PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ

ESCOLA DE CIÊNCIAS DA VIDA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

ENIO FRANCISCO MOURA

CLINICAL AND GENETIC STUDY OF CANINE X-LINKED HYPOHIDROTIC ECTODERMAL DYSPLASIA, INCLUDING NEXT GENERATION SEQUENCING OF THE *EDA* GENE

(**Estudo genético e clínico da displasia ectodérmica hipoidrótica ligada ao X canina, incluindo sequenciamento de nova geração do gene *EDA***)

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal, área de concentração Saúde, Tecnologia e Produção Animal, da Escola de Ciências da Vida da Pontifícia Universidade Católica do Paraná, para obtenção do título de Doutor em Ciência Animal.

Orientador(a): Prof.ª Dr.ª Cláudia Turra Pimpão

CURITIBA

2020

TERMO DE APROVAÇÃO

Pontifícia Universidade Católica do Paraná
Programa de Pós-Graduação em Ciência Animal
Câmpus Curitiba

PUCPR
GRUPO MARISTA

ATA Nº 018 E PARECER FINAL DA DEFESA DE TESE DE DOUTORADO EM CIÊNCIA ANIMAL DO ALUNO ENIO FRANCISCO MOURA

Aos dezenove dias do mês de fevereiro do ano de dois mil e vinte, às 13:00 horas, realizou-se na sala de Pós 02, 2º andar, Bloco Amarelo, da Pontifícia Universidade Católica do Paraná, localizada no Campus de Curitiba, Rua Imaculada Conceição, nº 1155, Prado Velho – Curitiba – PR, a sessão pública de defesa da tese do doutorando Enio Francisco Moura, intitulada: **"Estudo genético e clínico da displasia ectodérmica ligada ao X canina, incluindo sequenciamento de nova geração do gene *EDA*"**. O doutorando concluiu os créditos exigidos para obtenção do título de Doutor em Ciência Animal, segundo os registros constantes na secretaria do Programa. Os trabalhos foram conduzidos pela Professora orientadora e Presidente da banca, Dra. Claudia Turra Pimpão (PUCPR), auxiliada pelos Professores Doutores Marconi Rodrigues de Farias (PUCPR), Jair Rodini Engracia Filho (PUCPR), Carlos Eduardo Larsson (USP) e Thaís Andrade Costa Casagrande (UP). Procedeu-se à exposição da tese, seguida de sua arguição pública e defesa. Encerrada a fase, os examinadores expediram o parecer final sobre a tese, que foi considerada Aprovada.

MEMBROS	ASSINATURA
Profa Dra Claudia Turra Pimpão - Orientador	
Prof Dr Marconi Rodrigues de Farias (PUCPR)	
Prof Dr Jair Rodini Engracia Filho (PUCPR)	
Prof Dr Carlos Eduardo Larsson (USP)	
Prof Dra Thaís Andrade Costa Casagrande (UP)	

Proclamado o resultado, a Presidente da Banca Examinadora encerrou os trabalhos, e para que tudo conste, eu Caroline Nocera Bertton, confiro e assino a presente ata juntamente com os membros da Banca Examinadora.

Curitiba, 19 de fevereiro de 2020.

Caroline Nocera Bertton
Caroline Nocera Bertton
Secretária do Programa de Pós-Graduação em Ciência Animal

Renata Ernlund Macedo
Profa. Dra. Renata Ernlund Freitas de Macedo
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DEDICATÓRIA

À minha mulher e às minhas filhas, **Marga**, **Ana Luísa** e **Manoela Maria**, que são a razão da minha vida.

Aos meus pais, **Ives** e **Leonor** (*in memoriam*), pela dedicação aos seus filhos.

Ao meu irmão **Hélio** pelo apoio fraterno que garantiu meu início acadêmico.

Aos Professores **Newton Freire-Maia** (*in memoriam*) e **Rui Fernando Pilotto**, pioneiros da genética médica brasileira, pelo conhecimento, grandeza de alma e amizade que motivaram minha dedicação à genética clínica.

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Prof. **Jaime Marinero**
Prof. Dr. **Marconi Rodrigues de Farias**
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Prof. Dr. **Antônio Felipe Paulino de Figueiredo Wouk**
Prof. Dr. **Jair Rodini Engracia Filho**
Prof. Dr. **Saulo Henrique Weber**
Prof.ª Dr.ª **Silvana Maris Cirio**

FORMATO DA TESE

A presente tese é composta de quatro capítulos. O capítulo 1 apresenta uma caracterização genética e clínica da displasia ectodérmica hipoidrótica ligada ao X (DEHLX) canina com base em cinco casos espontâneos e na literatura, além dos objetivos deste estudo. O capítulo 2 é um artigo a ser submetido ao periódico *Veterinary Dermatology* e que apresenta uma análise do gene *EDA* de um cão com fenótipo clínico de DEHLX por meio de sequenciamento de nova geração (NGS). O capítulo 3 é um artigo aceito para publicação no periódico *Topics in Companion Animal Medicine* e que analisa por meio de inferência bayesiana um caso isolado de displasia ectodérmica hipoidrótica em uma família de cães Yorkshire terrier, demonstrando que teve origem por mutação nova. Os capítulos 2 e 3 estão formatados de acordo com as normas de cada revista. O capítulo 4 contém as conclusões gerais e considerações finais com sugestões que podem gerar estudos futuros. As referências do capítulo 1 encontram-se ao final desta tese.

RESUMO GERAL

Displasia ectodérmica hipoidrótica ligada ao X é uma condição clínica causada por mutação recessiva do gene *EDA*, que se localiza no cromossomo X e é importante para o desenvolvimento dos derivados ectodérmicos. Manifesta-se principalmente por defeitos nas glândulas sudoríparas, glândulas sebáceas, pelos e dentes. Os afetados geralmente são machos e apresentam alopecia e hipotricose congênitas com distribuição típica, além de dentes conoides e oligodontia. Outros derivados ectodérmicos podem apresentar redução de número e/ou função, como as glândulas traqueobrônquicas e as glândulas lacrimais e meibomianas, o que aumenta o risco de doenças respiratórias e oculares, respectivamente.

Em humanos, são conhecidas mais de 300 mutações diferentes no gene *EDA*, sendo a grande maioria mutações de ponto e indels, com uma parte significativa tendo origem por mutação nova. Em cães, apenas três mutações são conhecidas e, com exceção de um caso, os dados publicados não são suficientes para comprovar origem por mutação nova.

Neste estudo, além de uma caracterização genética e clínica baseada na literatura e em cinco afetados, foi realizado sequenciamento de nova geração (NGS) do gene *EDA* de um deles para identificar a mutação causadora e análise bayesiana da família de outro para testar se sua doença teve origem por mutação nova.

O NGS não mostrou nenhuma mutação de ponto nem indels nos éxons e sítios de splice, porém, mostrou uma transição no íntron 6 (c.779-1188C>T) e uma transversão na região 3'UTR (c.*1018T>A). A inferência bayesiana chegou a uma probabilidade 99,99% de a mãe do afetado não ser portadora, confirmando que ele teve origem por mutação nova. Esses resultados evidenciam que a causa da displasia ectodérmica hipoidrótica do cão que teve o gene *EDA* sequenciado não é uma mutação nos éxons ou sítios de splice e que cães com sinais desta displasia podem ter outros tipos de mutação, incluindo mutações intrônicas profundas, mutações em regiões não traduzidas (UTR) ou mutações em outros genes da via de sinalização EDA, como os genes *EDAR* e *EDARADD*. Evidenciam também que os casos esporádicos de displasia ectodérmica hipoidrótica canina podem ter origem por mutação nova, como é comum em humanos.

Palavras-chave: Alopecia, cão, gene *EDA*, hipotricose, oligodontia.

ABSTRACT

X-linked hypohydrotic ectodermal dysplasia is a clinical condition caused by recessive mutation of the *EDA* gene, which is located in the X chromosome and plays an important role in the development of ectodermal derivatives. It manifests mainly by defects in sweat glands, sebaceous glands, hair and teeth. Affected individuals are usually male and have congenital alopecia and hypotrichosis with typical body distribution, in addition to conoid teeth and oligodontia. Other ectodermal derivatives may have reduction in number and/or function, such as the tracheobronchial glands and the lacrimal and meibomian glands, increasing the risk of respiratory and eye disease, respectively.

In humans, over 300 different mutations in the *EDA* gene are known, the vast majority being point mutations and indels, with a significant portion originating from new mutations. In dogs, only three mutations are known, and except for one case, enough data have yet to be published to prove origin by new mutation.

In this study, in addition to a clinical and genetic characterization based on the literature and five affected individuals, a new generation sequencing (NGS) of the *EDA* gene of one of them was performed to identify the causative mutation as well as a Bayesian analysis of the family of another to test whether its disease originated from a new mutation.

The NGS showed no point mutation or indels in the exons and splice sites but showed a transition in the intron 6 (c.779-1188C>T) and a transversion in the 3'UTR region (c.*1018T>A). The Bayesian inference reached a 99.99% probability that the affected mother was not a carrier, confirming that it originated from a new mutation. These results evidence that the cause of hypohidrotic ectodermal dysplasia in the dog whom *EDA* gene was sequenced is neither a point mutation nor a small deletion or insertion in the exons or splice sites, and dogs with signals of this dysplasia may have other types of mutations, including deep intronic mutations, untranslated region mutations (UTR) or mutations in other genes in the EDA signaling pathway, such as the *EDAR* and *EDARADD* genes. They also evidence that sporadic cases of canine hypohidrotic ectodermal dysplasia may arise from new mutations, as often occurs in humans.

Keywords: Alopecia, dog, *EDA* gene, hypotrichosis, oligodontia.

CAPÍTULO 1

1. A DISPLASIA ECTODÉRMICA HIPOIDRÓTICA LIGADA AO X

1.1 **Definição**

Displasia é uma anormalidade em que as células e outros componentes de um tecido estão desorganizados, causando alterações na forma de estruturas corporais (Spranger et al., 1982). Se uma displasia afeta tecidos derivados da ectoderme como glândulas da pele, pelos e dentes é chamada de displasia ectodérmica (Freire-Maia e Pinheiro, 1984). Displasia ectodérmica hipoidrótica ligada ao X (DEHLX) é a displasia causada por mutação recessiva de um gene ligado ao cromossomo X, manifestando-se principalmente por anormalidades nas glândulas sudoríparas, glândulas sebáceas, pelos e dentes (Freire-Maia e Pinheiro, 1984). O adjetivo “hipoidrótica” é uma referência ao número reduzido de glândulas sudoríparas e, consequentemente, à produção deficiente de suor (hipoidrose).

1.2 **Aspectos históricos**

O primeiro relato desta displasia em cães, considerada como tal, foi feito por Selmanowitz et al. (1970). Mais tarde, os animais desse primeiro relato foram cruzados e os resultados sugeriram herança ligada ao X recessiva (Selmanowitz et al.,1977). A confirmação definitiva do padrão de herança ligado ao X só ocorreu em 1997, quando uma colônia para pesquisa foi fundada na Universidade da Pensilvânia (Casal et al., 1997). Em 2004, novos casos espontâneos de DEHLX canina foram publicados, incluindo critérios para o diagnóstico clínico (Moura e Cirio, 2004). Em 2005, os pesquisadores da Universidade da Pensilvânia identificaram a mutação causadora da DEHLX nos cães da sua colônia, confirmando que, assim como ocorre em humanos, esta genodermatose é causada por mutações no gene que codifica a ectodisplasina A (Casal et al., 2005a). Posteriormente, também realizaram ensaios terapêuticos pós-natais, utilizando ectodisplasina recombinante, com resultados significativos (Casal et al., 2007; Mauldin et al., 2009; Margolis et al., 2019). De 2016 até 2019, três estudos foram publicados. Em um deles, a análise de DNA dos afetados não detectou nenhuma mutação (Waluk et al.,

2016). Nos outros dois, duas diferentes mutações foram encontradas (Hadji Rasouliha et al., 2018; Vasiliadis et al., 2019).

1.3 **Raças caninas em que a ocorrência da DEHLX já foi registrada**

Em cães, confirmadamente há registro de sua ocorrência em pastor alemão, poodle, dachshund, Yorkshire terrier e em cães SRD, incluindo mestiço pinscher e mestiço pequinês (Selmanowitz et al., 1970; Casal et al., 1997; Moura e Cirio, 2004; Ríos, 2010; Waluk et al., 2016; Hadji Rasouliha et al., 2018; Vasiliadis et al., 2019). Há relatos que provavelmente são de DEHLX (mesmo fenótipo), mas foram publicados como “hipotricose congênita” ou “alopecia congênita” em whippet, cocker spaniel, pastor belga, Labrador retriever e bichon frise (Thomsett, 1961; Kral e Schwartzman, 1964; Muller e Kirk, 1976; Kunkle,1984; Grieshaber et al.,1986). Outros casos também publicados como hipotricose congênita (basset hound, buldogue francês, rottweiler) mostram características que não correspondem ao fenótipo esperado para DEHLX (Chastain e Swayne, 1985; Marks et al., 1992; Ihrke et al., 1993).

1.4 **Outras espécies em que a ocorrência da DEHLX foi registrada**

Em humanos, é a mais frequente das displasias ectodérmicas e sua ocorrência espontânea é amplamente documentada, sendo chamada também de displasia ectodérmica anidrótica e, às vezes, de síndrome de Christ-Siemens-Touraine (OMIM 305100).

Em animais, tem sido descrita em murinos, bovinos e caninos, recebendo diferentes denominações, tais como, fenótipo Tabby em camundongos, hipotricose com anodontia em bovinos, defeito ectodérmico congênito, displasia ectodérmica ligada ao X e displasia ectodérmica hipoidrótica ligada ao X em cães (Selmanowitz et al., 1970; Srivastava et al., 1997; Drögemüller et al., 2002; Moura e Cirio, 2004; Moura et al., 2019). Há também um possível caso em cavalo e um caso presumido em foca do atlântico oriental (Ramzan et al., 2001; Kahle et al., 2018)

1.5 **Etiologia**

A DEHLX é causada por mutações recessivas no gene *EDA*. O nome deste gene deriva do inglês “ectodermal dysplasia, anhidrotic” (Casal et al.,

2005a; Hadji Rasouliha et al., 2018; Vasiliadis et al., 2019). O gene *EDA* é altamente conservado nos vertebrados e, nos mamíferos, localiza-se no cromossomo X. Ele codifica a ectodisplasina A (EDA), uma proteína de sinalização celular necessária para a formação dos derivados ectodérmicos, inclusive dentes (Sadier et al., 2014; Lefebvre e Mikkola, 2014). A maioria dos casos de DEHLX canina, provavelmente, surge por mutação nova, como ocorre com os casos humanos (Casal et al., 1997; Zonana, 1997). O gene *EDA* canino é ortólogo do gene *EDA* humano (Gene Database, NCBI).

