZGER et al., 2017). Entretanto, não encontramos nenhum equino da raça Mini-Horse com nanismo e com genótipo *D3**/*D3** para avaliação histológica em nosso estudo.

Além dos achados fenotípicos e de exames complementares discutidos anteriormente, o grupo de pesquisa conseguiu sequenciar 89% do RNAm

(RefSeq XM_005602799.2) do gene *ACAN*, utilizando a metodologia descrita nos capítulos anteriores. Infelizmente, não foi possível o sequenciamento de dois fragmentos do RNAm do gene *ACAN*, ambos localizados no éxon 11 e posicionados próximos um ao outro (intervalo de 318 pb entre eles), sendo o primeiro com 102 pb e o segundo com 748 pb. Ressalta-se que o segundo fragmento (748 pb), localiza-se em região altamente repetitiva (por exemplo, a sequência de 80 pb: CCTTCTGGAGGAGAGATCCATCTCGAGCCCACTGCCTCTGGAGTAGAGGA CCTCGGTGGACTTCCTTCTGGAGGAGAGAT, repete-se três vezes). A incapacidade de sequenciamento dessa região também foi encontrada no estudo realizado por Eberth et al. (2018). Apesar disso, foram encontradas algumas alterações genéticas quando comparadas com o RNAm do gene alvo (RefSeq XM_005602799.2) e descritas nos capítulos anteriores. O SNP c.6465A>T (g.95271115A>T; p.Leu2155Phe - RefSeq XM_005602799.2; XP_005602856.2 – EquCab3.0) foi avaliado *in silico* e considerado potencialmente associado ao nanismo condrodisplásico em equinos da raça Mini-Horse, embora uma variante causal possa estar localizada na região não sequenciada do éxon 11 e c.6465A>T pudesse simplesmente ser um marcador em desequilíbrio ligado com a outra variante funcionalmente importante e presumivelmente causadora da doença.

Infelizmente, nenhuma região homóloga ao domínio CS do agrecano de equinos foi encontrada nos bancos de dados disponíveis. Portanto, foi impossível modelar a região da proteína contendo a substituição de aminoácidos p.Leu2155Phe e, consequentemente, analisar os efeitos do SNP c.6465A>T na estrutura e função do domínio CS. No entanto, a substituição de um aminoácido apolar alifático (Leu) por um aminoácido aromático (Phe) tem um potencial sugestivo de induzir modificações estruturais e funcionais significativas no domínio CS do agrecano.

Visto que o SNP c.6465A>T foi encontrado em heterozigose em equinos pertencentes às outras quatro raças avaliadas e em homozigose em dois cavalos Mangalarga Marchador (MM), sugere-se que mais estudos sejam necessários para analisar a potencial associação dessa variante com o nanismo condrodisplásico em Mini-Horses e a função biológica de *D1*, *D2*, *D3** e *D4* em cavalos de raças grandes. Aparentemente, os Mini-Horses são

filogeneticamente mais relacionados aos MM em comparação com as outras raças testadas (*Warmblood*, Puro Sangue Inglês e Quarto de Milha) (PETERSEN et al., 2013). Isso poderia explicar as maiores frequências das variantes do gene *ACAN* em MM em relação às outras raças avaliadas. Como a mutação *D2* foi encontrada em heterozigose em um MM, apesar de o pequeno número de animais amostrados, sugere-se que, com esse achado, a potencial associação entre c.6465A>T e o nanismo condrodisplásico continua a ser considerada relevante, uma vez que c.6465A>T possui evidências de predição populacional, genética e de bioinformática para que possa ser associada como potencial variante causal dessa enfermidade em equinos da raça Mini-Horse (CHEN et al., 2015). De fato, uma mutação nem sempre causa os mesmos efeitos e pode levar a diferentes fenótipos da mesma doença em diferentes indivíduos (WANG et al., 2017; DOMINGO et al., 2019). Existem diversos modificadores genéticos que podem produzir fenótipos inesperados da variante primária classificada como causadora da doença (RAHIT e TARAILO-GRAOVAC, 2020) e o contexto genético de equinos da raça Mini-Horse e pôneis Shetland, que foram selecionados por tamanho ao longo dos anos, é diferente dos cavalos de raças grandes (MAKVANDI-NEJAD et al., 2012; METZGER et al., 2013; FRISCHKNECHT et al., 2015; METZGER et al., 2018) e pode predispor à condição caracterizada como nanismo devido às mutações no gene *ACAN*.

A frequência cumulativa das variantes (*D1*, *D2*, *D3**, *D4* e c.6465A>T) na população estudada foi de 0,392 (q), o que sugere uma taxa de heterozigotos (2pq) de 47,7% e uma taxa para equinos afetados (q^2) de 15,4% no rebanho nacional da raça Mini-Horse. Separadamente, a variante *D4* foi a mais prevalente (0,244), seguida por c.6465A>T (0,112); *D2* (0,017); *D3** (0,016) e *D1* (0,003). Nos EUA, a frequência cumulativa dos alelos associados ao nanismo condrodisplásico foi de 0,163 e a taxa prevista de animais afetados (q^2) foi de 2,7% (EBERTH et al., 2018). Tal estudo utilizou amostras de 361 equinos que foram submetidas a testes genéticos pelos proprietários. A variante *D2* foi a mais prevalente (0,09), seguida por *D1* (0,03); *D4* (0,03) e *D3** (0,02) (EBERTH et al., 2018). A taxa predita de equinos da raça Mini-Horse com nanismo condrodisplásico no Brasil (15,4%) é mais influenciada pela variante *D4*, diferentemente do rebanho nos EUA. Portanto, a ocorrência da enfermidade no Brasil pode ser mais influenciada pelas outras raças que foram utilizadas na

