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ESTUDO DO TRANSCRIPTOMA RELACIONADO À OCORRÊNCIA DE HÉRNIAS UMBILICAIS EM SUÍNOS

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ESTUDO DO TRANSCRIPTOMA RELACIONADO À OCORRÊNCIA DE HÉRNIAS UMBILICAIS EM SUÍNOS

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ESTUDO DO TRANSCRIPTOMA RELACIONADO À OCORRÊNCIA DE HÉRNIAS UMBILICAIS EM SUÍNOS

Elaborada por
MAYLA REGINA SOUZA

como requisito parcial para obtenção do grau de
Mestre em Zootecnia

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Chapecó, 30 de julho de 2019.

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"Eu sei que os sonhos são pra sempre
Eu sei aqui no coração
Eu vou ser mais do que eu sou
Para cumprir as promessas que eu fiz
Porque eu sei que é assim
Que os meus sonhos dependem de mim"
(*Marina Elali e Dudu Falcão*)

RESUMO
Dissertação de Mestrado
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

ESTUDO DO TRANSCRIPTOMA RELACIONADO À OCORRÊNCIA DE HÉRNIAS UMBILICAIS EM SUÍNOS

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Chapecó, 30 de julho de 2019

A hérnia umbilical (HU) é caracterizada como uma protusão anormal do conteúdo abdominal para o exterior da cavidade na região do umbigo, que ocorre geralmente por um defeito no músculo ou no fechamento do anel umbilical. Os animais afetados por esta anomalia possuem menores taxas de crescimento, pior conversão alimentar e maior índice de morbidade. Apesar de alguns estudos terem sido desenvolvidos para identificar regiões cromossômicas relacionadas a essa condição, nenhum trabalho envolvendo o sequenciamento de RNAm, que possibilita a descoberta de genes, foi realizado. Como a frequência de HU ainda é um problema nos rebanhos e sua etiologia permanece desconhecida, este estudo tem como objetivo identificar genes relacionados a esse defeito em suínos utilizando análise de transcriptoma. Para isto, 10 fêmeas Landrace, cinco normais e cinco afetadas com hérnia umbilical, adquiridas de uma mesma granja núcleo, com aproximadamente 90 dias, foram utilizadas. A necropsia foi realizada na Embrapa Suínos e Aves onde foram coletados tecidos da região umbilical dos animais herniados e não herniados e imediatamente colocados em nitrogênio líquido para posterior extração do RNA e preparo das bibliotecas de RNA-Seq. As amostras foram submetidas ao sequenciamento em HiSeq2500 Illumina (2x100pb). O controle de qualidade (QC) das sequências foi realizado com o Seqyclean, as *reads* foram mapeadas contra o genoma suíno v11.1 usando o STAR e contadas usando HTSeq. A análise de expressão diferencial foi realizada com o EdgeR e a validação de um conjunto de genes diferencialmente expressos (DE) foi feita por qPCR. As análises funcionais foram realizadas com a base de dados DAVID 6.8. Uma média de 21,7 milhões de *reads*/amostra foram obtidas após o QC, compreendendo 13.216 genes expressos no tecido herniário. Um total de 230 genes foi DE entre animais normais e afetados (FDR <0,05). Destes, 145 foram menos expressos e 85 mais expressos nos animais afetados em relação aos normais e, de acordo com a análise funcional, a maioria deles está relacionada a sistema imune, desenvolvimento anatômico, adesão celular e matriz extracelular. Seis genes DE: *ACER2, SLC2A6, PTGS1, LGALS3, KANK3* e *FOS* foram identificados em regiões de QTL previamente associadas a hérnia umbilical. Dos genes DE chama-se atenção para os novos genes candidatos *ACAN, MMPs*, *EPYC* e *VIT*, que atuam na remodelação da matriz extracelular e na produção, integridade e resistência do colágeno. Já o *CCBE1* atua nas ligações de cálcio e colágeno e já foi associado ao aparecimento de hérnias umbilicais em humanos. Acredita-se que o desbalanceamento da expressão desses genes possivelmente candidatos para esta anomalia, causa flacidez, enfraquecimento e lesão do tecido, podendo levar a formação de HU. Este estudo contribui para o maior conhecimento dos fatores genéticos envolvidos na ocorrência da HU, porém, mais estudos são necessários para confirmar a atividade desses genes como causa do problema e também melhorar a compreensão dessa condição em suínos.
Palavras-chave: expressão gênica, RNA-Seq, suinocultura.

ABSTRACT
Master's Dissertation
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

TRANSCRIPTOME ANALYSIS RELATED TO THE OCURRENCE OF UMBILICAL HERNIAS IN PIGS

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Chapecó, 30 de julho de 2019

Umbilical hernia (UH) is characterized as an abnormal protrusion of the abdominal contents to the exterior of the umbilical region, which occurs due to a defect in the muscle or umbilical ring closure. The affected animals have lower growth rates, worse feed conversion and higher morbidity than normal pigs. Although some studies have been performed to identify chromosomal regions related to this condition, no study involving RNA sequencing, which allows gene discovery, was performed. As the UH frequency is still a problem in pig production and its etiology remains unknown, this study aimed to identify genes related to this defect using transcriptome analysis. Therefore, ten Landrace females, five normal and five UH-affected, from the same nucleus farm, with approximately 90 days of age, were used. Necropsy was performed at the Embrapa Swine and Poultry, where tissue from the umbilical region was collected and immediately placed in liquid nitrogen for subsequent RNA extraction and preparation of the RNA-Seq libraries. Samples were sequenced on HiSeq2500 Illumina (2x100pb). Sequences quality control (QC) was performed with Seqyclean, reads were mapped against the pig genome v11.1 using STAR and counted using HTSeq. Differential expression analysis was performed using EdgeR and the validation of differentially expressed (DE) genes was conducted with qPCR. The functional analysis was performed using DAVID 6.8 database. An average of 21.7 million reads/sample was obtained after QC, comprising 13,216 genes expressed in the herniary tissue. A total of 230 genes were DE between normal and UH-affected pigs (FDR <0.05). From those, 145 were downregulated and 85 upregulated in the affected animals compared to the normal pigs. According to the functional analysis, most of them were related to immune system, anatomical development, cell adhesion and extracellular matrix. Six DE genes: *ACER2, SLC2A6, PTGS1, LGALS3, KANK3* and *FOS* were identified in QTL regions previously associated to UH in pigs. The DE genes: *ACAN, MMPs, EPYC* and *VIT*, can be highlighted as novel candidates genes for triggering UH since they act in the remodeling of the extracellular matrix and in the production, integrity and resistance of the collagen. *CCBE1* acts on calcium and collagen binding and has been associated with the appearance of umbilical hernias in humans. It is known that the imbalance of the expression of these genes can cause weakness and tissue damage, which can lead to the UH formation. This study contributes to increasing the knowledge of the genetic factors involved in the occurrence of UH. Nevertheless, studies are still needed to confirm the activity of these genes as the cause of the problem, and also to improve the understanding of this condition in the pig production.

Key-words: gene expression, RNA-Seq, pig production.

SUMÁRIO

1 CAPÍTULO I

REVISÃO DE LITERATURA

1.1 SUINOCULTURA

A suinocultura é uma das atividades produtivas mais antigas estabelecidas mundialmente; isto pode ser observado diante do crescimento da produção mundial de suínos. No ano de 1996, cerca de 78,5 milhões de toneladas de carne suína foram produzidas no mundo, enquanto que no ano de 2017, estimou-se uma produção mundial de 111,0 milhões de toneladas (ABIPECS, 2000; USDA, 2018). A produção de suínos brasileira também é expressiva e o último relatório anual da Associação Brasileira de Proteína Animal (ABPA, 2018) estimou uma produção em torno de 3,78 milhões de toneladas de carne suína, colocando o Brasil como quarto maior produtor mundial, perdendo apenas para China, União Europeia e Estados Unidos.

Para chegar nesse patamar produtivo, a suinocultura mundial passou por diversas mudanças, as quais ocorreram principalmente nas áreas de nutrição, genética, manejo, sanidade e ambiência, pois estas áreas são as responsáveis por influenciar positivamente ou negativamente o desempenho dos suínos (HECK, 2009). Essas modificações levaram ao aperfeiçoamento destas áreas e consequentemente ao aumento de tecnologia empregada.

Com o avanço no conhecimento na área da genética, passou-se a entender que a avaliação dos animais não deveria ser apenas pelo seu fenótipo, pois isso poderia caracterizá-lo de forma errônea, visto que o seu fenótipo pode ser muito diferente da sua composição genética. Isto ocorre principalmente porque algumas características apresentam interação dos genes com o meio em que o animal está inserido (MAFESSONI, 2006). Com estes conhecimentos, descobriu-se também que o ganho genético é de ordem estável e permanente, ou seja, passa de uma geração para a outra, e isto levou a um aumento do interesse pela área do melhoramento genético, pois era um campo de trabalho que resultaria em um bom retorno econômico no futuro (PEREIRA, 2008). Porém, mesmo com todos esses avanços na cadeia produtiva de suínos, ainda existem lacunas que precisam ser estudadas visto os novos desafios que apareceram. Entre os problemas produtivos na suinocultura, chama-se atenção para um problema recorrente e de caráter genético e ambiental: as hérnias, as quais são consideradas um dos defeitos congênitos com maior frequência na suinocultura (MATTSSON, 2011).