Até agora, três mutações foram encontradas em cães, uma substituição e duas pequenas deleções. A substituição (c.910-1G > A) ocorreu na sequência de consenso AG do sítio aceptor de splice do último íntron. Em consequência, um sítio aceptor de splice críptico dentro do éxon seguinte foi usado nos afetados, resultando em um desvio da matriz de leitura (*frameshift*) e um códon de terminação prematuro, que encerrou o processo de tradução (Casal et al., 2005a). As deleções são de um único par de bases (c.842delT e c.458delT) e foram encontradas em cães Dachshund de famílias diferentes. Em ambos os casos, a deleção também resultou em uma mutação *frameshift* e um códon de terminação prematuro, que causou o encerramento do processo de tradução e a produção de uma ectodisplasina truncada (Hadji Rasouliha et al., 2018; Vasiliadis et al., 2019).

1.6 **Fenótipo clínico**

O aspecto mais evidente da DEHLX é presença de alopecia ou hipotricose simétricas com distribuição corporal característica, desde o nascimento, associada com anormalidades dentárias, tanto na dentição decídua quanto na definitiva (Moura e Cirio, 2004; Mecklenburg, 2009; Lewis et al., 2010; Moura et al., 2019).

A alopecia se sobressai na área frontoparietal, região sacra, região ventral do pescoço e do tronco e metades proximais dos quatro membros locomotores. Nas regiões sacra e ventral do tronco pode haver hipotricose em lugar de alopecia (Figura 1). A extensão da alopecia/hipotricose em todas essas regiões pode apresentar variação conforme o indivíduo. De qualquer modo, em todos os afetados, são sempre as mesmas regiões que apresentam ausência ou

diminuição do número de pelos (Selmanowitz et al., 1970; Casal et al., 1997; Moura e Cirio, 2004). Heterogeneidade alélica (existência de diferentes mutações do gene *EDA*) é uma causa possível das variações na extensão das áreas de alopecia/hipotricose observadas em cães (Moura e Cirio, 2004). Nas áreas pilosas, não existem pelos secundários ou são muito raros e os pelos primários tendem a ser mais finos. A pelagem geralmente é seca e tem menos brilho que o normal por causa do número reduzido de glândulas sebáceas e sudoríparas (Selmanowitz et al., 1970; Casal et al., 1997; Moura e Cirio, 2004).

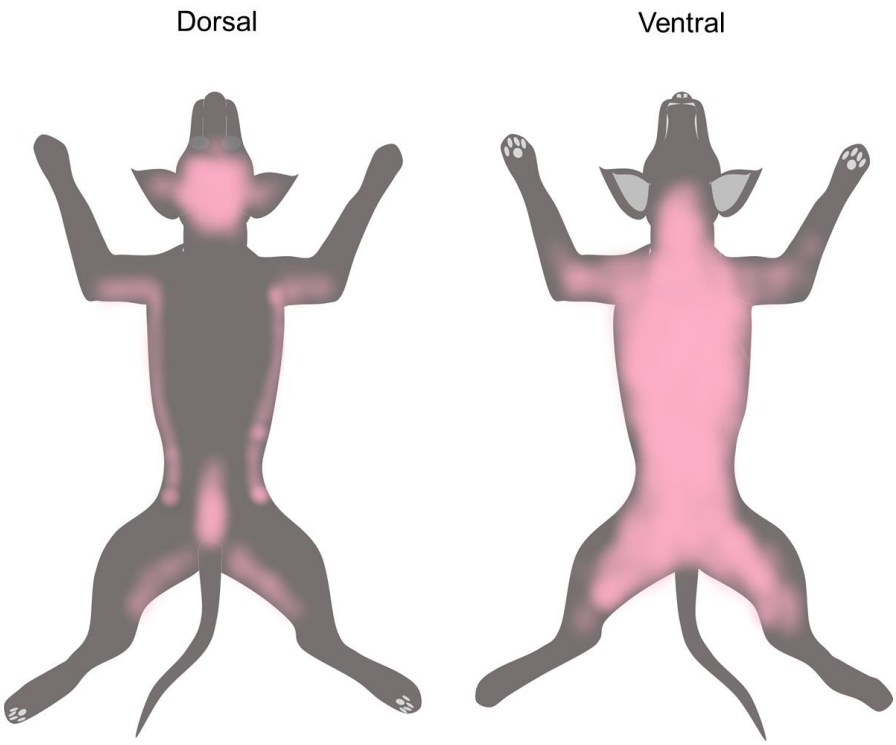

Figura 1. **Distribuição típica da alopecia e hipotricose na DEHLX canina**. As áreas rosadas representam os locais de alopecia e hipotricose. Elas estão presentes desde o nascimento e em todos os afetados a localização é sempre a mesma. A pele é fina e, nos animais novos, a vasculatura pode estar vísivel.

Nos cães recém-nascidos ou com poucos meses de idade, as áreas alopécicas apresentam-se com cor rosada ou rosa-acinzentada e a vasculatura pode estar visível. Já nos cães com mais idade, estas áreas tornam-se gradualmente hiperpigmentadas. A pele é fina, seca e pode apresentar episódios de descamação (Selmanowitz et al., 1970; Casal et al., 1997; Moura e Cirio, 2004). Em nível microscópico, chama a atenção a ausência ou

redução congênitas das unidades piloglandulares. Nas áreas alopécicas, não existem glândulas sudoríparas, glândulas sebáceas, pelos e músculos piloeretores. Nas áreas hipotricóticas, essas estruturas estão reduzidas e displásicas. Independentemente da quantidade de pelos, com o tempo surgem hiperpigmentação, hiperceratose ortoceratótica e focos de espongiose, mantendo-se normais as fibras colágenas (Moura e Cirio, 2004). Outros sinais menos óbvios podem ocorrer, tais como sensibilidade ao frio e pele mais sujeita a escoriações, piodermites e dermatomicoses (Moura e Cirio, 2004; Moura et al., 2019). Sinais oculares como olho vermelho, fotofobia e secreção mucopurulenta são frequentes por causa de conjuntivite recorrente (Moura e Cirio, 2004; Casal et al., 2005b). A recorrência de conjuntivite é atribuída à falta ou diminuição do número de glândulas meibomianas e à diminuição da produção lacrimal que é cerca de 25% menor do que a dos cães normais e que pode evoluir para ceratoconjuntivite seca (Casal et al., 2007; Margolis et al., 2019), porém, há indivíduos em que os valores do teste de Schirmer mostraram-se dentro da normalidade (Moura e Cirio, 2004). Também são frequentes infecções respiratórias como rinite, sinusite e broncopneumonia devido à ausência das glândulas da mucosa respiratória necessárias para o *clearence* mucociliar (Moura e Cirio, 2004; Mauldin et al., 2009).

Hipertermia, uma complicação frequente apresentada por humanos afetados e que oferece risco de vida em crianças mais novas devido a sudorese insuficiente ou gravemente reduzida (Lefebvre e Mikkola, 2014), não acontece em cães porque a dissipação de calor nesta espécie não é dependente das glândulas sudoríparas (Moura e Cirio, 2004).

1.7 **Padrão de herança**

A DEHLX apresenta padrão de herança ligada ao X recessiva (Selmanowitz et al., 1977; Casal et al., 1997). Neste padrão, o pai nunca transmite o gene para os filhos, porém, transmite para todas as filhas. Isto ocorre porque os filhos herdam somente o cromossomo Y do pai (o Y não contém o gene *EDA*), enquanto as filhas herdam o cromossomo X. Assim, se um macho afetado se unir com uma fêmea normal, todos os descendentes serão normais, mas, todas as filhas serão portadoras, ou seja, heterozigotas (Figuras 2). Essas portadoras de modo geral são normais porque herdam o

alelo dominante da mãe. Quando uma mãe portadora se une com um macho normal, ela transmite o gene para metade dos filhos e metade das filhas, que serão afetados e portadoras, respectivamente (Selmanowitz et al., 1977; Casal et al., 1997; Thomas, 2004). Fêmeas portadoras tendem a ser normais, porém, se houver um desvio na inativação aleatória do cromossomo X, elas podem apresentar sinais clínicos com gravidade proporcional ao desvio (Sofaer, 1981; Zonana, 1997). Considerando que animais afetados geralmente são excluídos da reprodução pelos criadores, o cruzamento com chance de originar afetados e que é mais provável de ocorrer é aquele entre uma fêmea portadora e um macho normal. As chances para os descendentes são de um quarto de fêmeas normais homozigotas, um quarto de fêmeas normais heterozigotas (portadoras), um quarto de machos normais e um quarto de machos afetados. Portanto, metade das fêmeas deve ser portadora e metade dos machos deve ser afetada. Uma vez que se trata de herança ligada ao sexo recessiva, quase sempre a DEHLX é vista em machos (Figuras 2 e 3). Deve-se lembrar, que nos casos originados por mutação nova, não há história familial da doença (Zonana, 1997).

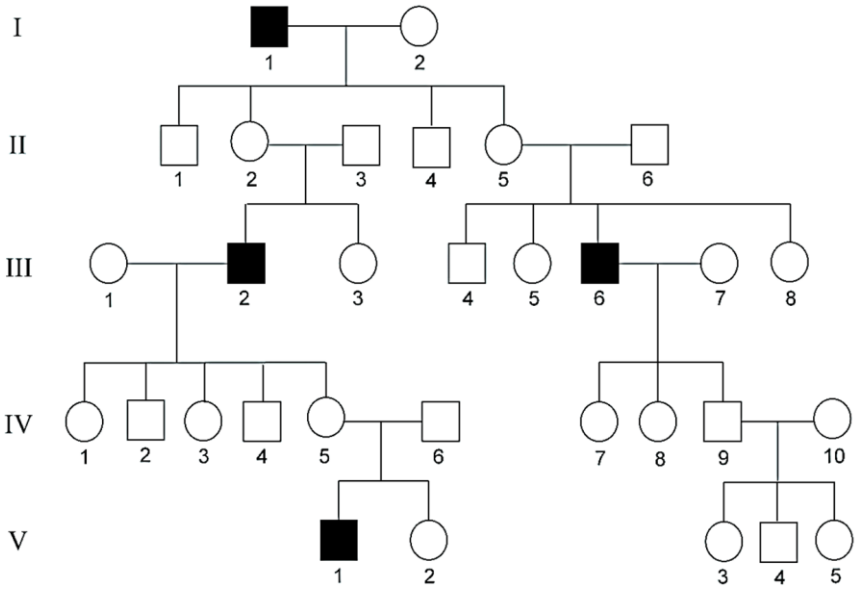

Figura 2. **Heredograma hipotético representando o padrão de herança recessiva ligada ao X**. Na DEHLX, assim como em qualquer doença ligada ao X recessiva, quando a mutação está segregando ao longo das gerações, a doença ocorre predominantemente em machos e salta gerações. Note que os afetados nunca transmitem para os filhos, apenas para as filhas, as quais serão portadoras.

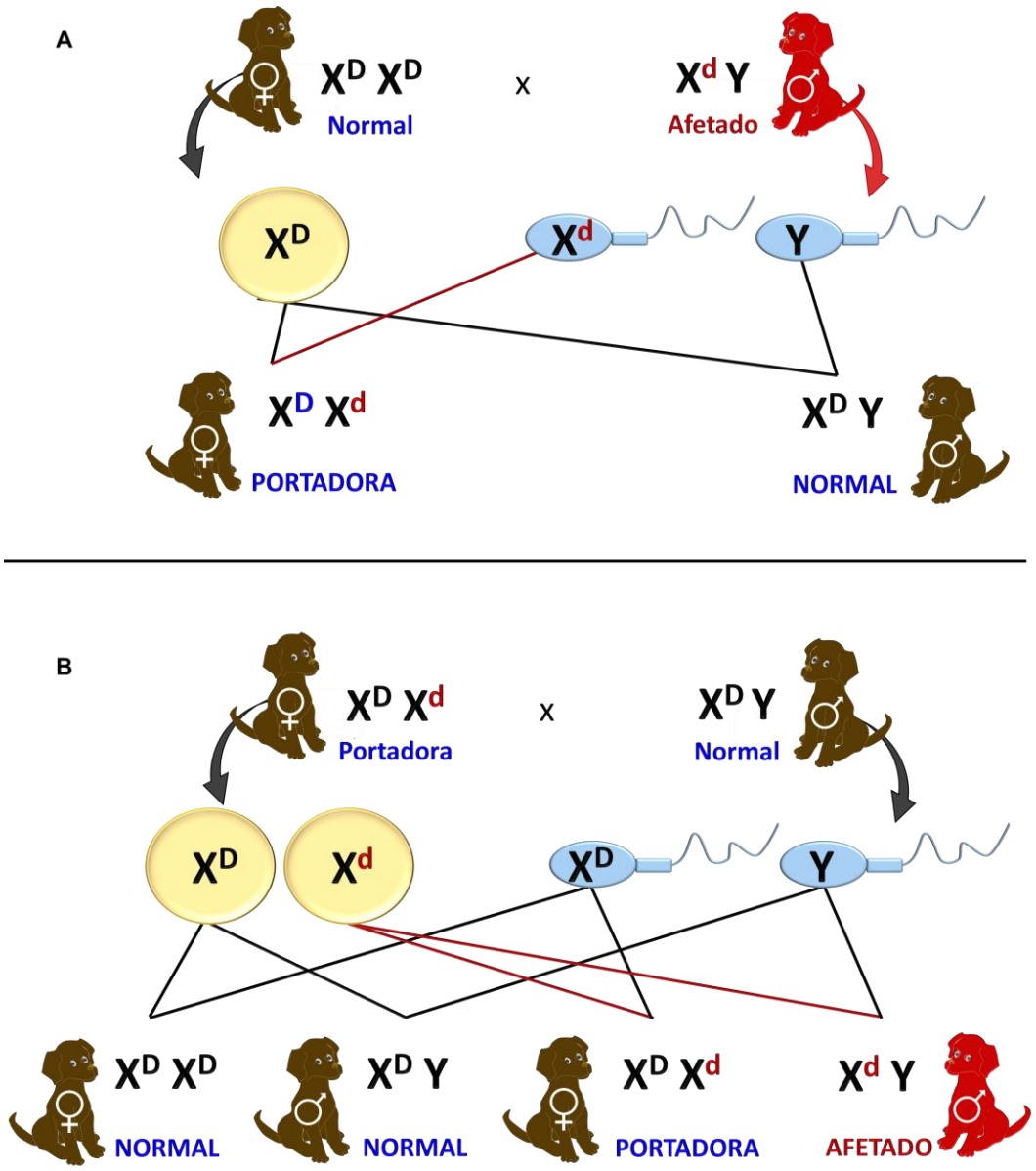

Figura 3. **Representação do mecanismo de herança da DEHLX**. A) Se um afetado for cruzado com uma fêmea normal terá todas as filhas portadoras (heterozigotas) e todos os filhos normais. As portadoras tendem a ser normais, mas podem também apresentar grau variável de expressão clínica. B) Se uma portadora for cruzada com um macho normal, haverá uma probabilidade de 25% de nascer um filho afetado e 25% de nascer uma portadora, ou seja, metade dos filhos será afetada e metade das filhas será portadora.