origem e formação dos cavalos Mini-Horse brasileiros do que pelos cavalos registrados na AMHA, que também fizeram parte da origem do cavalo Mini-Horse nacional, entretanto são mais utilizados para refrescamento de sangue. Além disso, as diferenças de prevalência das variantes podem ser explicadas porque diferentes animais fundadores da raça foram utilizados nos dois países e/ou porque a população brasileira de Mini-Horses pode apresentar maior consanguinidade do que a população de *American Miniature horses* nos EUA.

Como limitações do estudo, aponta-se a incapacidade de sequenciamento gênico da região inicial do éxon 11 do gene *ACAN* de acordo com a metodologia utilizada, a impossibilidade de modelagem do domínio CS do agrecano dos equinos da raça Mini-Horse homozigotos para c.6465A>T e o pequeno número de equinos de raças grandes testados para *D1*, *D2*, *D3** e *D4*. Por fim, os resultados obtidos estão devidamente justificados e discutidos no corpo dos respectivos artigos que compõem essa tese quando comparados aos dados já apontados na literatura consultada. Entretanto, reitera-se que a medida de controle mais efetiva para o nanismo condrodisplásico em equinos das raças pônei é a seleção dos acasalamentos, com base nos genótipos dos reprodutores e matrizes, com a finalidade de diminuir a probabilidade de nascimentos de animais afetados ou de abortamentos decorrentes dessa doença.

Conclusões gerais

Os achados clínicos de potros afetados cujos genótipos eram *D4*/*D4* ou homozigotos para c.6465A>T foram caracterizados. As variantes causais (*D1*, *D2*, *D3** e *D4*) do nanismo condrodisplásico foram verificadas na população estudada de equinos da raça Mini-Horse. Além disso, outra variante potencialmente associada a essa enfermidade foi encontrada (c.6465A>T; g.95271115A>T; p.Leu2155Phe) no gene *aggrecan* (RefSeq XM_005602799.2; XP_005602856.2 – EquCab3.0). A frequência alélica cumulativa das cinco variantes foi de 0,392 na população estudada. Portanto, espera-se que medidas de controle com base em seleção dos acasalamentos de acordo com testes genéticos, uma vez que estes foram padronizados, possam ser consideradas e adotadas para minimizar perdas econômicas e nascimento de potros fenotipicamente anões.

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ANEXOS

Atestado - Comissão de Ética no Uso de Animais (CEUA)

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

fmvz - unesp
Faculdade de Medicina Veterinária e Zootecnia
Campus de Botucatu

ATESTADO

Atesto que o subprojeto intitulado "Avaliação das mutações causadoras do nanismo do tipo condrodisplásico em equinos da raça Mini-Horse no Brasil" faz parte do Projeto "Estudo clínico e molecular do nanismo do tipo condrodisplásico em equinos da raça Mini-Horse no Brasil", **Protocolo CEUA 219/2016**, aprovado em 09/02/2017, a ser conduzido por **Danilo Giorgi Abranches de Andrade**, para fins de pesquisa científica, e encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal – CONCEA.

Vigência do projeto	20/03/2017 a 29/02/2020
Finalidade	Pesquisa Científica
Espécie/Linhagem	Equina/*Equus caballus*
Raça	Mini Horse
Nº de animais	200
Peso/Idade	10 kg/Variadas
Sexo	Indefinido
Origem	Proprietários

Botucatu, 09 de março de 2017

Profª.Ass.Drª. Ibiara Correia de Lima Almeida Paz

Presidente da CEUA da FMVZ, UNESP - Campus de Botucatu

Declaração de comunicação de invenção

UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
AGÊNCIA UNESP DE INOVAÇÃO

DECLARAÇÃO DE COMUNICAÇÃO DE INVENÇÃO

Declaramos ter recebido dos inventores Jose Paes de Oliveira Filho, Danilo Giorgi Abranches de Andrade, Alexandre Secorun Borges, a Comunicação de Invenção intitulada "MÉTODO DE DETECÇÃO DA MUTAÇÃO g.95271115A>T NO GENE AGGRECAN ASSOCIADA AO NANISMO CONDRODISPLÁSICO EM EQUINOS E KIT DE DETECÇÃO APROPRIADO PARA TAL", representada pelo código da Agência UNESP de Inovação 19CI108.

Pelo exposto, constata-se que a Comunicação de Invenção compreende as condições necessárias para compor o portfólio de invenções da Agência UNESP de Inovação e será submetida à avaliação das áreas de Propriedade Intelectual e Transferência de Tecnologia.

São Paulo, 31 de agosto de 2020.

RENAN PADRON ALMEIDA:33778301896
Assinado de forma digital por RENAN PADRON ALMEIDA:33778301896
Dados: 2020.08.31 15:38:30 -03'00'

Renan Padron Almeida
Agência UNESP de Inovação
Gerente de Propriedade Intelectual

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