1.2 HÉRNIAS

Na suinocultura existem diversos tipos de doenças congênitas, mas entre as mais comuns estão as hérnias umbilicais (HU), inguinais (HI) e escrotais (HE), cuja incidência nos suínos varia de 1,7 a 6,7% (SEARCY-BERNAL; GARDNER; HIRD, 1994; THALLER; DEMPFLE; HOESCHELE, 1996). Contudo, podem ultrapassar os 7% de incidência, dependendo da linhagem e do manejo utilizado (SOBESTIANSKY; CARVALHO, 2007). Sabe-se que as hérnias possuem caráter hereditário e acredita-se que, possivelmente, as hérnias umbilicais possuem mecanismos de transmissão poligênicos (SOBESTIANSKY; CARVALHO; BARCELLOS, 2012). Um estudo de Thaller, Dempfle e Hoeschele (1996) mostrou uma herdabilidade de 0,25 para hérnias umbilicais em suínos. Já Sevillano et al. (2015), demonstraram estimativa de herdabilidade para hérnias escrotais/inguinais de 0,31, confirmando que há uma influência genética no aparecimento destas anomalias. Entretanto, ainda não se sabe quantos e quais genes estão envolvidos no desenvolvimento das mesmas.

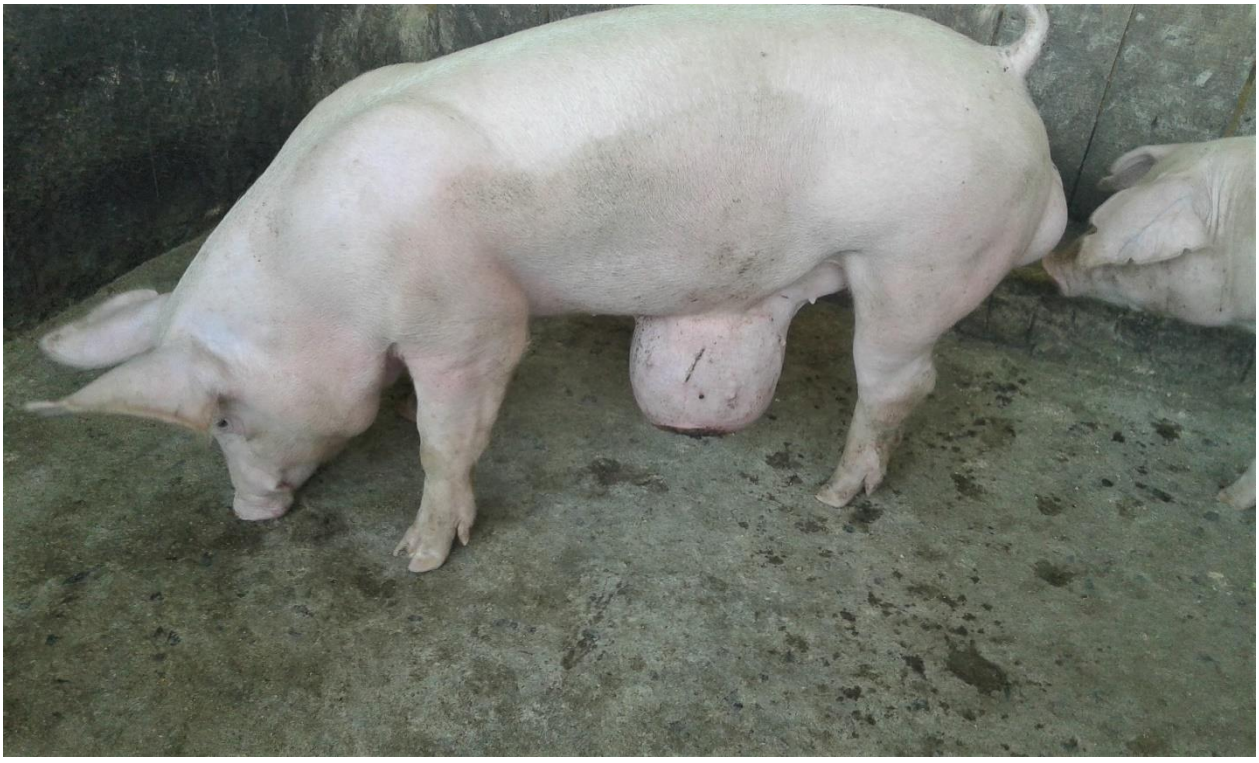
Sabe-se que as hérnias escrotais afetam somente os machos, já as inguinais podem afetar tanto machos como fêmeas, porém, ela se manifesta mais frequentemente em machos (POMMEREHN, et al., 2014). O mesmo ocorre com hérnias umbilicais, pois afetam ambos os sexos, mas são mais incidentes em machos (TIRANTI et al., 2002). Acredita-se que as hérnias podem ser decorrentes de um fator predisponente ao sexo (POMMEREHN, et al., 2014). Para animais acometidos por hérnias, a recomendação é que seja realizado o abate precoce dos mesmos, ou ainda, realizar um procedimento cirúrgico para o reparo do canal aberto (SOBESTIANSKY; CARVALHO; BARCELLOS, 2012). Caso o produtor mantenha o animal nestas condições, ele pode receber até 50% a menos pelo produto, ou pior, o animal pode morrer por complicações causadas pelo estrangulamento das alças intestinais (PERROTT, 2003). De qualquer forma, o produtor terá perdas econômicas pelo aparecimento destas anomalias, seja pelo decréscimo da produção ou aumento do custo produtivo.

Mesmo com todos esses indícios sobre as perdas econômicas causadas pelas hérnias, estes defeitos ainda persistem nos rebanhos. A dificuldade para eliminar este problema da produção está relacionada ao fato da etiologia e da causa das hérnias umbilicais ainda serem desconhecidas. Dessa forma, o estudo das hérnias, na atualidade, é muito importante para aumentar o conhecimento de suas causas e consequências na produção de suínos.

1.3 HÉRNIA UMBILICAL

As hérnias umbilicais são caracterizadas como uma protrusão anormal do conteúdo abdominal presente na região do umbigo (Figura 1), que ocorre geralmente por um defeito no músculo (MILLER et al., 1995) ou também quando não acontece o completo fechamento do anel umbilical, podendo ser causado por uma infecção no local ou por modificações nesta musculatura (SEARCY-BERNAL; GARDNER; HIRD, 1994; YOUNG; ANGUS, 1972). A protrusão que é caracterizada como hérnia consiste basicamente de gordura, omento e partes do intestino delgado (SUTRADHAR et al., 2009). A hérnia umbilical pode não ser identificada no início da vida do animal e ocorrer somente no período de crescimento, ou ainda, pode ser visível no nascimento e aumentar conforme o passar da idade (SUTRADHAR et al., 2009). Isso faz com que o descarte dos animais ou o controle desta anomalia seja difícil de ser realizado.

Figura 1: Suíno comercial acometido por hérnia umbilical.



Fonte: Igor Savoldi, 2018.

Um estudo de Muschaweck (2003) relacionado à ocorrência de hérnias umbilicais congênitas em humanos, demonstrou que existe duas formas distintas de desenvolvimento das hérnias umbilicais no período gestacional. Uma na fase embrionária (antes da oitava semana de gestação), em que ocorre uma falha no fechamento da parede abdominal, acarretando uma união

do cordão umbilical ao abdômen, causando a herniação do intestino. Já na fase fetal, o intestino deve voltar a cavidade abdominal, mas quando isso não acontece, o desenvolvimento não ocorre adequadamente e isso gera um defeito na parede que deveria sustentar o órgão. Ainda, neste mesmo estudo, Muschaweck (2003) relata o aparecimento de hérnias umbilicais somente em adultos e que poderiam ser explicadas pela cicatrização de tecidos na região umbilical, principalmente devido ao aumento da pressão intra-abdominal, a qual pode ser resultante do maior peso.

Outro trabalho mais antigo de Evans e Sack (1973), com suínos, aponta que a partir dos 21 dias de gestação se dá início ao processo herniário umbilical.

As hérnias também podem ser confundidas com outras patologias, como tumores ou abscessos. Desta forma, para a sua confirmação deve-se saber que elas ocorrem somente pelo canal umbilical e são de característica mole e redutível. Uma forma melhor de diagnóstico é através da palpação do local, considerando-se que devem ser observados três fatores: o saco herniário (peritônio), o conteúdo (vísceras - geralmente o intestino) e o anel herniário. Em situações normais de hérnias, o saco herniário e conteúdo dele (intestino) são livres e deslizam entre si (SOBESTIANSKY; CARVALHO; BARCELLOS, 2012).