1.8 **Base molecular e patogênese**

O processo de desenvolvimento dos derivados ectodérmicos originando estruturas perfeitamente formadas depende em grande parte da via de sinalização EDA (Sadier et al., 2014). Esta via é representada por um conjunto

de moléculas de sinalização necessárias para as interações mesenquimoepiteliais que regulam a formação dos derivados ectodérmicos (Figura 4). As três principais moléculas são EDA (*ectodysplasin A*), EDAR (*ectodysplasin A receptor*) e EDARADD (EDAR *associated death domain*). A via EDA interage com várias outras e compartilha a jusante vários componentes com a via de sinalização TNF (Zonana, 2008; Sadier et al., 2014; Trzeciak e Koczorowski , 2016). A EDA é uma proteína transmembrana tipo II, (um único domínio transmembrana com terminal N citosólico) pertencente à superfamília do fator de necrose tumoral (TNF). É um ligante formado por um curto domínio intracelular, um domínio transmembrana e um domínio extracelular (Pispa e Thesleff, 2003). A atividade biológica da EDA depende da clivagem realizada por uma proproteína-convertase (furina) no domínio extracelular, permitindo sua ligação à molécula receptora (EDAR) (Ezer et al., 1999; Zonana, 2008). A EDAR é uma proteína transmembrana tipo I (um único domínio transmembrana com terminal C citosólico) que tem domínios extracelulares de receptor TNF, possibilitando sua ligação à isoforma A1 da ectodisplasina (Zonana, 2008). Na sua porção intracelular, ela tem um domínio de morte (DD) que garante sua ligação à EDARADD, uma proteína de adaptação citosólica que, por sua vez, ativa direta ou indiretamente proteínas de outras vias sinalizadoras a jusante, como a NF-kB (Zonana, 2008; Lefebvre e Mikkola, 2014).

Em humanos, mutações em qualquer um dos genes que codificam as três proteínas principais da via de sinalização EDA causam a sua disrupção e, consequentemente, formas clinicamente indistinguíveis de displasia ectodérmica hipoidrótica (DEH), porém, com padrões de herança diferentes. As mutações do gene *EDA* apresentam herança ligada ao X recessiva; as do gene *EDAR* apresentam herança autossômica dominante ou recessiva dependendo da mutação; e as do gene *EDARADD* apresentam herança autossômica recessiva e eventualmente dominante (Bal et al., 2007; Zonana, 2008; Schneider, 2008). Em cães, até agora, somente a forma ligada ao X recessiva foi registrada. A displasia ectodérmica que caracteriza raças sem pelo (*Chinese crested dog*, *Peruvian and Mexican hairless dog*) não deve ser confundida com a DEHLX. Nestas raças a herança é autossômica dominante (a rigor,

semidominante) e causada por haploinsuficiência do gene *FOXI3*. Os cães sem pelo são heterozigotos e a homozigose do alelo mutante causa letalidade embrionária (Drögemüller et al., 2008; Kupczik et al., 2017). Mutações em outros genes além desses podem também causar formas mais raras de DEH, porém, estas são caracteristicamente associadas a outras anormalidades. Por exemplo, mutações no gene *NF-kB1A* causam DEH autossômica dominante com imunodeficiência de células T (Zonana, 2008).

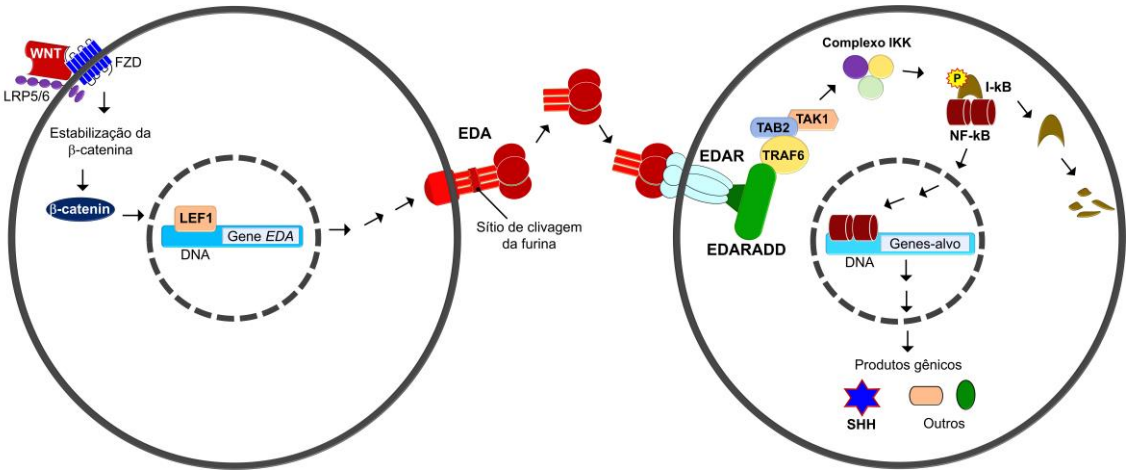

Figura 4. **Representação simplificada da via de sinalização celular EDA**. As três principais proteínas dessa via são a EDA (vermelho), EDAR (azul-claro) e EDARADD (verde). A WNT ativa o gene *EDA* por meio do fator de transcrição LEF1. A EDA é clivada por proteases (furina e semelhantes), permitindo que seu domínio extracelular se ligue à EDAR e provoque a formação de um complexo molecular (EDARADD e outras moléculas) adiante. Outras moléculas ativam a formação de um complexo com a cinase I-kB. A degradação da I-kB fosforilada libera o fator de transcrição NF-kB que entra no núcleo celular e ativa genes-alvo. A interação mesenquimoepitelial que gera os placoides de vários derivados ectodérmicos (pelos, glândulas sudoríparas, dentes, etc.) e regula o seu desenvolvimento depende dessa via em grande parte. Mutações em qualquer um dos genes que codificam as três proteínas principais da via EDA causam formas de displasia ectodérmica hipoidrótica que se distinguem unicamente pelo padrão de herança (Modificado de Moura et al., 2019).

1.9 **Diagnóstico**

O diagnóstico da DEHLX é relativamente fácil e pode ser feito apenas com base no quadro clínico característico (distribuição da alopecia/hipotricose e anormalidades dentárias). A confirmação do diagnóstico clínico é feita por

meio de exame histopatológico (Moura e Cirio, 2004; Mecklenburg, 2009; Lewis et al., 2010; Moura et al., 2019). Atualmente, também é possível a realização de diagnóstico molecular, porém, o custo é proibitivo para uso rotineiro.

1.10 **Tratamento**

Camundongos e cães com DEHLX têm servido como modelo animal em pesquisas para o desenvolvimento de um tratamento para a DEHLX humana. Os primeiros passos foram dados em experimentos com camundongos Tabby utilizando ectodisplasina recombinante (Fc-EDA). Esta molécula é formada por uma fração de imunoglobulina (Fc) mais o domínio de ligação ao receptor da EDA. Em um estudo publicado em 2003, a administração intravenosa de Fc-EDA em fêmeas gestantes causou a reversão do fenótipo Tabby na sua prole (Gaide e Schneider, 2003). Mais tarde, foi testada a administração intra-amniótica da Fc-EDA, com igual sucesso (Hermes et al., 2014).

A EDA recombinante também foi testada em cães, porém, utilizando administração intravenosa em recém-nascidos afetados porque no cão a passagem transplacentária de imunoglobulinas é praticamente nula (Casal et al., 2007). O melhor resultado foi alcançado com um protocolo de 2 mg de Fc-EDA1 com injeção repetida a intervalos de três dias (de 2 a 14 dias de idade), totalizando cinco injeções. Nenhum efeito colateral ocorreu. Dos cinco cães submetidos a este protocolo, quatro apresentaram correção significativa do fenótipo clínico. Eles desenvolveram dentição permanente com características próximas do normal, desenvolveram também a capacidade de suar e a lacrimação normal, além de não apresentarem infecções respiratórias (Casal et al., 2007). Em um estudo complementar, três de quatro cães que receberam as doses mais altas (injeções de 2 mg) desenvolveram, além da dentição permanente, glândulas esofágicas e um número de glândulas traqueais e bronquiais semelhante ao número normal, mostrando significativa melhora do *clearence* mucociliar. Os cães tratados não apresentaram episódios de doenças respiratórias, diferentemente do que comumente acontece com os afetados sem tratamento (Mauldin et al., 2009).

Esses estudos em camundongos e cães levaram ao desenvolvimento de um protocolo de administração intra-amniótica de Fc-EDA em humanos

(Huttner, 2014; Schneider et al., 2018). As crianças tratadas desenvolveram glândulas sudoríparas e foram capazes de suar normalmente. Por ocasião da publicação do trabalho, em 2018, elas estavam com idade entre 14 e 22 meses sem apresentar qualquer episódio de hipertermia e de problemas respiratórios (Schneider et al., 2018).

Recentemente, um protocolo semelhante foi testado em cães. Injeções únicas (100mg/kg) de Fc-EDA foram aplicadas no saco amniótico aos 32, 45 ou 55 dias de gestação ou duas injeções aos 32 e 45 dias de gestação (Margolis et al., 2019). Diferentemente do que ocorreu com o tratamento pós-natal testado por Casal et al. (2007) e Mauldin et al. (2009), não houve melhora do clearence mucociliar nem da dentição permanente. Houve aumento na quantidade de pelos, mas sem atingir o que seria normal. Entretanto, As glândulas sudoríparas das patas se desenvolveram similarmente ao observado em cães normais, a dentição decídua se desenvolveu melhor do que nos cães não tratados e houve um significativo desenvolvimento das glândulas meibomianas em todos os grupos de cães afetados tratados em comparação com os não tratados, prevenindo as complicações oculares frequentes nessa doença (Margolis et al., 2019). As diferenças entre os resultados do tratamento pré-natal e pós-natal é explicada pela variação entre os momentos em que os diferentes derivados ectodérmicos se desenvolvem, sugerindo que a ectodisplasina é necessária em diferentes fases da gestação e no início do período pós-natal (Margolis et al., 2019).

1.11 **Prognóstico** e **bem-estar dos afetados**

Apesar de a DEHLX ser irreversível, o prognóstico quanto à vida e à validez é bom, uma vez que as eventuais comorbidades como piodermites, micoses cutâneas, conjuntivites e infecções respiratórias geralmente respondem bem a medidas terapêuticas específicas. Ceratoconjuntivite seca pode ser suavizada com terapêutica apropriada. Os cães não desenvolvem hipertermia, complicação comum em humanos, pois não dependem das glândulas sudoríparas para perder calor. Atualmente, hipodontia ou oligodontia poderiam ser parcialmente corrigidas com implantes dentários, mas ainda não há relatos da sua utilização em pacientes com DEHLX (Moura e Cirio, 2004; Moura et al., 2019)

Por causa da alopecia/hipotricose e falta de pelos secundários, os cães com DEHLX são mais sensíveis ao frio e, por causa da diminuição do número de glândulas cutâneas, têm pele seca (xerose). Medidas simples, como mantê-los aquecidos em dias frios (roupas, cobertores, aquecedores) e aplicação de cremes hidratantes em áreas mais ressecadas da pele, sobretudo no inverno, proporcionam-lhes uma boa qualidade de vida. Considerando as anormalidades dentárias, os alimentos devem ser oferecidos em recipientes que facilitem preensão e devem ser cortados em pedaços pequenos (ou podem ser rações macias) para facilitar a mastigação e a deglutição.

1.12 **Prevenção**

Assim como se recomenda em casos de outras doenças genéticas, os afetados não deveriam ser cruzados. Casais normais que tiveram filhos afetados não deveriam ser novamente cruzados. Lembrando que em doenças ligadas ao X recessivas, como a DEHLX, todas as filhas de um afetado geralmente são normais, porém, são portadoras (heterozigotas) e não deveriam ser cruzadas mesmo que seja com machos normais.

1.13 **Outras considerações**

Por ser uma doença rara, a DEHLX é pouco conhecida dos clínicos e pode ser considerada uma doença órfã dentro da medicina veterinária. Uma maior divulgação das suas principais características contribui para que seja corretamente diagnosticada e os pacientes sejam atendidos adequadamente e, assim, contribui também para torná-la menos órfã.

É provável que uma grande parte dos casos caninos, a exemplo do que ocorre em humanos, tenha origem por mutação nova. É igualmente provável que os mesmos tipos de mutação encontrados em casos humanos ocorram em cães. À medida que novos casos forem reconhecidos e os recursos atuais de diagnóstico molecular forem utilizados, essa afirmação deve ser confirmada.

2. CASUÍSTICA E JUSTIFICATIVAS

Caso 1: Cão mestiço pinscher com fox paulistinha/Brazilian terrier (Figura 5-A); *Caso 2*: Cão sem raça definida (Figura 5-B); *Caso 3*: Cão mestiço

pequinês (Figura 5-C). Estes três casos foram publicados em 2004 (Moura e Cirio, 2004); *Caso 4*: Cão poodle cujo estudo está detalhado no Capítulo 2 desta tese; *Caso 5*: Cão Yorkshire terrier cujo estudo está detalhado no Capítulo 3 desta tese. Todos os cinco cães são do sexo masculino.

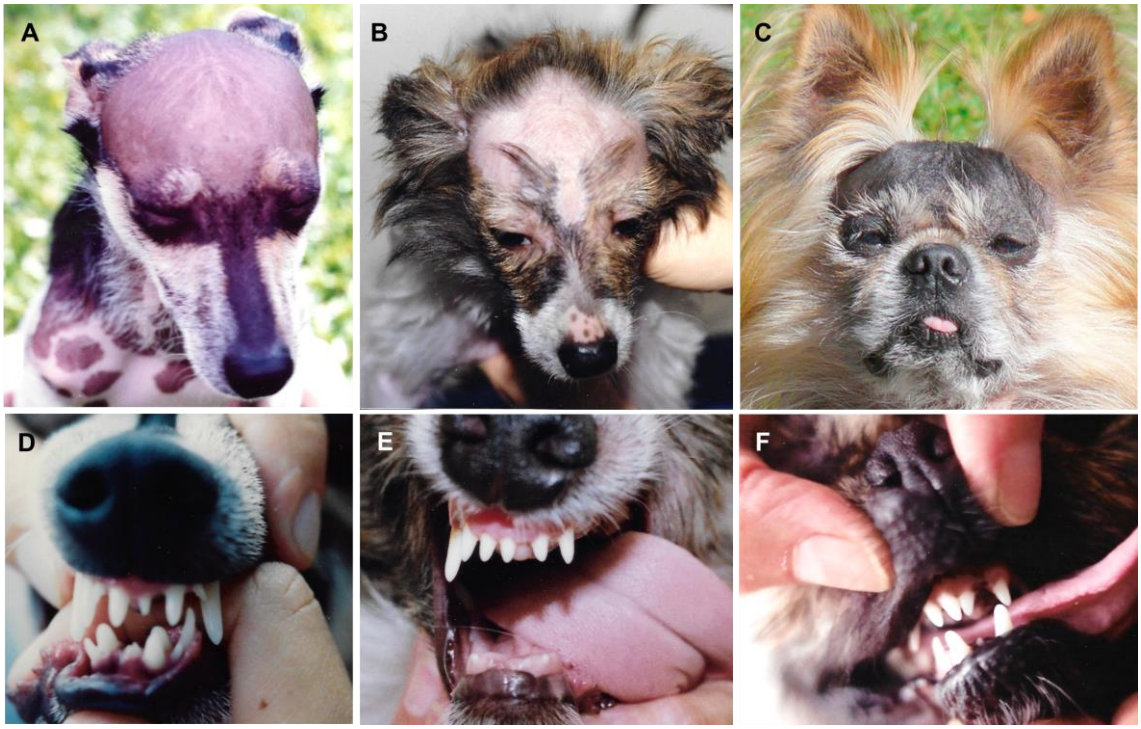

Figura 5. **Fenótipo clínico de três cães da causística**. A região frontoparietal é uma das regiões do corpo que caracteristicamente mostra alopecia na DEHLX. Note a diferença no grau de extensão da alopecia entre os cães vistos em (A) e (B). O cão visto em (B), aos quatro meses de idade, ainda tinha a pele rosada. Com o passar do tempo ela se tornou hiperpigmentada, como também ocorreu com o cão mostrado aos nove anos de idade em (C). D) Dentição de (A) aos dois anos. E) Dentição de (B) aos oito meses. Note a persistência de dentes decíduos na arcada inferior. F) Dentição de (C) aos 5 anos. Em todos eles, há oligodontia e os dentes são conoides.