Searcy-Bernal et al. (1994) identificaram a incidência de hérnias umbilicais em 1,5% em suínos do estado da Califórnia - EUA. Já, Keenliside (2006) observou uma ocorrência de 0,4% a 1,5% de hérnias umbilicais em suínos do Canadá, da Alemanha e da Holanda. Mais tarde, Petersen et al. (2008) encontraram uma prevalência de 0,82% de hérnias umbilicais em suínos da Dinamarca. Mais recentemente, Grindflek et al. (2018) observaram incidência de 0,55% de hérnias umbilicais em suínos Landrace da Noruega. Porém, sabe-se que a incidência pode variar de acordo com o lote de produção, linhagens e manejos utilizados (SOBESTIANSKY; CARVALHO, 2007).

Os animais afetados por hérnia umbilical possuem um risco maior de perdas produtivas e econômicas (YUN et al., 2017), pois acabam sendo descartados ou vendidos por um preço inferior (SEARCY-BERNAL; GARDNER; HIRD, 1994). Ainda, animais que sofrem com esta anomalia possuem menores taxas de crescimento e maior mortalidade em relação aos animais saudáveis (STRAW; BATES; MAY, 2009). Ademais, há também a preocupação com o bem-estar animal, pois animais com hérnia umbilical sofrem com dor e desconforto abdominal, principalmente quando há compressão do nervo e/ou infecção (MILLER et al., 1995). As hérnias ainda dificultam a mobilidade dos animais, o que aumenta o risco de mortalidade (SEARCY-

BERNAL; GARDNER; HIRD, 1994; STRAW; BATES; MAY, 2009).

Animais acometidos por hérnia umbilical causam prejuízo para o produtor, por causa do descarte ou reparo do problema, por isso indica-se um processo de seleção para o descarte de reprodutores que possam ter indícios de possíveis genes causadores de algum tipo de hérnias (SOBESTIANSKY; CARVALHO; BARCELLOS, 2012). Contudo, ainda existe carência de estudos relacionados as hérnias umbilicais, assim como de dados genéticos sobre essa anomalia.

1.4 ESTUDOS GENÔMICOS ENVOLVENDO HÉRNIA UMBILICAL

Muitos estudos na área genômica são voltados para a identificação de regiões de QTL (*Quantitative trait loci*), pois as mesmas são caracterizadas como regiões possivelmente responsáveis pela expressão de algumas características fenotípicas. Em busca realizada no QTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/SS/index - 11/02/2019) foi verificada a existência de 54 QTLs associados ao aparecimento das hérnias umbilicais. Estes QTLs constituem-se desde regiões menores e mais especificas até outras mais amplas do genoma.

Um dos primeiros estudos genômicos nesta área foi realizado por Ding et al. (2009) que, por meio de análise não paramétrica, identificaram 11 regiões associadas a hérnia umbilical nos cromossomos SSC1, 2, 3, 6, 7, 8, 10 e 11. Já, pelo teste de transmissão/desequilíbrio foram identificadas dez regiões nos cromossomos SSC1, 2, 4, 7, 10, 13, 14 e 15. Ainda, além de detectar vários *loci* de susceptibilidade para a ocorrência de hérnias umbilicais, eles também indicaram os *locus SWR1928* no SSC7 e o *SW830* no SSC10 como os mais relacionados a esta anomalia. Mais recentemente, novos estudos conseguiram identificar *loci* relacionados a susceptibilidade para hérnias umbilicais, além de variações no número de cópias (CNVs) e mutações que poderiam contribuir para esta desordem. Um deles, Liao et al. (2015) detectaram SNPs (*single nucleotide polymorphism*) significativos em dois *loci* nos cromossomos 2 e 17 para predisposição a hérnias umbilicais na raça Duroc. Porém, os mesmos SNPs não foram constatados nas raças Landrace e Large White, as quais não tiveram nenhum SNP associado a esta condição. Além disso, o gene *SRC* (*Proto-Oncogene, Non-Receptor Tyrosine Kinase*) foi caracterizado como possível candidato ao aparecimento das hérnias umbilicais devido a sua posição no genoma e também suas funções biológicas (LIAO et al., 2015). Em outro trabalho, Long et al. (2016) identificaram oito regiões de CNVs associadas ao risco de hérnias umbilicais nas raças Duroc, Landrace e Yorkshire, além de uma CNV no gene *NUGGC* (*Nuclear GTPase, Germinal Center Associated*) em Duroc. Dessa forma, concluiu-se que essas CNVs e o gene *NUGGC* contribuem para a ocorrência de hérnias umbilicais em suínos.

Outro trabalho foi o de Fernandes et al. (2018), o qual utilizaram a metodologia de GWAS com 325 suínos de linhagem comercial que possibilitou identificar cinco SNPs e quatro genes candidatos: *TBX15* (*T-Box 15*), *WARS2* (*Tryptophanyl TRNA Synthetase 2, Mitochondrial*), *LIP1* (*Lipase 1*) e *RBM11* (*RNA Binding Motif Protein 11*) associados a hérnia umbilical. Ainda, neste mesmo ano, Grindflek et al. (2018) encontraram um QTL e 62 SNPs significativos no cromossomo 14 de suínos Landrace e concluíram que esse QTL detectado no cromossomo 14, próximo a 50 Mb, é altamente correlacionado com hérnia umbilical, explicando cerca de 8,6% da variância fenotípica para HU. Ainda, nesta região de QTL, estes autores identificaram os genes *Leukemia inhibitory factor* (*LIF*) e *Oncostatin M* (*OSM*) como genes candidatos para essa anomalia. Corroborando com estes achados, mais recentemente, Li et al. (2019) identificaram no cromossomo 14 de suínos um SNP no gene *CAPN9* (*Calpain 9*) significativamente associado às hérnias umbilicais e mais dois SNPs significativos nos cromossomos SSC9 e 16. Neste mesmo trabalho, também foi identificado que os animais afetados com hérnia umbilical possuíam uma mutação no exon 10 do gene *CAPN9*, a qual não existia em animais sem hérnias.

Assim, alguns genes foram relacionados ao aparecimento das hérnias umbilicais nos suínos, mas apesar desses achados, ainda não se tem um conhecimento aprofundado dos mecanismos genéticos envolvidos no aparecimento deste problema. Por isso, estudos realizados com outras metodologias, como o sequenciamento do RNA, poderão auxiliar para o melhor entendimento dessa desordem.

1.5 ESTUDOS DE TRANSCRIPTOMAS

Os estudos de transcriptomas consistem basicamente em verificar alterações nas quantidades de transcritos (HAIDER; PAL, 2013), em um estágio fisiológico específico e/ou fase de desenvolvimento, sendo necessários para o maior conhecimento dos elementos funcionais do genoma, os constituintes moleculares e o desenvolvimento de algumas doenças (WANG; GERSTEIN; SNYDER, 2009).

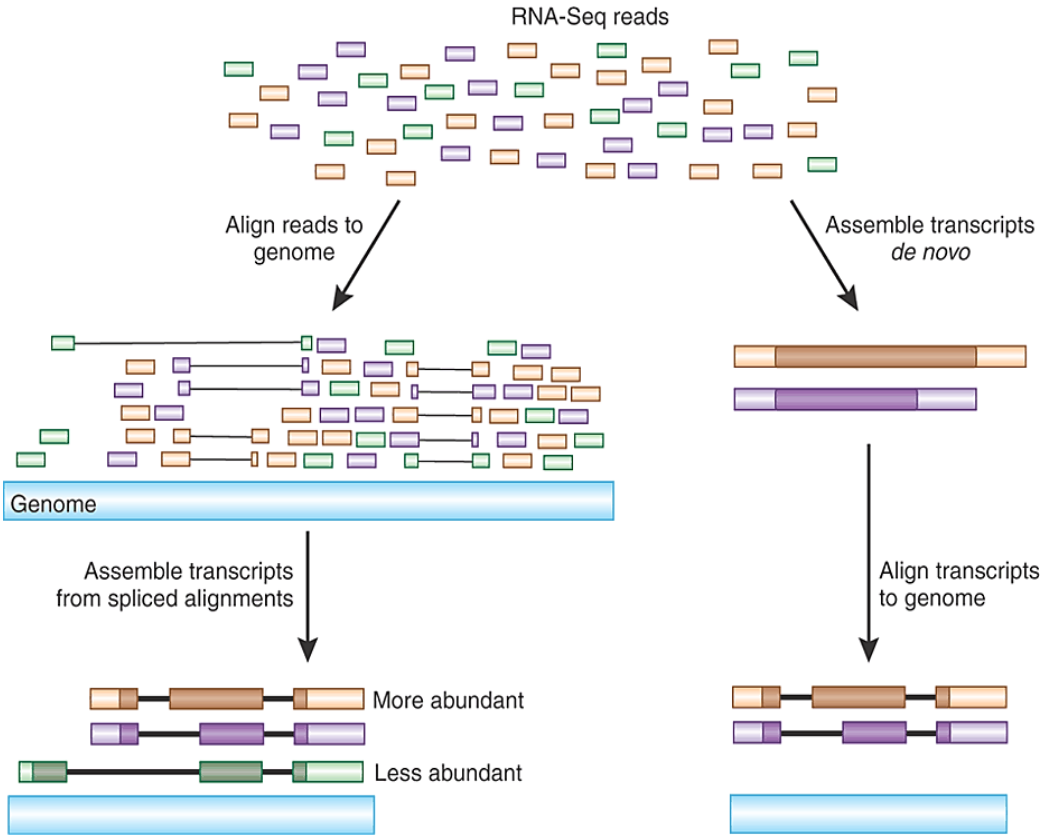
Dentre os vários objetivos propostos com o estudo do transcriptoma, os principais consistem na identificação de novas espécies de transcritos (RNAm, RNAs não codificadores e RNAs pequenos), padrões de *splicing*, determinação estrutural dos genes e a diferença de expressão entre eles (WANG; GERSTEIN; SNYDER, 2009, HAIDER; PAL, 2013). Nas décadas anteriores, para a realização de trabalhos nesta área, eram utilizadas metodologias como sequências expressas (ESTs) para sequenciar o RNAm (ADAMS et al., 1993), o sequenciamento de Sanger para sequências de DNA complementar (cDNA) e para expressão gênica, a

metodologia de microarranjos era a mais usada (TRAPNELL; PACHTER; SALZBERG, 2009). Atualmente, para conseguir alcançar com mais êxito o objetivo proposto em um estudo de transcriptoma, foi necessário que as metodologias antigas fossem aperfeiçoadas e que novas metodologias fossem criadas. Entre essas, surgiu a metodologia de RNA-Seq.

A metodologia de sequenciamento total do RNA (RNA-Seq) é uma ferramenta de análise revolucionária e fundamental para o estudo do transcriptoma. Ela é assim considerada pois exige uma menor quantidade de animais e utiliza uma tecnologia de sequenciamento de nova geração, que possibilita um estudo amplo de todos os transcritos expressos em determinada idade ou estado fisiológico (WANG; GERSTEIN; SNYDER, 2009). Outro diferencial desta metodologia é que ela não depende do conhecimento prévio do transcriptoma de referência (RAZ et al., 2011). Além disso, os genes que possuem altos níveis de expressão necessitam de uma quantidade menor de RNA total para quantificação, permitindo que o transcriptoma possa ser estudado quantitativamente e com uma abundância de informações (WANG; GERSTEIN; SNYDER, 2009; VAN VLIET, 2010).

Na técnica de RNA-Seq converte-se o RNA isolado em DNA complementar (cDNA). Após isso, o cDNA é sequenciado por meio do sequenciamento de nova geração (MORTAZAVI et al., 2008). Após o sequenciamento, as sequências oriundas dos transcritos expressos em um determinado tecido podem ser alinhadas com um genoma de referência ou transcritos de referência, ou ainda podem ser montadas *de novo* para montar um novo mapa de transcritos (WANG; GERSTEIN; SNYDER, 2009) (Figura 2). Assim, é possível quantificar os níveis de expressão gênica relacionados a determinado problema ou condição de interesse (MARGUERAT; BÄHLER, 2010; NAGALAKSHMI; WAERN; SNYDER, 2010).

Figura 2: Ilustração demonstrativa dos dois métodos de alinhamento e montagem das leituras do RNA-Seq.



Fonte: HAAS, B. J.; ZODY, M. C. Advancing RNA-Seq analysis. **Nature Biotechnology**, v. 28, n. 5, p. 421–423, 2010.

Vários estudos de RNA-Seq vêm sendo realizados em suínos nas mais diversas áreas. Du et al. (2014) estudaram o transcriptoma do tecido placentário e testicular de suínos para entenderem mais sobre as características reprodutivas e, através disto, identificaram 5.516 novos transcritos na placenta e 9.061 nos testículos. Destes, 159 transcritos foram específicos para placenta e 252 para testículos. Com este trabalho foi possível demonstrar que esses novos transcritos foram enriquecidos em regiões de QTL para características reprodutivas de suínos. Mesmo com esse avanço nas pesquisas, os autores afirmam que ainda há necessidade de explorar melhor os dados e de realizar novos estudos para compreender melhor os aspectos da fisiologia que estão relacionados ao desemprenho reprodutivo.

Ropka-Molik et al. (2014) estudaram mudanças globais no transcriptoma do tecido muscular de duas raças suínas distintas (Pietrain e Landrace Polonês), pois as mesmas possuíam índices distintos para musculosidade, taxa de crescimento e reprodução. Neste estudo, foram

identificados 35 genes diferencialmente expressos entre as raças, estando envolvidos principalmente na codificação de proteínas do ribossomo, citoesqueleto e ligações de cálcio (contração muscular, sinalização e transporte de cátions). Nos animais Landrace, um maior número de genes reguladores de funções importantes relacionadas à reprodução foi observado, enquanto nos animais da raça Pietrain foi identificada maior relação com genes de apoptose, migração celular e resposta imune.

Ainda, um estudo mais recente de Horodyska et al. (2019), utilizando a metodologia de RNA-Seq no fígado de suínos, buscou elucidar as mudanças no metabolismo, crescimento hepático e resposta imune de suínos com diferentes eficiências alimentares. Neste estudo, foram identificados 922 genes diferencialmente expressos entre os animais de alta e baixa eficiência alimentar, dos quais vários tinham funções e vias biológicas relacionadas a crescimento hepático, resposta imune, metabolismo lipídico, proteico e de carboidratos. Ademais, suínos com alta eficiência alimentar apresentaram maiores níveis de expressão dos genes relacionados à absorção hepática de carboidratos e colesterol, à síntese de ácidos biliares e ao aumento da concentração de glicose e da resposta a estímulos inflamatórios.

O avanço tecnológico é evidente e com as novas descobertas é possível realizar trabalhos com mais enfoque em genômica funcional. Assim, o RNA-Seq é uma metodologia inovadora e essencial para a identificação de novos genes e mutações causais que possam estar expressos nos animais. Nesse contexto, a utilização do RNA-Seq para a avaliação do transcriptoma do anel umbilical é primordial para o estudo de hérnias umbilicais em suínos. Sabe-se que as HU são de grande complexidade, causadas por múltiplos genes e mesmo os genes candidatos já conhecidos precisam ser melhor investigados para se identificar marcadores ou variantes que possam ser utilizados na seleção genética para a diminuição da ocorrência de hérnias umbilicais (LIAO et al., 2015).

1.6 METODOLOGIA DE VALIDAÇÃO POR qPCR

A análise de qPCR (reação em cadeia da polimerase quantitativa em tempo real) é uma metodologia quantitativa e muito utilizada atualmente nos estudos, pois por meio dela se consegue mensurar a expressão gênica nos tecidos de forma rápida e confiável (REBOUÇAS et al., 2013). Essa técnica obtém resultados através da amplificação do DNA/cDNA das amostras extraídas. Um dos métodos atuais utilizados para quantificação de expressão gênica é a quantificação relativa dos genes, o qual possibilita melhor averiguação dos níveis de expressão de RNAm e não se faz necessário uma curva de calibração, pois nesta metodologia se usa genes

referência para a comparação com os genes alvos (SCHMITTGEN; LIVAK, 2008).

Os genes constitutivos, também chamados de genes referência, são usados para a análise de qPCR, pois são caracterizados como genes de pouca variabilidade, seja em diferentes tecidos ou estados fisiológicos, mas principalmente porque os fatores experimentais não causam mudanças na sua expressão (KOZERA; RAPACZ, 2013). Mesmo que estes genes possuam essa definição, existe a necessidade de se realizar estudos antes de utilizá-los, para que se escolham os genes mais estáveis e melhores para aquela determinada situação fisiológica ou idade dos animais, a fim de evitar problemas na execução ou resultado do experimento.

Um estudo de Lorenzetti et al. (2018) validou alguns genes referência para hérnias escrotais em suínos MS115 de 30 dias e suínos Landrace de 60 dias de idade. Os genes mais estáveis foram *RPL19* (*Ribosomal Protein L19*), *RPL32* (*Ribosomal Protein L32*) e *H3F3A* (*H3 Histone Family Member 3A*) para os MS115 de 30 dias e *PPIA* (*Peptidylprolyl Isomerase A*) e *RPL1A* (*Ribosomal Protein L10A*) para os Landrace de 60 dias de idade. Como visto anteriormente, já existem alguns genes referência relacionados a tecidos musculares e hérnias, porém com relação as hérnias umbilicais nada ainda foi visto na literatura.