Apesar de o número de casos ser pequeno, ele pode ser considerado um número relativamente grande uma vez que a DEHLX é uma doença rara. Representa a maior casuística de casos espontâneos e oriundos de famílias diferentes obtida por um grupo de pesquisadores, levando mais de 35 anos para ser constituída. Os demais estudos envolvem casos únicos ou casos dentro de uma mesma família. Na colônia da universidade de Pensilvânia todos os indivíduos derivam de um mesmo fundador e, portanto, apresentam a mesma mutação.

Foram obtidas amostras para extração de DNA dos cinco cães. Quatro amostras de pele preservada em formalina 10% e uma amostra de sangue venoso. O DNA obtido das amostras de pele não apresentou qualidade suficiente para garantir resultados fidedignos no sequenciamento. Por esta razão, apenas o DNA extraído do sangue foi devidamente sequenciado.

3. **OBJETIVOS**

3.1 Caracterizar clínica e geneticamente a DEHLX canina com base na literatura e em cinco casos espontâneos da casuística do grupo de pesquisa do autor;

3.2 Identificar por meio de sequenciamento de nova geração a mutação de um dos afetados;

3.3 Verificar por meio de estatística bayesiana se um dos afetados teve origem por mutação nova.

CAPÍTULO 2

(Artigo científico a ser submetido para publicação no periódico *Veterinary Dermatology*)

Obs.: O periódico *Veterinary Dermatology* orienta os autores para que os artigos sejam submetidos em espaço simples, apesar disso, aqui está em espaço 1,5 para facilitar a leitura.

X-linked hypohidrotic ectodermal dysplasia in dogs — Other causal possibilities beyond point mutations in exons and splice sites

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Running title: Hypohidrotic ectodermal dysplasia in dogs

X-linked hypohidrotic ectodermal dysplasia in dogs — Other causal possibilities beyond point mutations in exons and splice sites

Background – X-linked hypohidrotic ectodermal dysplasia (XLHED) is a rare genodermatosis that affects humans, dogs, cattle, mice and probably other mammals. In humans, over 300 mutations are known, but in dogs, only three.

Hypothesis/Objectives – To verify whether a dog with a clinical and histopathological diagnosis of X-linked hypohidrotic ectodermal dysplasia presents one of the three known mutations in the *EDA* gene or whether the mutation is a new one.

Animals – A male dog with no family history presenting alopecia, hypotrichosis and characteristic dental abnormalities of XLHED, and a normal male dog.

Methods – The DNA of the affected dog was submitted to next generation sequencing and the result was compared with the reference sequence (CanFam3.1 NC_006621.3) and with the result of the next generation sequencing of the normal dog's *EDA* gene.

Results – No point mutation or indels were found in the exons and splice sites of *EDA* gene, but a transition (c.779-1188C>T) and a transversion (c.*1018T>A) were found in the intron 6 and 3' UTR region respectively.

Conclusions and clinical importance – Dogs with signs of XLHED may have other types of mutation, including deep intronic mutations and untranslated region mutations in the *EDA* gene, or mutations in other genes of the EDA signaling pathway.

Keywords: Alopecia, conical teeth, dog, EDA, EDAR, EDARADD, genodermatosis, hypotrichosis.

INTRODUCTION

X-linked hypohidrotic ectodermal dysplasia (XLHED) is a genetic disorder that affects ectoderm derivatives and is mainly manifested through structural and numerical abnormalities in the teeth, hair, sweat glands and sebaceous glands, transmitted from one generation to the next in accordance with the X-linked recessive inheritance pattern.[1]

It occurs in human beings at an estimated frequency of approximately 1:100,000 births, with variations according to the population, and is considered a rare disease, however, it is the most frequent ectodermal dysplasia.[2, 3] In animals, the spontaneous occurrence of XLHED has been confirmed in mice, cattle and dogs.[4-8] Although the highest number of spontaneous cases has been registered in dogs, this number remains low and there are not sufficient data to estimate the frequency of occurrence in this species.

Affected dogs present alopecia and congenital hypotrichosis, with typical body distribution encompassing the frontoparietal region, mediocaudal region of the thoracic and pelvic limbs, an area of the sacral region and the entire ventral region of the trunk. In these regions, the hair follicles and cutaneous glands are absent or fewer in number than normal. The regions of alopecia/hypotrichosis are always the same but may present variations in terms of extension and, in the hairy regions, there are no secondary hairs.[6-8] Furthermore, the affected animals present hypodontia or oligodontia and the teeth are conical and misaligned.[9,10] Other ectodermal derivatives may be reduced in number and/or function, such as the tracheobronchial glands and the lacrimal and meibomian glands. These abnormalities increase the risk of respiratory and ocular diseases, respectively, in both dogs and humans.[11-13] Regarding other aspects, affected individuals are normal.[6-13]

XLHED is caused by recessive mutations in the *EDA* gene, which is highly conserved in tetrapods and, in mammals, it is located in the X chromosome.[14] This gene encodes ectodysplasin A, a type II membrane protein that belongs to the tumor necrosis factor (TNF) superfamily and is part of an important cellular signaling pathway for the development of ectodermal derivatives. Mutations in the *EDA* gene cause disruption in the EDA signaling pathway, whose integrity is

necessary to promote the adhesion of cells to the extracellular matrix and ensure the mesenchymal-epithelial interaction that regulates the formation and development of the ectodermal derivatives.[15-17]

Currently, over 300 different mutations in the human *EDA* gene are known, with the vast majority being point mutations and small deletions or insertions.[18] In the canine gene, only three mutations are known, one substitution and two different deletions of a single pair of bases. The former was found in a research colony and derived from an affected male German shepherd.[7, 19] The latter two were found in two different families of Dachshunds.[20, 21] In 2016, a study of three affected males, two of whom were siblings, found no mutation and the cause was probably a defect in the splice mechanism.[22]

In the present study, in order to verify whether one of the three known gene mutations had occurred, we analyzed the *EDA* gene of a dog with a characteristic phenotype of XLHED by next generation sequencing.

MATERIALS AND METHODS

Animals and samples

A male poodle, six months old and with an unknown family history was forwarded to us for diagnosis of its clinical condition. It had areas of alopecia and hypotrichosis, in addition to dental abnormalities. It underwent physical examination and was monitored for 8 years. Recently, samples of alopecic skin and blood were collected for further examination. For comparison, a blood sample was collected from a normal male dog. The skin sample was taken from the thorax and prepared for histopathological examination in accordance with the routine protocol for this purpose, being stained with hematoxylin and eosin.[23] The blood sample (10 ml) was taken from the cephalic vein and stored in Vacutainer tubes with EDTA and used for the extraction of genomic DNA. Blood was collected from the normal dog following the same procedure. The DNA of both samples was submitted to next generation sequencing for analysis of the *EDA* gene as described in the following section. All these procedures were conducted in compliance with ethical principles for the handling of animals

and approved by institutional Committee for Ethical Use of Animals in Research.

Next generation sequencing (NGS)

Panel design. The panel was designed on the Ion AmpliSeq Designer platform (Thermo Fisher Scientific), available at www.ampliseq.com. The genomic coordinates inserted into the panel design in accordance with the reference sequence NC_006621.3 covered the entire coding region of the canine *EDA* gene and its flanking regions containing the splice sites, in addition to the 5'UTR and 3'UTR regions. The final design resulted in a panel with 1 multiplex primer pool (74 amplicons) between 125 and 275 bp. The final coverage involved the coding and flanking region of all the exons, in addition to the entire intronic region from exon 4 to 8, a large part of the 5'UTR region and the entire 3'UTR region. The reference sequence (CanFam3.1 NC_ 006621.3) is available at the NCBI website in the Genome database.

DNA Extraction. The DNA was extracted using the ReliaPrep™ Blood gDNA kit (Promega), following the manufacturer's protocols. The quality of the extracted DNA was verified using the Qubit dsDNA BR Assay kit and Qubit Fluorometric Quantitation equipment (Thermo Fisher Scientific) and viewing in agarose gel, in addition to being amplified with specific primers for dogs.

Preparation of libraries and next generation sequencing. The genomic libraries were prepared from the extracted DNA using the Ion AmpliSeqLibrary kit 2.0 (Thermo Fisher Scientific) together with the AmpliSeq Panel for the amplification of the regions of interest of the canine EDA gene. The emulsion PCR was then prepared with the reagent Ion 520™ & Ion 530™ Kit-Chef and the Chip (Ion 530™ Chip - Thermo Fisher Scientific) was loaded using Ion Chef System equipment (Thermo Fisher Scientific). The loaded chip was then submitted to NGS on the Ion S5 System platform (Thermo Fisher Scientific).

Data analysis. The files generated by NGS were aligned with the reference sequence of the canine genome (CanFam 3.1 NC_006621.3) and the variants were called by the variantCaller plugin available on the S5 Torrent Server (Thermo Fisher Scientific). Later, an analysis of the coverage of the sequenced regions was conducted using the data generated by the coverageAnalysis

plugin available on the S5 Torrent Server (Thermo Fisher Scientific) and the sample of the affected dog was compared with that of the normal dog. Each variant called by the variantCaller plugin was checked by individual analysis on the Integrative Genomics Viewer (IGV).

RESULTS

Physical examination. Male with congenital alopecia in the frontoparietal regions, mediocaudal region of the thoracic and pelvic limbs and the entire ventral region of the trunk. Hypotrichosis in the sacral region and the pinnae. All of these regions were lightly pigmented (Figure 1a - d). Secondary hair was absent all over the body and the skin was thin and dry. Abnormal permanent dentition (conical teeth and oligodontia) and persistence of deciduous teeth. Clinical signs of bilateral keratoconjunctivitis sicca. Throughout the period of monitoring, the skin became hyperpigmented, the persistent deciduous teeth fell (Figure 2) and the keratoconjunctivitis sicca became more severe (Figure 3-a and b). No other abnormality was found.

Histopathological examination. Presence of mild orthokeratotic hyperkeratosis and abundant deposition of melanin in the epidermis (hyperpigmentation). Lack of piloglandular units in the areas of alopecia and reduction of the number in the areas of hypotrichosis. Normal collagen fibers (Figure 3-c).

Next generation sequencing. The NGS assay achieved the expected reading, integrity and coverage parameters for the equipment and to ensure quality. The sequencing did not detect any point mutation, small deletion or insertion in the exons or splice sites of the affected dog's EDA gene. Likewise, no changes were found in the *EDA* gene of the normal dog. In the intron 6 and 3'UTR region of the affected dog's *EDA* gene a transition (c.779-1188C>T) and a transversion (c.*1018T>A) were detected respectively (Figure 4), and both were confirmed using IGV.

DISCUSSION

The sex of the patient (male), the typical distribution of alopecia and hypotrichosis, the thin and dry skin, the dental abnormalities and the absence of piloglandular units in the areas of alopecia confirmed by histopathological examination meet the criteria for the diagnosis of XLHED.[8, 10, 24] However, the absence of point mutations or small deletions in the coding regions and the splice sites of the *EDA* gene disclosed by the NGS is discordant. Although this result contradicts the clinical and histopathological diagnosis, similar situations are well known in human cases of hypohidrotic ectodermal dysplasia (HED).[25-27]

Our aim was to verify whether the patient in this study presented one of the mutations known so far in dogs,[19-21] and next generation sequencing is a powerful tool for molecular diagnosis that enables the detection of substitutions and small deletions or insertions (indels) efficiently and quickly.[28] That being the case and seeing that the result of the NGS does not correspond to the result of the clinical and histopathological examinations, it falls to the following hypotheses to explain the etiology of the hypohidrotic ectodermal dysplasia in this case: 1) it is caused by a mutation other than point mutations or indels in the exons and splice sites; 2) it is not caused by a mutation in the *EDA* gene; 3) the NGS did not detect the mutation; 4) it is caused by a deep intronic mutation, untranslated region mutation or both.

Considering that canine and human *EDA* genes are orthologous and encode ectodysplasin A (EDA), a protein whose integrity is essential for the smooth functioning of the EDA cell signaling pathway, and also considering that this pathway is highly conserved in vertebrates and allows the development of ectodermal derivatives, such as mammalian teeth, sweat glands and hair,[29] lessons on XLHED learned from cases in other species, especially humans, can be applied to canine cases. Furthermore, the degree of homology between the *EDA* genes of the four species (man, mouse, dog and cattle) in which the spontaneous occurrence of XLHED has been confirmed is quite high.[19] Thus, the mutation types are also expected to be quite similar in these mammals.