A técnica de qPCR é considerada de alta precisão, sensibilidade e velocidade (GACHON et al., 2004), corroborando para que essa seja considerada uma técnica de maior precisão e confiabilidade para validação de dados obtidos de outras metodologias (KOZERA; RAPACZ, 2013). Em razão disto é que essa técnica está sendo muito utilizada para validação dos genes identificados na metodologia de RNA-Seq.

Ropka-Molik et al. (2014) também utilizaram a metodologia de qPCR para validar os resultados obtidos por RNA-Seq de amostras de tecido muscular de suínos da raça Pietrain e Landrace. Quatro genes identificados por RNA-Seq e diferencialmente expressos entre as duas raças foram selecionados para validação: *CISH* (*Cytokine Inducible SH2 Containing Protein)*, *SPP1* (*Secreted Phosphoprotein 1*), *XIAP* (*X-Linked Inhibitor Of Apoptosis*) e *CLIC5* (*Chloride Intracellular Channel 5*) sendo que valores semelhantes na expressão gênica entre as técnicas foram observados com uma correlação de Pearson de 0,7 e $p \leq 0,006$, confirmando os resultados encontrados pela metodologia de RNA-Seq.

Wang et al. (2016) utilizaram o RNA-Seq para identificar novos genes relacionados à fase de pré-implantação no endométrio de suínos. Neste estudo foi avaliado o perfil de expressão tecidual do endométrio nos dias 9, 12 e 15 de gestação e no dia 12 para não gestantes da raça Yorkshire. Este trabalho identificou 2.376 novos genes (301 não codificadores e 2.015 RNAm) e destes, 10

foram escolhidos para validação por qPCR: *TCONS_01729386*, *TCONS_01325501*, *FGF7* (*Fibroblast Growth Factor 7*), *NMB* (*Neuromedin B*), *FGF9* (*Fibroblast Growth Factor 9*), *VEGFC* (*Vascular Endothelial Growth Factor C*), *VEGFA* (*Vascular Endothelial Growth Factor A*), Muc1 (*Mucin 1*), *ESR1* (*Estrogen Receptor 1*) e *RBP4* (*Retinol Binding Protein 4*). A correlação identificada entre os resultados de expressão gênica das duas metodologias para cada gene usado na validação variou de 0,7 a 1,0, sendo considerada de boa concordância pelos autores.

Ainda, um estudo mais recente de Guo et al. (2019), também com a metodologia de RNA-Seq, buscou identificar genes no músculo *longissimus dorsi* de suínos Large White e Mashen que estavam relacionados ao crescimento. Neste trabalho foram identificados 697 genes diferencialmente expressos entre as raças, os quais estavam envolvidos principalmente no crescimento e no desenvolvimento do músculo esquelético, ligações de proteínas e formação de organelas. Destes genes identificados, 10 deles: *ITGAL* (*Integrin Subunit Alpha L*), *MCM4* (*Minichromosome Maintenance Complex Component 4*), *LPIN1 (Lipin 1*), *SFRP2* (*Secreted Frizzled Related Protein 2*), *EMC2* (*ER Membrane Protein Complex Subunit 2*), *HK1* (*Hexokinase 1*), *ACTR2* (*Actin Related Protein 2*), *KDM6A* (*Lysine Demethylase 6A*), *CLEC2D* (*C-Type Lectin Domain Family 2 Member D*) e *OGT* (*O-Linked N-Acetylglucosamine (GlcNAc) Transferase*) foram escolhidos para a validação pela metodologia de qPCR, os quais também foram diferencialmente expressos entre os dois grupos estudados e tiveram valores semelhantes entre as duas metodologias, confirmando os resultados encontrados no RNA-Seq.

A utilização da metodologia de RNA-Seq, em conjunto com a técnica de qPCR, é uma forma eficiente para a descoberta de genes envolvidos no desenvolvimento de características de interesse e doenças ainda pouco exploradas na produção animal e principalmente na suinocultura. Com isto, abre-se perspectivas de melhorar o conhecimento sobre o mecanismo genético e os processos biológicos envolvidos na ocorrência de hérnias umbilicais, possibilitando novas estratégias para reduzir este problema.

1.7 OBJETIVOS

1.7.1 Objetivo geral

Identificar genes associados à manifestação de hérnia umbilical em suínos.

1.7.2 Objetivos específicos

- Gerar o transcriptoma do anel umbilical de animais normais ou afetados por hérnia umbilical;
- Identificar um conjunto de genes diferencialmente expresso associado à hérnia umbilical utilizando a técnica de RNA-Seq;
- Confirmar, por meio da técnica de qPCR, a expressão diferencial de alguns genes identificados por RNA-Seq.
- Identificar rotas metabólicas envolvidas no desenvolvimento de hérnia umbilical em suínos.

2 CAPÍTULO II

MANUSCRITO

Os resultados desta dissertação são apresentados na forma de um manuscrito, com sua formatação de acordo com as orientações da revista PLoS ONE.

2.1 MANUSCRITO I

Transcriptome analysis identifies genes involved with the development of umbilical hernias in pigs

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De acordo com normas para publicação em:

PLoS ONE

Transcriptome analysis identifies genes involved with the development of umbilical hernias in pigs

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Abstract

Umbilical hernia (UH) is one of the most frequent defects affecting pig production, however, it also affects humans and other mammals. UH is characterized as an abnormal protrusion of the abdominal contents to the umbilical region, causing pain, discomfort and reduced performance in pigs. Some genomic regions associated to UH have already been identified, however, no study involving RNA sequencing was performed when umbilical tissue is considered. Therefore, here, we have sequenced the umbilical ring transcriptome of five normal and five UH-affected pigs to uncover genes and pathways involved with UH development. A total of 13,216 transcripts were expressed in the umbilical ring tissue. From those, 230 genes were differentially expressed (DE) between normal and UH-affected pigs (FDR <0.05), being 145 downregulated and 85 upregulated in the affected compared to the normal pigs. A total of 68 significant biological processes were identified and the most relevant were extracellular matrix, immune system, anatomical development, cell adhesion, membrane components, receptor activation, calcium binding and immune synapse. The results pointed out *ACAN*, *MMPs*, *COLs*, *EPYC*, *VIT*, *CCBE1* and *LGALS3* as strong candidates to trigger umbilical hernias in pigs since they act in the extracellular matrix remodeling and in the production, integrity and resistance of the collagen. We have generated the first transcriptome of the pig umbilical ring tissue, which allowed the identification of genes that had not yet been related to umbilical hernias in pigs. Nevertheless, further studies are needed to identify the causal mutations, SNPs and CNVs in these genes to improve our understanding of the mechanisms of gene regulation.

Key-words: gene expression, RNA-Seq, swine production.

Introduction

Pig husbandry has become one of the most important activities in livestock production and increment in pig production in the last years has been observed [1, 2]. However, at the same time that production has increased some physiological problems have emerged, causing economic losses and affecting animal welfare. The umbilical hernia (UH), an anatomic defect characterized by the protrusion of abdominal content through the umbilical ring, is one of the most frequent defects affecting pig production. HU prevalence in pigs ranges from 0.40 to 2.25%, varying according to the breed, farm and production system [3, 4]. Animals affected with UH experience pain and discomfort, and generally have reduced performance, with low growth rates, poor feed conversion and low meat quality [5].

The development of umbilical hernia can be caused by external factors, such as physical lesions, high pressure in the abdomen, inappropriate removal of the umbilical cord, infections and management [5]. Meanwhile, it has been observed that related animals with the same management practices could present different phenotypes, i.e., being normal or affected with hernias [5], indicating that a genetic factor is also present. Moreover, the heritability estimate of 0.25 for UH in pigs [6] and 0.40 in cattle [7] reinforce the genetic component regulating this trait. However, the mode of inheritance and its etiology remain unclear.

Several quantitative trait loci (QTL) associated with UH have already been reported for pigs. In a search in the pig QTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/SS/index - 11/02/2019), 54 QTLs for umbilical hernia have been found in several pig breeds, being located in chromosomes SSC1, SSC2, SSC3, SSC4, SSC6, SSC7, SSC8, SSC9, SSC10, SSC11, SSC13, SSC14, SSC15, SSC16 and SSC17 [8, 9, 10, 11]. Ding et al. (2009) [8] performed the first genomic study with UH and found some chromosomic regions related to the appearance of this condition. More recently, a copy number variation (CNV) polymorphism on SSC14 was found to be related to UH [9] and a highly significant QTL for this trait was detected in Norwegian Landrace pigs also in SSC14 [10]. This QTL explained approximately 8.6% of the phenotypic variance for UH, and the LIF Interleukin 6 Family Cytokine (*LIF*) and Oncostatin M (*OSM*) genes were located within this QTL, being considered candidates for functional studies [10]. Recently, in a genome-wide association study (GWAS) with crossbred pigs, SNPs in the chromosomes SSC4, SSC6, SSC11 and SSC13 were associated with umbilical hernia. In these regions, novel genes: *TBX15* (T-box 15) and *WARS2* (tryptophanyl-tRNA synthetase 2) in SSC4,

and *LIPI* (lipase I) and *RBM11* (RNA Binding Motif Protein 11) in SSC13, were identified as possible candidates to the UH development [12].

Although some genomic regions associated with UH have already been identified, no functional studies with the umbilical ring tissue were performed in pigs. Therefore, knowing the complexity of this disorder, in this study, we have sequenced the umbilical ring transcriptome of normal and affected pigs to discover genes and pathways involved with the development of umbilical hernia in a swine purebred line using RNA-Seq.

Material and Methods

Animals and Sampling

In this study, 10 unrelated Landrace purebred females from the same nucleus farm, with high sanitary status, located in Santa Catarina State, south of Brazil, with approximately 90 days of age were used. From those, 5 were affected with umbilical hernia and 5 were normal, being the later chosen from families with no history of hernias. For each affected animal, a contemporary normal pig was chosen. The animals were transported to the Embrapa Swine and Poultry National Research Center, located in Concórdia-SC, to be evaluated and necropsied. Euthanasia was performed by electrocution desensitization for 10 seconds, followed by bleeding, according to the practices recommended by the Embrapa Swine and Poultry National Research Center Ethics Committee on Animal Utilization, approved under protocol #011/2014. Necropsy was performed and tissue samples were collected from the umbilical ring of herniated and non-herniated animals. Samples were placed in 4% paraformaldehyde buffer for histopathological analysis and those for gene expression analysis were immediately frozen in liquid nitrogen and, subsequently, stored at -80 °C for further RNA extraction.

Histological analyses of umbilical ring tissue

Tissues from the umbilical ring region of the herniated or non-herniated animals were dehydrated in a series of crescent ethanol concentration, diaphanized with xilol and embedded in paraffin. Tissue sections with 2 to 5 μm thickness were cut with an automatic microtome, stained by the hematoxylin & eosin method and analyzed with optical microscopy. The cell types were

evaluated in a 10x eyepiece with 5x to 100x objectives, following a routine histopathological analysis.

RNA extraction, library preparation and sequencing

For RNA extraction, about 100 mg of each sample were macerated in liquid nitrogen using 1 mL of Trizol (Invitrogen, USA), according to the manufacturer's instructions. Next, 200 μL of chloroform was added, shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. Centrifugation at 11,000 xg at 4 °C for 15 minutes was performed and 600 μL of the aqueous phase were transferred to a new tube containing 600 μL of 70% ethanol. This volume was then added to an RNEasy mini silica column (Qiagen, Germany) following the manufacturer's instructions. The quality and quantity of the total RNA were evaluated in Biodrop spectrophotometer (Biodrop, UK), 1% agarose gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA). Samples with 260/280 nm ratio above 1.9 on Biodrop and with RNA integrity number (RIN) greater than 8.0 were used for preparing the RNA-Seq libraries.

To prepare the libraries, 4ug of total RNA from each sample were used with the TruSeq Stranded mRNA Kit (Illumina, Inc.; San Diego CA, USA) following the manufacturer's recommendation. The size of the libraries was evaluated in the Bioanalyzer obtaining an average size of 300 bp for each sample. After checking the concentration and size of the libraries, the paired-end (2x100 bp) RNA sequencing was performed in the Illumina HiSeq2500 equipment (Illumina, Inc.; San Diego CA, USA) at the Center for Functional Genomics of ESALQ/USP. All samples (normal and affected pigs) were placed and sequenced in the same lane.

Quality control, assembly and differential expression analysis

For the quality control, the SeqyClean tool [13] was applied for the removal of short reads (<70bp), low quality reads (QPhred <24), adapter sequences, contaminants (phiX) and poly A/T tails. After, the sequences were mapped against the pig reference genome (Sus scrofa, assembly 11.1), available in the Ensembl database version 94 (www.ensembl.org), using the STAR program [14]. The reads counting was performed with the HTSeq-count program [15]. The EdgeR package [16] from R [17] was used for identifying differentially expressed (DE) genes between normal and UH-affected pigs. The significance threshold to declare genes as DE was set

to a False Discovery Rate (FDR) ≤ 0.05 after multiple correction tests to reduce type I error, following the Benjamini and Hochberg (BH) method [18]. The Multi-Dimensional Scaling (MDS) plot was created with R using the LogFC values of each expressed gene in the umbilical ring tissue of the normal and UH-affected pigs. The heatmaps were generated with the plots package from R [17] using the expression data of each sample for each DE gene. The FASTQ files were deposited at the SRA database with BioProject number PRJNA445856 and biosample numbers: SAMN08801040, SAMN08801041, SAMN08801042, SAMN08801043, SAMN08801044, SAMN08801045, SAMN08801046, SAMN08801047, SAMN08801048 and SAMN08801049.

Validation of DE genes using quantitative PCR (qPCR)

The qPCR analysis was used to confirm the results found in the RNA-Seq, using the same tissue samples from the normal and affected pigs. The RNA extraction was performed as previously mentioned and the cDNA synthesis was carried out using 3 μg of total RNA and the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen, USA) standard protocol. For validation, the following 12 DE genes were chosen according to their functions: Matrix metallopeptidase 13 (*MMP13*), Vitrin (*VIT*), Alkaline ceramidase 2 (*ACER2*), Molecule CD3D (*CD3D*), Galactin 3 (*LGALS3*), Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*), collagen and calcium binding EGF domains 1 (*CCBE1*), Plakophilin 3 (*PKP3*), Epiphycan (*EPYC*), S100 calcium binding protein A2 (*S100A2*), Aggrecan (*ACAN*) and Microtubule associated protein 1 light chain 3 gamma (*MAP1LC3C*). In addition, ten candidate reference genes were tested to select the appropriate reference genes to be used in the qPCR analysis (Table 1), as described by Lorenzetti et al. [19]. The primers were designed in exon-exon regions using primer-blast tool [20] and their quality was evaluated in the NetPrimer program (http://www.premierbiosoft.com/NetPrimer) (Table 1). The qPCR reactions were performed in the Quantstudio 6 (Applied Biosystems, USA) with a final volume of 15 μL containing 1X GoTaq® qPCR Master Mix (2x) (Promega), 0.13 μM of the forward and reverse primers and 2.0 μL of 1:10 diluted cDNA. Reactions were performed in duplicates, with cycling 95º for 2 minutes, 40 cycles for 15 seconds of 95ºC and 60º for 30 seconds. Furthermore, negative control samples were included to detect contaminations.

To determine the stability of the ten candidate reference genes in the umbilical ring tissue for selecting the best gene(s) as normalizer(s) in the qPCR analyses, the endoGenes pipeline

(https://github.com/hanielcedraz/endoGenes) was used. This pipeline performs an automated analysis of the BestKeeper [21], geNorm [22] and NormFinder [23] tools and ranks the most stable genes with the RankAggreg package from R [24].

The Ct means of the 12 evaluated target genes were obtained and normalized using the most stable reference genes selected based on the previous step. After data normalization, the log2FC (Log_2 Fold Change) values obtained from both qPCR and RNA-Seq analyses were compared using the Pearson´s correlation analysis in the R program [17].

In silico functional analysis

Functional annotation of DE was performed using DAVID 6.8 database (https://david.ncifcrf.gov/). The clustering of biological processes (BP) was performed in Revigo (http://revigo.irb.hr/). A gene network was constructed with the BP in Cytoscape v3.7.1 [25]. Furthermore, it was verified whether the DE genes were in QTL regions for umbilical hernia occurrence in pigs using the Pig QTLdb from the Animal Genome Database (http://www.animalgenome.org/QTLdb/app).

Table 1: Primers for the 12 target genes and the 10 candidate reference genes used in qPCR analysis of the umbilical ring tissue in pigs.