In humans, most of the more than 300 mutations identified in the *EDA* gene are point mutations, followed by small deletions and insertions.[18, 30] However, there

have been reports of large deletions, some including one or more entire exons, and the complete deletion of the *EDA* gene, in addition to structural chromosomal abnormalities.[18,25,30-36] In dogs, of the three mutations identified so far, there is one substitution and two small deletions. The substitution (c.910-1G > A) occurred in the conserved AG consensus sequence of the splice acceptor site in the last intron. Consequently, a cryptic splice acceptor site within the exon downstream was used in the affected dogs, resulting in a frame shift and a premature termination codon, terminating the translation process.[19] The deletions are from a single base pair (c.842delT and c.458delT) and were found in Dachshunds from different families. In both cases, the deletion also resulted in a frame shift and a premature termination codon, causing the translation process to terminate and the production of a truncated ectodysplasin.[20-21]

Although point mutations and small deletions are the main causes of XLHED in humans and probably in dogs, other types of mutation need to be considered as causes of canine XLHED, as is done in human cases. Large deletions and insertions cause disruption of the *EDA* gene as well as structural chromosomal abnormalities.[18,30-36] Translocations involving the X chromosome and an autosome have been identified as the cause of XLHED in humans.[25] There are even records of women carriers with complete clinical signs of XLHED. Cytogenetic examination showed that they had a translocation between the X and 9 chromosomes, and preferential inactivation of the normal chromosome occurred in lyonization and not randomly as normally occurs.[25,32, 33] At the chromosome level, this phenomenon mimics the chromosome combination of males where there is only one X chromosome and being abnormal causes the disease. Translocations involving other autosomes have also been documented.[34-36] Wu et al. studied a pericentric inversion in the X chromosome segregating into one family and causing XLHED.[37] A defect in the splicing mechanism was considered the cause of XLHED in three mixed breed dogs. The whole-genome sequencing of the DNA of these dogs found no mutation in the gene sequence, but the RNA analysis showed an altered transcript and a truncated ectodysplasin would have originated in its translation.[22]

It is important to remember that ectodysplasin A is part of a cell signaling pathway (EDA pathway) whose core is formed by the following proteins: ectodysplasin A (EDA), EDA receptor (EDAR) and the EDAR associated death domain (EDARADD).[15-17] In humans, mutations in any one of the genes that encode these proteins cause clinically indistinguishable hypohidrotic ectodermal dysplasias (HED) but with distinct inheritance patterns.[25] Mutations in the *EDA* gene present recessive X-linked inheritance; mutations in the *EDAR* gene may have autosomal recessive inheritance or dominant inheritance depending on the type of mutation; and mutations in the *EDARADD* gene generally have autosomal recessive and eventually dominant inheritance.[25, 26] X-linked HED (*EDA* gene) is the most common of the three, autosomal recessive HED is much less common than X-linked HED, and autosomal dominant HED is very rare.[18,25, 38] Mutations in some other genes of the EDA signaling pathway also cause an HED phenotype, but one which is associated with some distinctive characteristics.[25] There are well documented cases of affected people without a family history of the disease (sporadic cases) in which no mutation of the *EDA* gene was found, but sequencing revealed a mutation in the *EDAR* gene.[39] The vast majority of spontaneous cases of HED in dogs are sporadic cases, but it is unlikely that any of these was caused by a mutation of the *EDAR* gene or the *EDARADD* gene, since despite the unknown family history all the cases were male dogs and, in two cases, the crossings confirmed that it was XLHED.[7, 40] The autosomal forms affect males and females with the same frequency. However, the possibility of mutations in these genes should not be ignored because, in addition to their occurrence in humans, they are also known to occur in mice and rats.[41-43]

The NGS assay used in this study was targeted sequencing, and it met all the parameters of reads, integrity and coverage necessary to ensure its quality. This kind of assay analyzes a specifically selected region, i.e., the coding regions and splice sites of the *EDA* gene. In this type of assay, the sequencing is generally very deep and very good at detecting single nucleotide variations (SNVs) and indels, but not at detecting copy number variations (CNVs) or structural variations (SVs).[28] Even so, an attempt to detect a CNV was made through a comparative coverage analysis between the affected dog and the

normal dog, but no difference was found. Abnormalities in the splicing mechanisms are not detected by targeted sequencing, and an appropriate type of assay is required (RNA-Seq assay).[28]

In principle, the transition (c.779-1188C>T) that we found in the intron 6 and the transversion (c.*1018T>A) in the 3'UTR region of the affected dog's *EDA* gene should not have any phenotypic effect. However, in humans, numerous deep intronic mutations are known, most of them substitutions, causers of mendelian diseases, including some linked to the X-chromosome.[44, 45] These mutations were located in at least 100 pb of the closest canonical splice site and led to the inclusion of a pseudoexon or disruption of transcriptional regulatory motifs and noncoding RNA genes. The inclusion of a pseudoexon is the most frequent phenomenon and generally involves the creation of a new donor splice site that activates a pre-existing noncanonical acceptor site. A new acceptor site may also be created. Another mechanism is the creation of enhancer elements or the disruption of splicing silencer elements.[44] Any one of these molecular alterations produces an abnormal transcript. However, targeted sequencing does not detect them and another type of NGS assay (RNA-seq assay) is required for this analysis.[28] Mutations in untranslated regions may modify regulatory elements impairing the interaction of the UTRs with proteins and microRNAs. The 3'UTR region plays an important role in the stability, localization and translation of the mRNA. Variants found in this region are often considered polymorphisms, but other ones affecting the stop codon, polyadenylation signal or secondary mRNA structure may dysregulate the translation and have been related to disease.[46,47] Thus, the two mutations that we found could be implicated in the etiology of this case of HED.

Irrespective of the reason, the absence of point mutations and indels in the exons and splice sites shows the importance of investigating other types of mutation. Among the possible causes are CNVs, SVs, mutations in other genes of the EDA pathway, deep intronic mutations and 3'UTR mutations with the two latter being the closest to our results. Abnormalities in the splicing mechanism, large deletions or insertions in the *EDA* gene and structural abnormalities involving the X chromosome should be considered in the XLHED etiology. In the absence of these alterations, it is also necessary to think of autosomal HED,

primarily those that may be caused by mutations in the *EDAR* gene and then those that may be caused by mutations in the *EDARADD* gene.

When clarifying the etiology of HED, in addition to the different NGS assays for DNA (targeted sequencing, whole-genome sequencing, whole-exome sequencing) and RNA (RNA-seq assay) it is convenient to use traditional methodologies such as Sanger sequencing. A G-banding karyotype examination should also be included, as well as molecular techniques such as fluorescent *in situ* hybridization (FISH) and array comparative genomic hybridization (array CGH).

CONCLUSION

Considering the information provided above, the case of hypohidrotic ectodermal dysplasia here reported is probably linked to X chromosome and caused by a deep intronic mutation, 3'UTR mutation or both.

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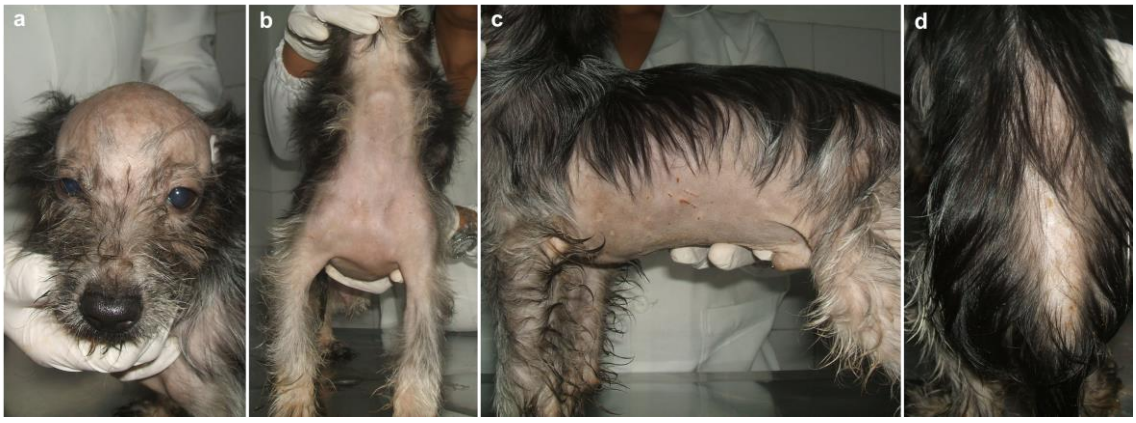

Figure 1. Clinical phenotype of the patient at six months of age. Characteristic distribution of alopecia and hypotrichosis. Skin lightly pigmented. Thin skin sensitive to any kind of friction, including scratching (c).

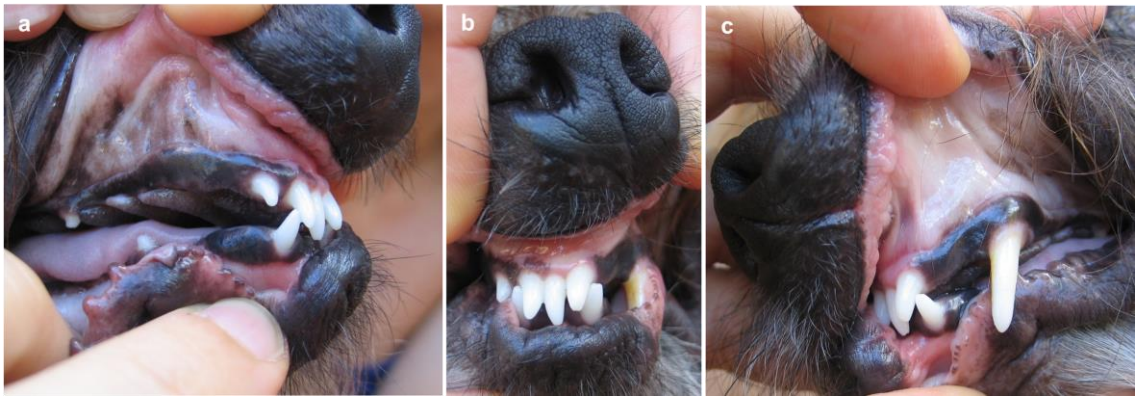

Figure 2. Permanent dentition of the patient at the age of five years. Oligodontia, conical teeth and malocclusion.

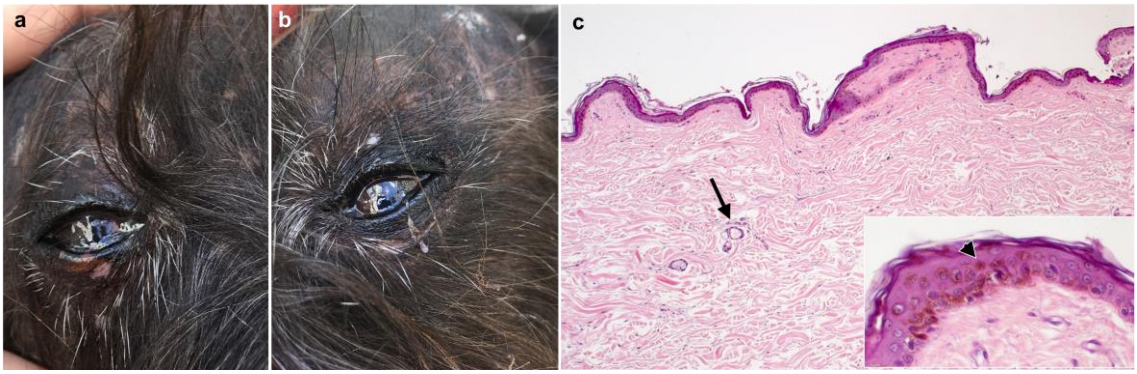

Figure 3. Eyes and skin of the patient at 8 years of age. (a-b) - Right and left eye, respectively, showing signs of severe keratoconjunctivitis sicca. Note the hyperpigmented skin of the frontoparietal region; (c) - Histological section of the skin showing mild orthokeratotic hyperkeratosis and absence of piloglandular units. Vestigial sebaceous glands can be observed (arrow) and collagen fibers are normal. Detail highlighting the abundant deposition of melanin (arrowhead) in the epidermis. Sample obtained from the thorax ventral region (Hematoxylin & eosin stain, x10; detail x63).

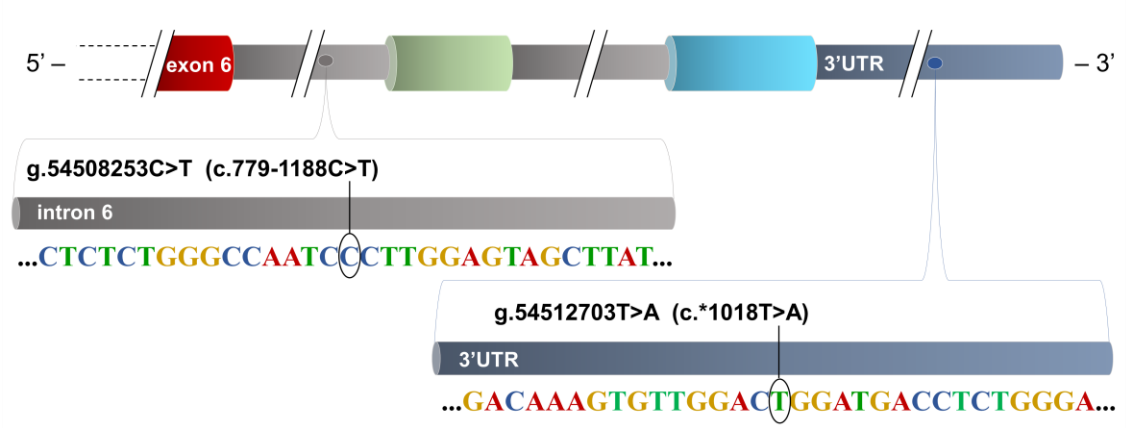

Figure 4. Partial representation of the *EDA* gene showing the location of variants (mutations) detected by NGS. The number preceded by “g.” indicates the position according to the dog’s linear genomic reference sequence (X chromosome, CanFam3.1 NC_006621.3); the number preceded by "c." indicates the position according to coding DNA reference sequence (canine *EDA* gene). At position indicated in the intron 6, the nucleotide C was substituted by T; in the 3'UTR region, the nucleotide T was substituted by A.

CAPÍTULO 3

(Artigo científico aceito para publicação no periódico *Topics in Companion Animal Medicine*)

A hypohidrotic ectodermal dysplasia arising from a new mutation in a Yorkshire terrier dog

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A hypohidrotic ectodermal dysplasia arising from a new mutation in a Yorkshire terrier dog

Abstract

Hypohidrotic ectodermal dysplasias (HED) constitute a group of genetic disorders that affect ectodermal derivatives such as sweat glands, sebaceous glands, hair and teeth. The vast majority of cases of HED is caused by a recessive mutation of the *EDA* gene located in the X chromosome. In these cases, affected individuals are usually male and have alopecia and hypotrichosis with characteristic distribution, in addition to malformed teeth and fewer than normal. From a canine HED isolated case (proband) and in order to verify if this emerged from a new mutation, it was possible to construct a pedigree with five generations and 93 individuals representing an extended and informative family. The proband's mother crossed with two different males and generated 33 descendants in nine gestations: 1 affected male (proband), 15 normal males and 17 normal females, which together can be considered as one sibship. Through Bayesian inference, it was possible to establish that this case originated from a new mutation, with a 99.99% probability of the mother of the proband not being a carrier.

Keywords: Bayesian inference, dog, new mutation, skin genetic disorders

Introduction

Hypohidrotic ectodermal dysplasia (HED) is a clinical condition resulting from abnormal ectodermal development caused by a mutation in genes of the EDA signaling pathway and exhibits abnormalities in the sweat glands, sebaceous glands, hair and teeth. In humans, there are rare autosomal forms due to

mutations in the *EDAR* and *EDARADD* genes, and an X-linked form caused by recessive mutations in the *EDA* gene, which accounts for the vast majority of cases of HED [1]. Mutations in other genes might eventually cause clinical conditions that share features with HED, but also show distinctive characteristics, as occurs in HED-immunodeficiency and odontoonychodermal dysplasia [1, 2]. In dogs, only the X-linked form has been reported so far [3-8].