Ensembl ID	Target genes	Chr.	Primer sequence (5' to 3')
ENSSSCG00000001832	*ACAN* Aggrecan	7	F:CAGGAGGGGTTGTGTTCCATTA R:CCTCCTCGAAAGTCAGTGAGTAG
ENSSSCG00000034213	*ACER2* Alkaline Ceramidase 2	1	F:AAGGAGGTGCGACAACGTG R:TAGGGGAAGTGGAAGGCAGAT
ENSSSCG00000034214	*CCBE1* Collagen and Calcium binding EGF domains 1	1	F:GGGGGACAAGTACCCCAATG R:GGGAGCAGGGCAATCTTCTG
ENSSSCG00000034215	*CD3D* CD3d molecule	9	F:CTCCCGAGTGAGCCCCTAT R:GATCCAGGATGCGTTTTCCCA
ENSSSCG00000034216	*EPYC* Epiphycan	5	F:CTGCTGTGACTGCCCCAA R:TCGATCTCAGCTGGACCCAT
ENSSSCG00000034217	*FOS* Fos proto-oncogene, AP-1 transcription factor subunit	7	F:GTGAAGACCATGCCAGGAGG R:TAGCTGGTCTGTCTCCGCTT
ENSSSCG00000034218	*LGALS3* Galectin 3	1	F:CCCCTTCTGGACCACTGAAT R:TGTTGTCCTCGTTGAAGCGT
ENSSSCG00000034219	*MAP1LC3C* Microtubule Associated Protein 1 Light Chain 3 gamma	10	F:TGGAAACAGCTGGAGGAATGAG R:CCTCTCTTCTGGTTGCTAAGCTC
ENSSSCG00000034220	*MMP13* Matrix Metallopeptidase 13	9	F:AAGAGCATGGAGACTTCTACCC R:GGAGGAAAAGCATGAGCCAA

ENSSSCG00000034221	*PKP3* Plakophilin 3	2	F:GCAGACAATAAGCTGGCCCT R:ATCCCTGTGACGTTCTTGCG
ENSSSCG00000034222	*S100A2* S100 calcium binding protein A2	4	F:ACAAGTACTCGGGCCAAGAAG R:TTCTCCCCTACAAAGCTGGG
ENSSSCG00000034223	*VIT* Vitrin	3	F:GTCGAAGCCACCCACACTG R:AAGTCAGGTTCCTCCCCCA
Ensembl ID	**Candidate reference genes***	**Chr.**	**Primer sequence (5' to 3')**
ENSSSCG00000023971	*H3F3A* H3 histone, family 3A	10	F: CTTTGCAGGAGGCAAGTGAG R: TGGCATGGATAGCACACAGG
ENSSSCG00000027637	RPL32 Ribosomal protein 32	13	F: CAAAATTAAGCGGAACTGGCGG R: GCACATTAGCAGCACTTCAAGC
ENSSSCG00000015108	*HMBS* hydroxymethylbilane synthase	9	F: AGGATGGGCAACTCTACCTGA R: ATGGATGGTGGCCTGCATAG
ENSSSCG00000017509	*RPL19* ribosomal protein L19	12	F: ACCGCCACATGTATCACAGTC R: TGTGCTCCATGAGAATCCGC
ENSSSCG00000004489	*EEF1A1* eukaryotic translation elongation factor 1 alpha 1	1	F: CCGCCAGGACACAGGT R: TTCCCATCTCCGCAGCCT
ENSSSCG00000003166	*RPL13A* ribosomal protein 13A	3	F: CCAAGCAGGTACTTCTGGGC R: GGCAGCATGCCTCGCA
ENSSSCG00000011213	*TOP2B* topoisomerase (DNA) II beta	13	F: AGAAGAGCTGCTGCTGAAAGG R: TCCCCGTCATTTGTCACAGG
ENSSSCG00000020686	*SDHA* succinate dehydrogenase complex flavoprotein subunit A	16	F: TTGTACGGAAGGTCTCTGCG R: GATGACTCCACGACACTCCC
ENSSSCG00000006062	*YWHAZ* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	4	F: ATCAGATTGGGTCTGGCCCT R: GGTATCCGATGTCCACAATGTC
ENSSSCG00000016737	*PPIA* peptidyl-prolyl cis-trans isomerase A	18	F: GCGTCTCCTTCGAGCTGTTT R: ACTTGCCACCAGTGCCATTA

Chr: chromosome; F: forward; R: reverse; *Lorenzetti et al. (2018).

Results

Histological analysis

The histopathological evaluation has shown that, in general, the umbilical ring tissue of animals affected with umbilical hernia was thickened by an abundant proliferation of dense connective tissue. On the other hand, a normal amount of collagen fibers of connective tissue interspersed with adipose tissue was found in the umbilical ring tissue of the normal pigs (Fig 1).

Fig 1. Hematoxylin & eosin stain histological section of the umbilical ring tissue sample from a normal (A) and a umbilical hernia-affected (B) piglet.

Sequencing and mapping

Sequencing of the umbilical ring tissue transcriptome produced about 24 million paired-end reads per sample. After the quality control, about 2.3 million reads were removed, remaining in average 21.7 million reads/sample. About 99.85% of the reads were mapped in the genome (Sus scrofa 11.1), with an average of 82% of the reads mapped in genes.

Differentially expressed genes

From the 25,880 genes annotated in the swine genome (Ensembl 94), a total of 13,216 was expressed in the umbilical ring tissue. From those, 230 genes were DE, being 145 (63.04%) downregulated and 85 (36.96%) upregulated in animals affected with umbilical hernia compared to the normal pigs (Supplementary Material S1 Table). A clear separation of samples from UH-affected and normal pigs was observed in both the heatmap (Fig 2) and the MDS plot (Supplementary Material S1 Figure), comparing the groups of animals used in this study.

Fig 2. Heatmap with 230 differentially expressed genes between animals affected with umbilical hernia (21A, 27A, 27A, 29A, 31A and 36A) and normal piglets (20C, 26C, 28C, 30C and 37C). The expression for each gene is shown in the rows and samples are visualized in the columns, showing a hierarchical clustering of genes and samples. Genes are upregulated (in green) and downregulated (in red) in the affected samples.

The 10 most down and upregulated genes based on the Log2FC of the UH-affected compared to normal pigs (Table 2) are mainly related to the organization of the extracellular matrix, morphogenesis, cartilage development and biosynthesis processes. Some of these transcripts identified in our study have not yet been characterized in Ensembl 94.

Table 2: Top 10 down and upregulated genes in the umbilical ring tissue of normal and umbilical hernia-affected piglets.

ENSEMBL ID	Gene symbol	Gene name	Log2FC
ENSSSCG00000037358	*SAA3*	Serum Amyloid A-3 Protein	-7,40
ENSSSCG00000014988	*MMP13*	Matrix Metallopeptidase 13	-7,31
ENSSSCG00000037009			-5,94
ENSSSCG00000036318			-5,75
ENSSSCG00000036203			-5,64
ENSSSCG00000004195	*ARG1*	Arginase 1	-5,53
ENSSSCG00000036127			-5,24
ENSSSCG00000040651			-5,21
ENSSSCG00000037141			-4,93
ENSSSCG00000036445	*CXCL13*	C-X-C Motif Chemokine Ligand 13	-4,85
ENSSSCG00000003509	*SH2D5*	SH2 Domain Containing 5	2,76
ENSSSCG00000016883	*ISL1*	ISL LIM Homeobox 1	2,78
ENSSSCG00000001832	*ACAN*	Aggrecan	2,81
ENSSSCG00000006021	*KCNV1*	Potassium Voltage-Gated Channel Modifier Subfamily V Member 1	2,99
ENSSSCG00000026780	*EDIL3*	EGF Like Repeats And Discoidin Domains 3	3,01
ENSSSCG00000003431	*NPPB*	Natriuretic Peptide B	3,05
ENSSSCG00000036566	*LY6G6C*	Lymphocyte Antigen 6 Family Member G6C	3,38
ENSSSCG00000038121	*TCHH*	Trichohyalin	3,76
ENSSSCG00000034838	*MAP1LC3C*	Microtubule Associated Protein 1 Light Chain 3 Gamma	3,95
ENSSSCG00000033927			4,26

Selection of reference genes and confirmation of RNA-Seq results with qPCR

From the 10 candidate reference genes tested, H3 histone 172 family 3A (*H3F3A*) and Ribosomal protein 32 (*RPL32*) were considered the most stable and therefore used in qPCR normalization. The qPCR analysis confirmed the RNA-Seq results with a high concordance between the Log2FC of the RNA-Seq and the qPCR analysis (Fig 3). The pairwise correlation analyses

248 between the Log2FC of the two methodologies showed that the results from RNA-Seq obtained
249 in this study were consistent (r=0.82, Fig 4).
250
251 **Fig 3. Comparison of Log2FC expressed values between the RNA-Seq and qPCR**
252 **methodologies for the 12 target genes chosen for validation.**
253
254 **Fig 4. Pearson`s correlation (r) between Log2FC values of RNA-Seq and qPCR analyses for**
255 **the 12 target genes selected for validation.**
256
257 ***In silico* analyses**
258 From the 230 DE genes found in this study, 161 were selected for the *in silico* analyses
259 considering the Log2FC interval from -1.5 to +1.5. From this set of genes, 91 were identified in
260 David 6.8 database, comprising BP, molecular functions (MF) and cellular components (CC). A
261 total of 68 significant BP ($p < 0.05$) were identified, which were grouped using Revigo in 20
262 superclusters: cell adhesion, lymphocyte activation, extracellular matrix organization, cell
263 activation, biological adhesion, regulation of cell proliferation, immune system, among others
264 (Fig 5, S2 Table). The main cell components were involved in the cell matrix, T cells and cell
265 membrane, comprising the molecular functions related to calcium activity, molecular
266 transduction and receptor activity (Fig 6).
267
268 **Fig 5**. **Significant biological processes of differentially expressed (DE) genes related to**
269 **umbilical hernia in pigs.** The X-axis shows the total number of genes that were DE in each
270 biological process, based on the genetic ontology using the David 6.8.
271
272 **Fig 6**. **Significant cell components and molecular functions of differentially expressed (DE)**
273 **genes related to umbilical hernia in pigs.** The X-axis shows the total number of genes that were
274 DE in each of them, based on the genetic ontology using the David 6.8 database.
275
276 **Gene network**
277 The constructed gene network (Fig 7) based on the DE genes, the BP and the cluster performed
278 with Revigo showed the probable bioprocesses that would be the most involved with the

umbilical hernia development. A set of 10 BP was selected for constructing the gene network: development of anatomical structure, biological and cellular adhesion, lymphocyte activation, leukocyte proliferation, extracellular matrix organization, development processes, and multicellular processes of the organism and regulation of stimulus responses. In this analysis, we were able to relate 53 genes distributed according to their interactions and importance in these processes.