X-linked hypohidrotic ectodermal dysplasia (XLHED) manifests mainly through defects in sweat glands, sebaceous glands, hair and teeth [9]. Its spontaneous occurrence has been known for a long time in humans and has been reported in cattle, mice and dogs, receiving different names such as anhidrotic or hypohidrotic ectodermal dysplasia (man), hypotrichosis with anodontia (cattle), Tabby phenotype (mice), congenital ectodermal defect, X-linked ectodermal dysplasia and X-linked hypohidrotic ectodermal dysplasia (dogs) [3-8, 10-12]. There is a possible case in a horse and a presumed case in an Eastern Atlantic harbor seal [13,14].

Spontaneous cases of canine XLHED have occurred in males of different breeds [3-8]. In all of these, the affected dogs showed characteristic alopecia and hypotrichosis from the time of birth, with an absence or lower than normal number of piloglandular units in the areas affected by alopecia or hypotrichosis, respectively [3-8]. These areas were pink at birth and became pigmented over time. In addition to the skin abnormalities, they had conoid teeth and hypodontia or oligodontia, in both their deciduous and permanent dentition [3-8, 15,16].

The first report of this dysplasia in dogs, considered as such, was published in 1970 [3], although some previous reports had already described canine cases with the characteristic phenotype under the name of congenital

hypotrichosis [5]. Later, the animals from this first report were crossed and the results suggested recessive X-linked inheritance [17]. The definitive confirmation of the X-linked inheritance pattern did not occur until 1997 [4], when a research colony was founded at the University of Pennsylvania. In 2004, new spontaneous cases of canine XLHED were published, including criteria for clinical diagnosis [5]. In 2005, researchers at the University of Pennsylvania identified that mutation that caused XLHED in the dogs at the colony, confirming that, as in humans, it is caused by mutations in the gene that encodes ectodysplasin A [18]. Ectodysplasin A is a type II transmembrane protein that regulates the mesenchymal-epithelial interactions necessary for the development of the ectodermal derivatives [19]. Later, they conducted post-natal therapeutic trials using recombinant ectodysplasin, with significant results [20-22].

XLHED has been widely studied in humans [10], a species in which a significant number of cases arise from a new mutation (*de novo* mutation) [23, 24]. In dogs, except for a case in which a family analysis led to the conclusion that it originated from a new mutation [4], sufficient data have yet to be published to prove the occurrence of this kind of phenomenon.

In this study, we present an extended family with a spontaneous occurrence of HED in a male Yorkshire terrier dog, and an analysis using Bayesian methodology to verify if this case was caused by a new mutation.

Material and methods

A three-month-old Yorkshire terrier with congenital alopecia/hypotrichosis and dental abnormalities underwent a physical and histopathological

examination to diagnose its clinical condition. In this study, the term alopecia means absence of hair, and hypotrichosis means presence of less hair than normal [25]. It was an isolated case recorded in a breeding kennel and monitored for 9 years. A skin sample was obtained for histopathological examination from the hypotrichotic area in the sacral region. A pedigree was constructed from this dog (proband) using the breeder's records and the results of the crossings were used to calculate the probability of the mother of an isolated case being a carrier using Bayes' theorem in accordance with the following formula [26]:

$$P(A|O) = \frac{P(A) \times P(O|A)}{P(A) \times P(O|A) + P(not\ A) \times P(O|not\ A)}$$

Where the probability of an event A given O is $P(A|O)$; the initial probability (a priori) of an event A occurring is denoted as $P(A)$, and of A not occurring as $P(not\ A)$; the conditional probability of an event O if A occurs is $P(O|A)$; and the conditional probability of an event O if A does not occur is $P(O|not\ A)$.

Results

Clinical phenotype of the proband. Male with congenital alopecia in the frontoparietal region, mediocaudal region of the thoracic and pelvic limbs and throughout the ventral region of the trunk, except in the area corresponding to the caudal half of the thorax where there was hypotrichosis. There was also hypotrichosis in the sacral region and in the pinnae (Fig. 1-A and B). All of these regions were clear at the time of birth but became pigmented over time (Fig. 2-A and B). All over the body there was an absence of secondary hairs and the skin was thin and dry. Deciduous dentition with conoid and spaced teeth (Fig. 1-C).

Permanent dentition with conoid and spaced teeth and oligodontia (only 13 teeth at the age of 5 years). There was retention of deciduous teeth (upper canine and lower incisors) until the age of two years. Presented episodes of pneumonia at the ages of 7 and 8, both of which were treated successfully.

[Figure 1]

Histopathological findings. Absence of piloglandular units in the areas of alopecia or a reduced number of these in the areas of hypotrichosis, where there were also dysplastic hair follicles. Hyperpigmentation, orthokeratotic hyperkeratosis, and normal collagen fibers (Fig. 2-C).

[Figure 2]

Pedigree. The construction of the pedigree resulted in five generations and 93 individuals. The female III-15 crossed with two different males and generated 33 descendants in nine gestations: 1 affected male, 15 normal males and 17 normal females, distributed over nine litters (litters *A* to *I,* of generation III), which together can be considered as one sibship, constituting an informative group (Fig. 3).

[Figure 3]

Bayesian inference. The probability of being a carrier: 0.001%; probability of not being a carrier: 99.99%. The Bayesian analysis of data and its conclusions are shown in Table 1.

Table 1. Bayesian inference

Probability	III-15 is a carrier	III-15 is not a carrier
Prior	½	½
Conditional		
1 affected male	½	$1 - \mu \approx 1$
15 unaffected males	$(½)^{15}$	$(1)^{15} = 1$
Joint	$½ \times ½ \times (½)^{15} = (½)^{17}$	½
Posterior	$(½)^{17} / (½)^{17} + ½ \approx 0.00001 =$ **0.001%**	$½ / (½)^{17} + ½ \approx 0.99999 =$ **99.99%**

DISCUSSION

The clinical phenotype of the proband and the histopathological findings meet the established criteria for a diagnosis of canine XLHED, i.e., the affected animal usually is a male and presents alopecia/hypotrichosis with typical distribution, dental abnormalities, thin and dry skin and, at the microscopic level, aplasia or dysplasia of piloglandular units, according to the examined area [5, 16, 27]. Nevertheless, it cannot be affirmed that this case is X-linked HED because when we gained access to the data that allowed the construction of the pedigree, unfortunately, the proband and its mother were no longer alive, making it impossible to conduct molecular tests to clarify the cause. In any respect, it is more likely to be a case of XLHED because, in humans, the autosomal forms are very rare [1, 28] and in dogs they have never been reported. Furthermore, the Bayesian analysis of our data indicates that the mutation is new. If this mutation were autosomal recessive, two copies would be necessary for the disease to manifest. Consequently, male III-16 would have to be heterozygous. The new mutation could be autosomal dominant, but in humans this is an extremely rare cause of HED [28].

Considering recessive X-linked disorders, when an affected male is born, there are three possibilities for the origin of its disease: its mother is heterozygous (carrier); its mother presents gonadal mosaicism, i.e., the ovaries have normal germinative cells and a percentage of cells with the mutation; or a new mutation occurred during the formation of the egg from which the affected individual came or early in embryogenesis [29]. All of the mutations of the *EDA* gene identified in humans, over 300 of them, are recessive [30]. The few known mutations in the *EDA* gene of other mammals are also recessive, including dogs [6-8, 11,12,18).

For an appropriate understanding of the genetic facts involved in this case, it is useful to recall the theoretical bases of X-linked inheritance (sex-linked inheritance). The *EDA* gene is located in the non-homologous region of the X chromosome, i.e., it is not present in the Y chromosome. As females have two X chromosomes, three genotypes are possible: two normal copies (homozygote and normal), one normal copy and another mutant (heterozygote, i.e., carrier) and two mutant copies (homozygote and affected) [16, 23, 29]. Carriers are normal or present a variable degree of clinical expression of XLHED because of the Lyonization [23]. As males only have one X chromosome, which is inherited from the mother, they are always hemizygote, normal or affected, according to whether they have a normal or mutant copy, respectively [16, 23, 29]. X-linked recessive mutations follow an inheritance pattern with the following characteristics: affected males do not pass the mutation to their male offspring, only to the female, which are carriers (heterozygotes); female carriers crossed with normal males pass the mutation to half of their male offspring (affected) and half of the females, which are carriers [16, 23, 29]. Thus, in families in

which the mutation is being transmitted from one generation to another, the individuals affected by XLHED are male and appear in alternate generations, save in those cases where an affected male is crossed with a carrier. In this situation, females with two copies of the mutation could be born and would be as affected as their father, but because it is a rare disorder, it is highly unlikely that this crossing would occur naturally. It is even more improbable that there would be a crossing of male and female that were both affected [16].

A significant part of the mutations that cause XLHED in humans emerge from new mutations, and the same should occur in canine XLHED, given that human and canine EDA genes are orthologous [23, 24, 31]. Added to this is the fact that reports of canine XLHED are generally of males that are isolated cases in their family history. New mutations emerge for the first time during an event of spermatogenesis or oogenesis of the parents of an individual or early in embryogenesis [32]. In cases of X-linked recessive inheritance and when the affected individual is a male, the latter two possibilities remain, as the father does not pass the X chromosome to his male offspring, only to the females.

Based on the theory of X-linked recessive inheritance, it can initially be admitted that the crossing that originated the proband would be of a carrier with a normal male (III-15 x III-16), because when a normal female has male offspring affected by an X-linked recessive disorder, the first hypothesis is that she is a carrier. However, in the present case, there is no family history of previous cases of XLHED and nor has there been in the siblings of the proband, which leads to the hypothesis of a new mutation.

If we admit that III-15 is a carrier, then the mutation would be transmitted at least since generation I as events of a new mutation in the spermatogenesis

of males I-3 or II-1, or as events of a new mutation in the oogenesis of I-4 or II-2; it could also be that the mutation is not a new mutation and would be transmitted down the maternal line at least from I-4 (I-4 > II-2 > III-15). We can also admit that III-15 is a carrier and that the mutation that originated her is new. With any of these assumptions, in the crossings of female III-15 with males III-14 and III-16, the expected number of affected males should have been 8, but the number observed was only 1. If female II-2 were a carrier, half of the males of generation III (3 out of a total of 6 males) should have been affected, but there are none. If the new mutation had occurred in male II-1 during the event of spermatogenesis that originated its female offspring III-15, she would mandatorily have been a carrier and half of her male offspring (8) should have been affected.

These observations indicate the occurrence of a new mutation during an event of oogenesis of female III-15 or in the early embryogenesis of the proband. The results of the Bayesian analysis are in keeping with this statement. The 99.99% probability of female III-15 not being a carrier corroborates the fact that the HED that affects the proband originated from a new mutation. Whether the mutation occurred in an event of oogenesis of III-15 or in the early embryogenesis of the proband cannot be determined. The possibility of III-15 being a gonadal mosaic is highly unlikely, as if it were so there should have been a recurrence of at least one case in the siblings [33]. Furthermore, in humans, no case of XLHED resulting from this type of mosaicism has ever been documented [34].

The Bayesian approach has been widely used in genetic counseling and is especially useful for determining the risk of a female being a carrier when in her

family there is an isolated case of a hereditary X-linked recessive disorder and for calculating the risk of recurrence of this disorder [26, 33]. In the present case, the a priori probability of the female being a carrier is equal to the probability of her not being one. In other words, there is a ½ probability of her being a carrier while the likelihood of her not being a carrier is also ½. From these probabilities, the calculations (Table 1) lead to a final (*a posteriori*) probability of 0.001% of III-15 being a carrier, and consequently 99.99% of her not being. The mutation rate (μ) estimated for different X-linked diseases varies from 1×10^{-5} to lower values [33]. As these values are extremely low and do not influence the result of the calculation, the mutation rate was not taken into account.

To avoid long calculations that would lead in the end to the same conclusion, individuals from generations I and II were not included in the Bayesian analysis; nor were the other individuals from generation III and their descendants (generations IV and V), all of whom were normal. If these data had been incorporated, the probability of III-15 not being a carrier would be even greater than the value obtained (99.99%). However, it would be unnecessary, given that this value has practically reached 100%. For the same reason, no calculations were made supposing that it was a case of autosomal dominant HED. In this hypothesis, all nine females resulting from the crossing of III-15 x III-16 should be included, in addition to the normal males, bringing the results even closer to 100%. In the hypothesis of autosomal recessive inheritance, the results would not be different.

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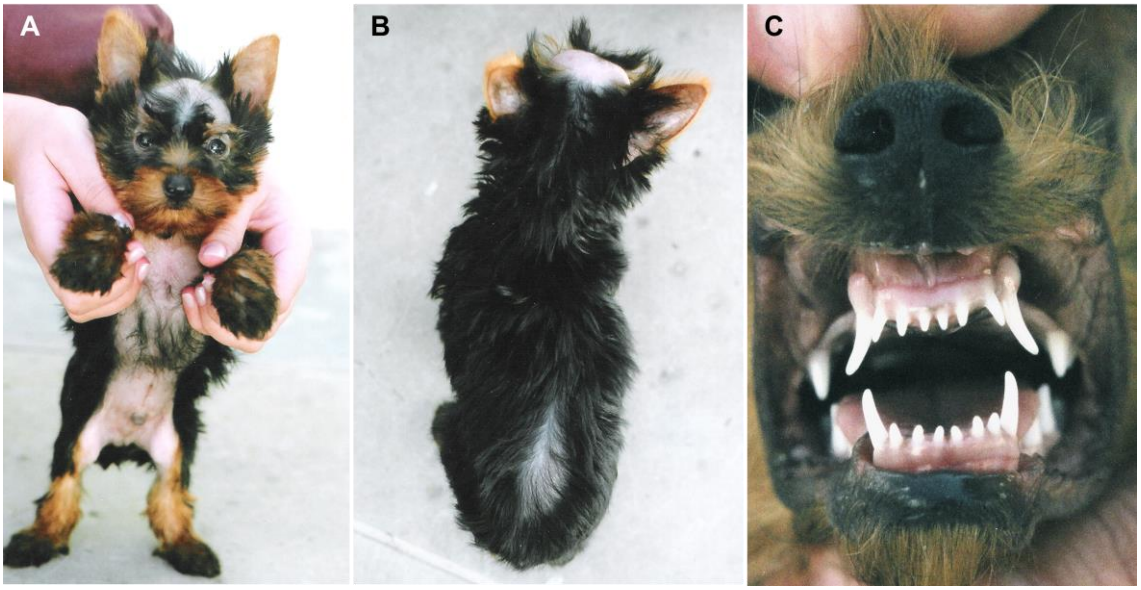

Fig. 1. Clinical phenotype of the proband. A and B) Characteristic distribution of the areas of alopecia and hypotrichosis. C) Deciduous dentition at 3 months of age showing malformed and abnormally spaced teeth.

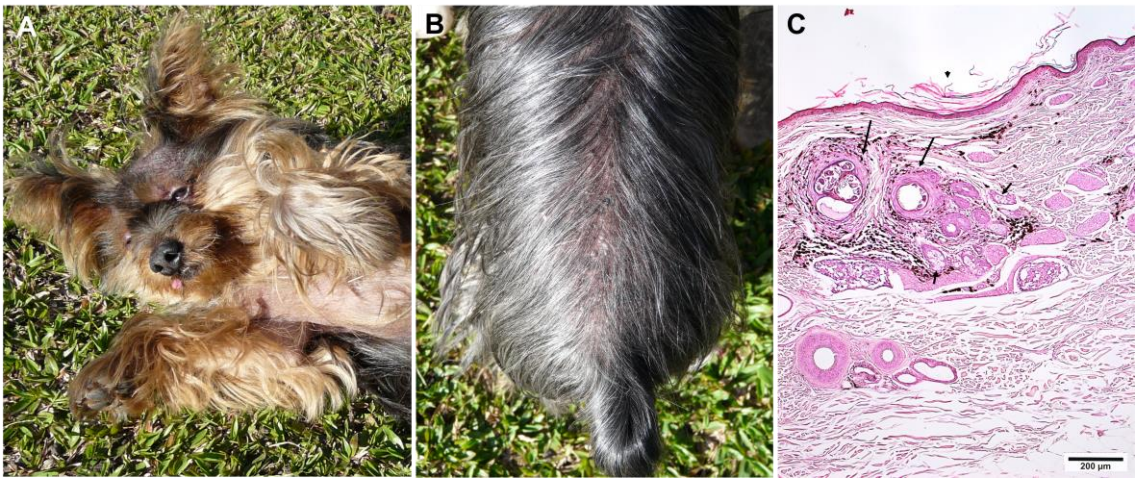

Fig. 2. Proband at 3 years of age. A) Hyperpigmented skin, principally in the region of the head (compare with Figure 1); note the tip of the tongue protruding on the right side of the mouth due to the lack of teeth, which was more severe on this side (oligodontia). B) Hypotrichotic and hyperpigmented sacral region. C) Histological section (sample obtained from the region shown in B) showing discrete hyperkeratosis (arrowhead), reduced number of piloglandular units, abundant melanin deposition, dysplastic hair follicles (long arrows), and sebaceous glands near to incompletely developed follicles (short arrows). Hematoxylin & eosin.

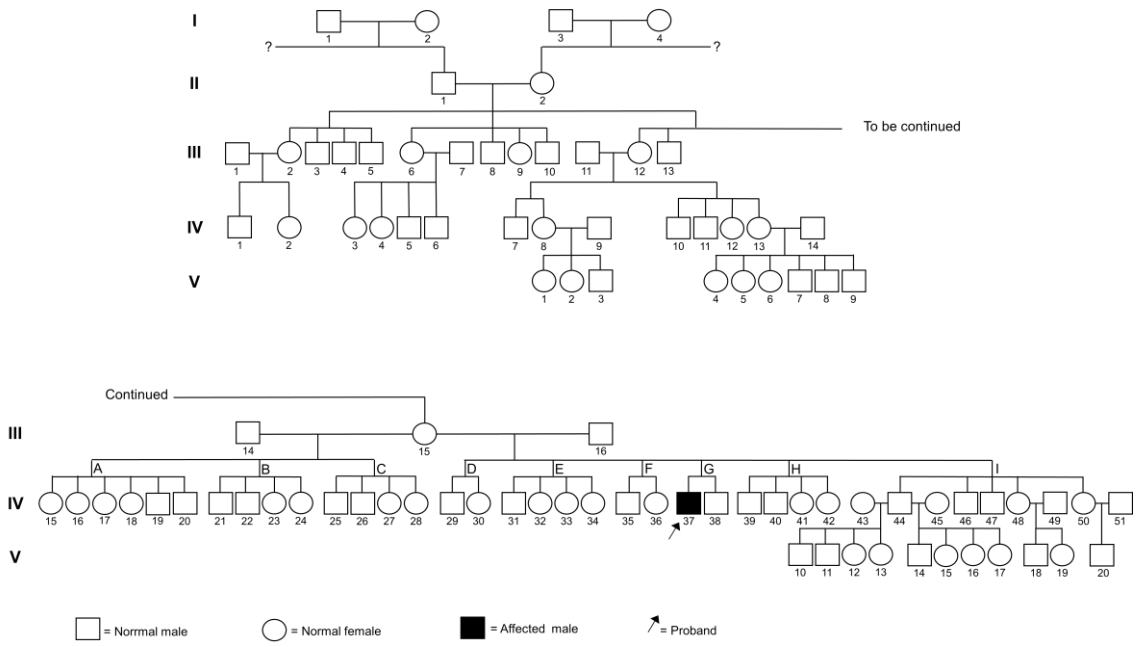

Fig. 3. Pedigree. It should be noted that there is no other case in addition to the proband in all the generations.

CAPÍTULO 4

CONSIDERAÇÕES FINAIS

O surgimento de ferramentas de amplificação e análise de DNA, como a reação em cadeia da polimerase (PCR) e, mais recentemente, as técnicas de sequenciamento massivo paralelo (NGS) garantiram um significativo aumento dos estudos de genética e genômica animal em várias partes do mundo. A clínica e a genética andando juntas mostraram que doenças antes identificadas apenas em humanos ocorrem também em animais. O cão doméstico em particular tornou-se um modelo espontâneo dessas doenças em razão da elevada similaridade genômica com a espécie humana e por compartilhar com ela os mesmos ambientes (Shearin e Ostrander, 2010; Switonski, 2014). A displasia ectodérmica hipoidrótica ligada ao X é um dos muitos exemplos dessas doenças. Os estudos com camundongos e cães resultaram em uma estratégia de tratamento para a mesma doença em humanos, mostrando que qualquer que seja a doença genética ou defeito congênito, o que se aprende com uma espécie pode beneficiar a outra (Moura e Pimpão, 2017).

Apesar de a DEHLX ser uma doença rara, a escassez de casos talvez esteja refletindo também a falta de diagnóstico e não apenas a raridade da doença. Por isso, é importante a divulgação das características clínicas e genéticas para torná-la mais conhecida dos clínicos em geral e, assim, ampliar o número de casos e facilitar a realização de novos estudos.

O fato de um caso de DEHLX canina não apresentar nos éxons ou nas regiões de *splice* nenhuma mutação de ponto nem indels (Capítulo 2), alerta para a existência de outras causas, tais como grandes deleções, inserções, anormalidades cromossômicas estruturais, mutações intrônicas profundas e mutações de regiões não traduzidas, além de anormalidades no *splicing*. Alerta também para a possibilidade de que alguns casos com fenótipo de DEHLX sejam, na realidade, de displasia ectodérmica autossômica causada por mutações no gene *EDAR* ou no gene *EDARADD*. A demonstração estatística de ocorrência de mutação nova (Capítulo 3) reforça a hipótese de que, como nos casos humanos, grande parte dos casos caninos tem origem por mutação nova.

Mais estudos devem ser realizados para que a etiologia da DEHLX possa ser totalmente esclarecida. Os diferentes fenômenos envolvidos poderão ser conhecidos por meio das atuais ferramentas moleculares associadas com as tradicionais. É conveniente utilizar além dos diferentes ensaios de sequenciamento de nova geração para DNA (targeted sequencing, whole-genome sequencing, whole-exome sequencing) e RNA (RNA-seq array), as metodologias tradicionais como o sequenciamento Sanger e exames de cariótipo com bandeamento G. Técnicas de citogenética molecular como a hibridização *in situ* fluorescente (FISH) e microarranjos de hibridização genômica comparativa (CGH-assay) também devem ser utilizadas.

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ANEXOS

ANEXO 1

Veterinary Dermatology - Guidelines

1. GENERAL

Veterinary Dermatology is a bi-monthly, peer-reviewed, international journal which publishes papers on all aspects of the skin of mammals, birds, reptiles, amphibians and fish. Scientific research papers, clinical case reports and reviews covering the following aspects of dermatology will be considered for publication:

Skin structure (anatomy, histology, ultrastructure)
Skin function (physiology, biochemistry, pharmacology, immunology, genetics)
Skin microbiology and parasitology
Dermatopathology
Pathogenesis, diagnosis and treatment, including prophylaxis, of skin diseases
New disease entities
Please carefully read the instructions below for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in Veterinary Dermatology. Authors are encouraged to visit the Blackwell Publishing Author Services site (http://authorservices.wiley.com) for further general information on the preparation and submission of articles and figures.

2. ETHICAL GUIDELINES

Veterinary Dermatology adheres to ethical guidelines given below for publication and research.

2.1. Authorship and Acknowledgements

Veterinary Dermatology adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE criteria authorship should be based on:

Substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, and
drafting the article or revising it critically for important intellectual content and
final approval of the version to be published.
All of the authors should meet conditions 1, 2 and 3.

Participation solely in the acquisition of funding or the collection of data (such as recruiting cases in multi-centre drug trials) does not justify authorship and, except in the case of complex large-scale or multi-centre research, the number of authors should usually not exceed six. It is a requirement that all authors have been accredited as appropriate upon submission of the manuscript. Contributors who do not qualify as authors should be mentioned under Acknowledgements, e.g. statisticians hired to analyse data; this may also include clinicians who recruit cases for multi-centre clinical trials.

Note to NIH Grantees: Pursuant to NIH mandate, Wiley-Blackwell will post the accepted version of contributions authored by NIH grant-holders to PubMed Central upon acceptance. This accepted version will be made publicly available 12 months after publication. For further information, see www.wiley.com/go/nihmandate

2.2. Ethical Approvals

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

The Journal reserves the right to reject any paper where there is reason to believe that animals have been subjected to unnecessary or avoidable pain or distress. Where animals have been used in a study, the relevant research ethical or animal welfare or institutional review authority, under which the work was conducted, must be stated. Furthermore, manuscripts describing prospective studies involving client-owned animals should also include documentation of informed client consent.

2.3 Clinical Trials

Randomised clinical trials (RCTs) and systematic reviews should be reported with due regard for the REFLECT guidelines available at http://www.reflect-statement.org/statement/ .

2.4 DNA Sequences and Crystallographic Structure Determinations

Papers reporting protein or DNA sequences will not be accepted without a Genbank accession number. Other supporting data sets must be made available on the publication date from the authors directly.

2.5 Conflict of Interest and Source of Funding

Conflict of Interest: Authors are required to disclose any possible conflict of interest, this may include financial support including consultancies, speaker's fees; any gift, income, funding or other material benefit, unsolicited or otherwise, from a commercial company or individual, even if it was not restricted to the project described in the submission. If in doubt please ask the editor for guidance about declaring a possible conflict. If the author does not include a conflict of interest statement in the manuscript then the following statement will be included by default: 'No conflicts of interest have been declared'.

Sources of funding: sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged.

The “conflict of interest” and “sources of funding” statements should be included immediately before the abstract section of your manuscript.

2.6 Appeal of Decision

Authors who wish to appeal the reviewers' decision and/or comments on their submitted paper may do so by e-mailing the editor with a detailed explanation for why they find reasons to appeal the decision.

2.7 Permissions

If the whole or part(s) of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers. Images that have been sold commercially should not be submitted for publication.

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2.9 Committee on Publication Ethics (COPE)
VDE is a member of, and subscribes to the principles of, the Committee on Publication Ethics (COPE) .

3. SUBMISSION AND ACCEPTANCE OF MANUSCRIPT

Manuscripts should be submitted electronically via the online submission site http://mc.manuscriptcentral.com/vde .

If assistance is needed (or if, for some reason, online submission is not possible) the Editorial Office can be contacted at VDEedoffice@wiley.com and will readily provide any help users need to upload their manuscripts.

4. MANUSCRIPT TYPES ACCEPTED

Papers are invited in the following categories: Reviews, Scientific Papers, Brief Communications, Case Reports, Letters to the Editor and Book Reviews (by invitation from the journal).

1. Reviews are by invitation from the journal or with approval from the Editor in Chief.
2. Scientific Papers are experimental or observational, ideally hypothesis-driven prospective studies.
3. Brief Communications are brief reports and will be limited to 1500 words (based on the text and not counting the words in the title, abstract and references) and up to four figures or tables or graphs. References should be limited to twelve.

For some articles the editor may require that the brief communication is reduced to less than 750 words, two figures and six references; the editor will annotate the abstract to several key points.

4. Case Reports (or case series) will be limited to 1500 words (based on the text and not counting the words in the title, abstract and references) and up to four figures. References should be limited to twelve. They will be considered for publication primarily if they add new and useful information for the discipline of veterinary dermatology; consideration will be given to:

a. Reports of new diseases or conditions, or variations on recently described diseases.

b. Reports of diseases that are of zoonotic importance or are highly contagious.

c. Reports that will make a significant change in how a disease is diagnosed or treated.

Supporting information containing additional text and figures may be allowed.

The editors may require that a case report is reduced to less than 750 words; two figures and six references; the abstract will be reduced to several key points by the editor.

5. Letters to the Editor: will be limited to 750 words, including references, and two (usually one) image / figure / table which may include one clinical image and one of histopathology; letters may cover a variety of topics and these may include but are not restricted to:

a. Briefly highlighting an issue with a previously published paper.

b. Seeking to generate discussion or awareness of a developing area.

6. Books for review should be sent to the Veterinary Dermatology Editorial Office at:

Veterinary Dermatology, Wiley-Blackwell, 9600 Garsington Road, Oxford, OX4 2DQ, UK

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Veterinary Dermatology operates a system of double-blinded review and the names of the authors will not be disclosed to the reviewers. Authors should therefore avoid including anything that could identify them within the text. This, for example, includes: the name of the institution at which the work was performed; initials of the authors; acknowledgements and names of institutions on illustrations, etc. To enable double-blinded review, contributors (including acknowledgements) should only be named on the title page or uploaded separately as a supplementary file, and not on the manuscript. Authors should also avoid statements that could identify them through references (e.g. instead of 'we have previously shown that black is white', authors should write 'previous studies have shown that black is white').

The manuscript (including references and figure legends) must be A4 or 8.5 x 11 inch format with 2.5 cm margins, single-spaced typed (please do not submit double line spaced), align text left, 12 point font using sans serif typeface such as Helvetica (Swiss), Arial or Verdana style (please do not use Times New Roman). Each line and page of the manuscript text should be numbered consecutively from the title page.

Authors are requested to write with the minimum of formatting and NOT to write over previous versions, which may contain hidden formatting. Do not enhance text and tables with unnecessary formatting (e.g. small capitals, headers). Software programmes that automatically create endnotes and footnotes should not be used.

Review Articles

In general, review articles are only by invitation and by approval of the Editor in Chief. The structure will vary depending on content. Authors should study the format used in previous issues of the journal for further guidance. Authors wanting to submit a review article should contact the Editor in Chief (via VDEedoffice@wiley.com) with a brief description of the article and outline.

Scientific Papers and Brief Communications

Manuscripts should be arranged as follows: title; acknowledgements; abstract; text with subdivisions as given below; references; legends for illustrations.

Case Reports

These are usually a chronological description of the case describing the history, physical findings, differential diagnoses, diagnostic tests, specialist diagnostic procedures, diagnoses, treatments and outcome.

Title Page

The title of the article should be concise but informative. Drug trade names will not be included in the title. The first name, middle initial(s), and last name of each author must be given. Professional affiliations of the authors at the time of the study should be indicated using the symbols *, †, ‡, §, ¶, then **, †† etc., in this order; these are not superscripts. Titles (e.g. professor) and qualifications (e.g. DipACVD) are not required.

Please provide full address details for all of the authors. If an author's affiliation has changed since the study was performed, the author's new affiliation should be identified. The name of the corresponding author, any conflicts of interest and sources of funding (see section 2.5) should be stated. If information in the text has been presented at a scientific meeting, this should be indicated. A short running title of no more than 40 characters (counting letters and spaces) should also be included. The short running title will be used in the journal at the top of the page, see current publications. Keywords are NOT required.

Abstract

The abstract should be no more than 250 words and must be constructed using the subheadings given below. While this format is most appropriate for scientific

studies, the authors of reviews, brief communications and case reports are encouraged to also provide a structured abstract using the following:

Background – A brief explanation of why the study was performed.
Hypothesis/Objectives – A statement of the principal hypothesis tested in the study, a brief statement of the major objectives, or both.
Animals – A concise description of the number of animals used in the study including the population from which they were drawn (e.g. research colony, hospital population) and any special characteristics of the animals (e.g. disease status).
Methods – A statement of overall study design (e.g. randomized, blinded, placebo-controlled clinical trial; retrospective study) and principal interventions or methods.
Results – Concise statement of important results including numerical description of critical variables and statement of statistical significance.
Conclusions and clinical importance – A summary of conclusions based on results of the study and statement of clinical importance of these conclusions. The results should not be restated.
Drug trade names will not usually be included in the abstracts

Introduction

State the purpose of the article. Summarize the rationale for the study or observation. Give only strictly pertinent references and do not review the subject extensively.

Materials and Methods

These should be described in sufficient detail to allow other workers to reproduce the results. References for study design and statistical methods should be to standard works (with pages stated) when possible rather than to papers where designs or methods were originally reported. Specify any statistics computer programs used. Report losses to observation (such as dropouts from a clinical trial).

The methods of data collection and use of statistical analysis will be checked by the referees, editors and, if necessary, a statistician. It is highly recommended that authors consult a professional statistician for advice on complex statistical analyses. It is also recommended that authors provide details of which statistical methods and the P-value, if relevant, have been used for each component of the data set (e.g. P = 0.08; ANOVA).

Drugs and therapeutic agents should be given in the format: drug ingredient (trade name; manufacturer name, city, (state), country), e.g. fenbendazole (Panacur; Intervet-Schering Plough, Milton Keynes, UK).

Drug names should follow the recommended International Non-Proprietary Names (rINN). Common examples include cefalexin, ciclosporin, meticillin and rifampicin.

Products such as equipment or methods should be given as: Product name (Company name; town or city, (state) and country); e.g. Datex CD 200-02 (Datex; Hatfield, UK); or SuperScript® III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA). The detailed information about drugs, therapeutic agents and products need only be given once.

Results

Present your results in a logical sequence in the text, tables and illustrations. Do not repeat in the text data in the tables or illustrations. In manuscripts describing more than one animal, all animals should be assigned a case number.

Discussion

The discussion should emphasize the new and important aspects of the study and the conclusions that follow from them. Include the implications of the findings and their limitations, including implications for future research. Relate the observations to other relevant studies. Link the conclusions with the goals of

the study but avoid unqualified statements and conclusions not completely supported by your data. Avoid claiming priority and alluding to work that has not been completed. State new hypotheses when warranted, but clearly indicate them as such.

Recommendations , when appropriate, may be included.

Acknowledgements (should be made on the title page or as a separate supplementary file and not included on the manuscript). These are to indicate support, advice or technical help that does not justify authorship. Please use first and second (family) names, e.g. The authors would like to thank Fred Flintstone for assistance with statistical analysis.

Funding sources should be included in the declared sources of funding (see section 2.5).

Language and style

The language of publication is English. Authors for whom English is a second language must have their manuscript thoroughly, and preferably professionally, edited by an English speaking person before submission to make sure that the English is of high quality. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp . All services are paid for and arranged by the author; use of one of these services does not guarantee acceptance or preference for publication.

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All units of measurement must follow the SI system. Concentrations of solutions should be given as molar concentrations (e.g. mmol/L). All other concentrations should be expressed as percentages. Drug dosages should be given as: e.g. mg/kg; µg/kg; also use ‘once daily’, ‘twice daily’ etc. Spell out numbers one to nine, keep 10 onwards as numerals. However, use Arabic numerals for numbers used with units of measure (e.g. 9 kg, 5 h, 10 mmol/L). Use h, min, s, for hour, minute, second, respectively. Abbreviations of biological, medical,

chemical and other terms should be used only when such abbreviations are both internationally recognized and unambiguous. The first use of an abbreviation must be explained by also giving the unabbreviated term.

All biological, medical, chemical and other names should be given in keeping with the latest international nomenclature. If an animal or micro-organism is being mentioned in the text for the first time, the binomial name should be given, e.g. carp (Cyprinus carpio). Thereafter, this can be abbreviated to C. carpio. Please check recent articles for information about the spelling of dog and cat breeds.

5.3. Figures (illustrations): Graphs, Tables, Clinical Photographs and Photomicrographs

Figure legends must be given at the end of the manuscript. Sufficient information should be included to allow the figure to be understood without reference to the text. Authors wishing to use any previously published figures must submit written permission from the copyright holder. Figure legends should be written in the following style:

1. Organ or tissue; animal identification, Case No. A sentence describing the change that is visible in the print. (For photomicrographs add: staining method with names of stains and counter stains and magnification, e.g. avidin–biotin–peroxidase complex method, Mayer's Haematoxylin counter stain, x40).

2. Graph or Table: statement of how data is expressed. Identification of symbols in table, graph, or photo: e.g. N, nucleus.

Examples:

1. Photomicrograph: Intra-epidermal, intact sub-corneal pustule showing small numbers of acantholytic cells and numerous neutrophils. H&E.

2. Table: Comparison of eosinophil counts over time between the control and treatment groups. Error bars indicate the mean ± SD.

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To ensure high-quality reproduction, symbols should be clear and even throughout and of sufficient size, that when reduced for publication, each item will still be legible. Graph axes should be labelled in sans serif (Helvetica or Arial) font. Letters, Numbers and Titles belong in the legends for illustrations, not on the illustrations themselves.

Software programmes such as GraphPad can be used to download graphs into a word document for submission as an image/figure file.

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5.3.3. Figures; Clinical Photographs & Photomicrographs (histopathology)
Such figures should be ideally be originally captured and submitted in a neutral data format such as TIFF or EPS. There is no requirement to convert an original uncompressed JPEG file to TIFF or EPS format. (JPEG format will be accommodated but must fulfil the format criteria given below and should be uncompressed). PowerPoint, PDFs and Word graphics are usually unsuitable for reproduction.

Figures should have a minimum resolution of 300 dpi; grey tone and line drawings require 600–1200 dpi. Photographic material should be of such quality that high-contrast that reproductions can be made. Poor-quality images may be

removed from a manuscript and where critical to the content may lead to rejection of a manuscript.

Graphics created in the CMYK colour palette (print colours) are preferable to those created in RGB (screen colours) to maximize the consistency of print reproduction. Images supplied in RGB will be converted to CMYK for printing; this may lead to some variations in colour representation. Immunofluorescence images may be submitted in RGB.

When symbols, arrows, numbers or letters are used to identify parts of the illustrations, identify and explain each one clearly in the legend.

Clinical and histopathology (photomicrograph) figures must be no more than 19 cm in width and must be submitted at a resolution of 300 dpi.

Limit figures to those that reduce or clarify the text. These should be free of extraneous material and, where possible, if portions of the handler such as fingers or hands are to be included, particularly adjacent to lesions, they should be gloved.

Montage (composite plates) figures are allowed and should have no tooling (space bars) to separate the individual images. Each part should be labelled in the top left-hand corner in black, (or if appropriate for clarity in white) in Arial, in lower case, with no brackets, starting with a, b, c, etc.

5.3.3.1 Photomicrographs

The microscope must be set up for Koehler illumination, so that the light is evenly dispersed in the image.

The epidermis should be at the top of the image and horizontal; the background above the epidermis should be bright and white.

Authors are not obliged to use length or scale bars, reviewers/editors may recommend their use because it is deemed to be critical to the understanding of

photomicrograph; they are required for electron micrograph images. Magnification (scale) bars should be black, approximately 1 cm long and placed in the lower right corner, 5 mm above the lower margin and with the right end 5 mm from the right margin. The overall magnification can be specified as, for example, (x40) where x10 eye lens x x4 lens = x40 overall.

For more information about the preparation of images please see the guidelines as attached here . While these guidelines were written some time ago and technology has moved on the general principles are still applicable. Please ensure that individual figures files are no larger than 5 MB. If your file is substantially bigger than this, please contact the Editorial Office: VDEedoffice@wiley.com; to discuss file saving and submission options.

5.4. References (Please note that EndNote™ and Refman™ software for the Journal of the American Veterinary Medical Association can be used for Veterinary Dermatology; please adapt to Vancouver style).

Software programs for creating reference lists may be used but they should be set up so that they generate in-text citations and reference lists according to the instructions and examples given below. Authors bear primary responsibility for the accuracy of all references. References must be limited to those that are necessary and must be cited in the text by superscript numbers in order of citation. Journal titles in the Reference section should be abbreviated in accordance with the National Library of Medicine (NLM website) and Index Medicus. For references with more than 3 authors, only the first 3 authors should be listed, followed by ‘ et al.’ The following is the style used for common types of references:

Article in journal

1. Müntener T, Doherr MG, Guscetti F et al. The canine hair cycle – a guide for the assessment of morphological and immunohistochemical criteria. Vet Dermatol 2011; 22: 383-395.

Book

2. Scott DW. Large Animal Dermatology. Philadelphia, PA: Saunders, 1988; 457–458.

Book chapter

3. Muir P, Johnson KA, Manley PA. Fractures of the pelvis. In: Birchard SJ, Sherding RG, eds. Saunders Manual of Small Animal Practice. 2nd edition. Philadelphia: W.B. Saunders Co., 2000; 1126–1132.

Proceedings

4. Kunkle G, Hillier A, Beale K et al. Steroid effects on intradermal skin testing in sensitized dogs. In: Proceedings of the American Academy of Veterinary Dermatology & American College of Veterinary Dermatology . Charleston, SC, USA: 1994; 54–55.

Electronic Material

5. Animal and Plant Health Inspection Service website. Bovine spongiform encephalopathy (BSE). Available at: www.aphis.usda.gov/lpa/issues/bse/bse.html. Accessed Feb 18, 2003.

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Animal experiments are to be undertaken only with the purpose of advancing knowledge and in a manner that avoids unnecessary discomfort to the animals by the use of proper management and laboratory techniques. They shall be conducted in compliance with federal, state and local laws and regulations, and in accordance with the internationally accepted principles and guidelines for the care and use of agricultural, laboratory or experimental animals. In the interests of the reproducibility of results, accurate information about any test animals used in the experiments (origin, inbreeding etc.), as well as information about the housing conditions (diet, environment etc.), should be given. For further information and guidance on how to report on animal experiments see: ARRIVE guidelines for reporting animal research see: ARRIVE guidelines for reporting animal research

6. AFTER ACCEPTANCE

Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor who is responsible for the production of the journal.

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The corresponding author will receive an e-mail alert containing a link to a website. A working e-mail address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site; the file should be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs. Proofs must be returned to the Production Editor within one week of receipt.

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ANEXO 2

Topics in Companion Animal Medicine - Guidelines

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Topics in Companion Animal Medicine is dedicated to providing the practitioner with the most recent advances in companion animal medicine. Each quarterly issue includes a comprehensive review of the latest developments and techniques regarding an important topic in veterinary medicine, guest edited by a leading expert in the field. The Journal also features peer-reviewed original research articles, case reports and review articles; as well as timely editorials addressing issues that affect the companion animal practitioner.

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3. Case Reports
4. Short Communication
5. Letters to the Editor

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ANEXO 3

Parecer de aprovação do CEUA

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação
Comitê de Ética em Pesquisa no Uso de Animais

Curitiba, 01 de Junho de 2016.

PARECER DE PROTOCOLO DE PESQUISA

REGISTRO DO PROJETO: **01039/2016 – 1ª versão**

TÍTULO DO PROJETO: Análise do gene EDA em cães afetados pela displasia ectodérmica hipoidrótica ligada ao (DEHLX)

PESQUISADOR RESPONSÁVEL: Enio Francisco Moura

EQUIPE DE PESQUISA: Claudia Turra Pimpão

INSTITUIÇÃO

Pontifícia Universidade Católica do Paraná

ESCOLA / CURSO:

Escola de Ciências da Vida / Medicina Veterinária

VIGÊNCIA DO PROJETO	07/2016 a 07/2017	**QUANTIDADE DE ANIMAIS**	Peças anatômicas
ESPECIE/LINHAGEM	*Cannis lupus familiaris*	**Nº SISBIO** (Somente animais de vida livre)	Não se aplica
SEXO	Não se aplica	**ATIVIDADES** (Somente animais de vida livre)	Não se aplica
IDADE / PESO	Não se aplica	**ESPECIÉ – GRUPO TAXONÔMICOS** (Somente animais de vida livre)	Não se aplica
ORIGEM DO ANIMAL	Pesquisas anteriores / arquivo pessoal	**LOCAL (IS)** (Somente animais de vida livre)	Não se aplica

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Atenciosamente,

Prof. Dra. Marta Luciane Fischer
Coordenadora - Comissão de Ética no Uso de Animais

Comissão de Ética no Uso de Animais PUCPR

Rua Imaculada Conceição, 1155 Prado Velho CEP 80.215-901 Curitiba Paraná Brasil
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ANEXO 4

Lista de figuras

ANEXO 5

Lista de abreviaturas

Obs.: Genes aparecem no texto/figuras em itálico. Proteínas recebem a mesma abreviatura, porém, sem itálico.

A = adenine/adenine
AG = adenina, guanina/ adenine/guanine
CGH = comparative genomic hybridization
CNV = copy number variations
DEH = displasia ectodérmica hipoidrótica
DEHLX = displasia ectodérmica hipoidrótica ligada ao X
DNA = deoxyribonucleic acid (ácido desoxirribonucleico)
EDA = ectodysplasin/ ectodisplasina A.
EDAR = EDA receptor.
EDARADD = EDAR associated death domain.
Fc-EDA – Ectodisplasina recombinante (fração de imunoglobulina e EDA)
FISH = fluorescent *in situ* hybridization
FOXI3 = forkhead box I3
FZD = frizzled class receptor
HED = hypohidrotic ectodermal dysplasia
IKK = inhibitor of nuclear factor kappa-B kinase
LEF1 = lymphoid enhancer binding factor 1
LRP5/6 = LDL receptor related protein 5/6
NF-kB = nuclear factor kappa B
RNA = ribonucleic acid (ácido ribonucleico)
SHH = sonic hedgehog
SNV = single nucleotide variation
SRD = Sem raça definida.
SV = structural variation
T = thymine/timina
TAB2 = TGF-beta activated kinase 1 binding protein 2
TRAF6 = TNF receptor associated factor 6

TRAK1 = trafficking kinesin protein 1

TNF = tumor necrosis factor

UTR = untranslated region

WNT = wingless type

XLHED = X-linked hypohidrotic ectodermal dysplasia