Fig 7. Gene network constructed with differentially expressed genes and main biological processes related to umbilical hernia using Cytoscape. Differentially expressed genes are visualized in the circles and biological processes in the rectangles. Node sizes indicate the number of predicted gene interactions. The edge colors indicate the betweenness of the edges (low values are in small size and in bright colors).

DE genes located in QTL regions for umbilical hernia

With the 230 DE genes, a query performed in the pig QTLdb pointed out that only six of these genes were located in QTL regions previously associated to UH. Four of them were mapped to SSC1: Alkaline ceramidase 2 (*ACER2*), Solute carrier family 2 member 6 (*SLC2A6*), Prostaglandin-endoperoxide synthase 1 (*PTGS*1) and *LGALS3*; one was located in SSC2: KN motif and ankyrin repeat domains 3 (*KANK3*) and the other in SSC7: *FOS*. Moreover, three other DE genes found in our study were located in QTL regions for scrotal/inguinal hernias: *ACAN* mapped in SSC7, Butyrylcholinesterase (*BCHE*) in SSC13 and *KANK3* in SSC2.

Discussion

Some studies have been performed to identify the genetic factors involved in the development of umbilical hernias, and QTL regions, SNPs and candidate genes associated with the appearance of this defect have been detected [5, 8, 9, 10]. In this study, using RNA-Seq, we sequenced the umbilical ring transcriptome to discover possible genes involved in the occurrence of umbilical hernias in pigs. A total of 230 DE genes were identified between normal and UH-affected animals (S1 Table). Twelve of them were selected to be validated by qPCR, which has confirmed the consistence of our RNA-Seq findings (Fig 3 and 4). Since the etiology of umbilical hernias is not yet fully understood, identifying genes and biological processes involved in the development

of this hernia is essential to find strategies to reduce this anomaly in pig production systems. From the 68 BPs found in our study, the following can be highlighted and will be further discussed: extracellular matrix, cell adhesion, development of the anatomical structure and immune system. This allowed a closer observation of the relationship of the DE genes with BPs in the organism.

Extracellular Matrix

The extracellular matrix (ECM) provides support and resistance to the tissues and organs of the whole body, acting in biochemical processes related to morphogenesis, differentiation and homeostasis of the tissues [26]. Also, in the ECM there are molecules responsible for adhesion, migration, proliferation, differentiation and cellular survival of the tissue [27]. This very organized structure is divided into molecules responsible for the formation of fibers (collagens, elastin and fibronectins) and the interfibrillar proteoglycans and glycoproteins bridges [28, 29]. In the fiber-forming group, the collagens can be highlighted, since they are responsible for the development and tissue resistance, and regulation of cell adhesion [30]. In the interfibrillar group, we highlight the proteoglycans that act in several functions, mainly related to binding, hydration, transport and resistance to force [26].

Two BPs were associated with ECM and both had the five clustered genes: *ACAN*, *MMP13*, Matrix metallopeptidase 9 (*MMP9*), Serine peptidase inhibitor, kunitz type 1 (*SPINT1*) and *VIT*. In addition, five cellular components related to matrix were found (Fig 6) and the most enriched genes were *ACAN*, *SPINT1*, *VIT*, *MMP13*, *MMP9*, Collagen type XI alpha 2 chain (*COL11A2*), *CCBE1*, *LGALS3*, Collagen type VI alpha 5 chain (*COL6A5*), cathepsin W (*CTSW*), S100 Calcium binding protein A4 (*S100A4*) and *EPYC*. The association of these genes in ECM bioprocesses and their location in extracellular matrix-related cell component (CC) reinforce their relationship with structural support and important biochemical signals in the cells and tissues of animals [31]. The identified genes involved in the maintenance of ECM clarify the relationship between ECM and umbilical hernias. It is known that problems in the connective tissue, such as disturb in collagen production, have already been associated with the appearance of hernias [32,33]. Furthermore, in the gene network it was possible to group genes that were enriched in ECM bioprocesses (Fig 7), such as *ACAN*, *MMP13*, *MMP9*, *SPINT1* and *VIT*, which were DE in our work.

Matrix metallopeptidase (*MMP*) family members are directly bound to collagen degradation and regulation. The *MMP13* gene is responsible for the degradation of type II collagen in cartilage and *MMP9* degrades type VI collagen [34, 35]. In the present study, the *MMP13* and *MMP9* genes were 7.3 and 3.9 times less expressed in animals affected by umbilical hernia than in normal animals. The downregulation of these genes may be related to tissue disorders due to their role in collagen production. Also, three other genes of the collagen family were downregulated in the affected pigs: *COL6A5* (-4,3); *COL11A2* (-3.7) and Collagen type II Alpha 1 chain (*COL2A1;* -3.4). The collagen family genes are involved with the production of fibers, structural organization and strength to the connective tissues in the animal organism [36, 37]. The relationship of metallopeptidase genes, such as *MMP1* and *MMP13*, with inguinal hernias in humans has already been verified [38]. Moreover, *MMP2*, *COL2A1*, *COL1A2* and *COL1A1* genes were associated to scrotal herniation in pigs [39]. In addition, mutations in *COL6A1*, *COL6A2* and *COL6A3* genes were considered causal mutations for congenital muscular dystrophy, a disease that affects connective and muscular tissue in humans [40]. Zhang et al. (2002) [41] reported that mutations in collagen genes *COL6A1*, *COL6A2* and *COL6A3* led to their mRNA decay. Moreover, Sabatelli et al. (2012) [42], demonstrated that *COL6A5* plays a key role in the tensile stress of connective tissue. Tagliavini et al. (2014) [43], verified that a defect in the *COL6A6* gene might contribute to collagen-related disorders.

There are several studies addressing MMP and collagen families as responsible for problems in the tissue and also for the manifestation of several types of hernias in different species [39-43]. Our findings are similar to those already reported, indicating that those genes related to ECM might be possibly triggering umbilical hernia in pigs. Thus, the downregulation of this set of genes could lead to problems related to the production of collagen, consequently causing tissue weakness and injury and, eventually, the formation of umbilical hernia.

Cell adhesion

The cell adhesion BP is highly related to biological adhesion (Fig 7), both allowing cellular connections and binding to organisms or substrate. In particular, cell adhesion is directly linked to adhesion to the extracellular matrix [28], tissue development and maintenance, cell differentiation, migration, communication, regulation and survival [44,45]. In our study, 19 DE genes were grouped in this BP: *ACAN*, molecule CD2 (*CD2*), *CD3D*, molecule CD5 (*CD5*),

molecule CD6 (*CD6*), molecule CD8A (*CD8A*), cadherin 7 (*CDH7*), desmoglein 2 (*DSG2*), indoleamine 2, 3-dioxygenase 1 (*IDO1*), interleukin 7 receptor (*IL7R*), Lymphocyte Cell-Specific Protein-Tyrosine Kinase (*LCK*), *LGALS3*, myelin protein zero like 2 (*MPZL2*), NLR Family Contingency Domain 3 (*NLRC3*), 5'-nucleotidase ecto (*NT5E*), *PKP3*, protein tyrosine phosphatase, type U receptor (*PTPRU*), transglutaminase 2 (*TGM2*) and *VIT* (Fig 7, S2 Table).

The *ACAN* gene belongs to the aggrecan proteoglycan family and was 2.8 times more expressed in the hernia-affected animals than in the normal pigs (Table 1). This gene has an important role in the cell adhesion process of the matrix, providing integrity, binding and resistance to cartilaginous tissue [46]. Polymorphisms in the *ACAN* have already been associated with hernias and cartilage degeneration [47,48]. However, there are no studies on the levels of *ACAN* gene expression for comparison. The observed *ACAN* upregulation in UH-affected pigs can lead to an exacerbated production of collagen. Association between abnormal amount of collagen and herniation was already reported [49]. Moreover, the *ACAN* has also been associated with other types of hernia, such as disc hernia [50].

The *VIT* gene was 3.7 times downregulated in the affected animals (S1 Table). *VIT* is responsible for encoding a protein related to the ECM and also participates in cell adhesion and cell migration [51,52]. The vitrin protein is similar to proteins that participate in the neural development and in the integrity of the extracellular matrix [53-55]. In the intact human cartilaginous tissues, higher levels of *VIT* were observed, whereas in tissues with cartilage problems the expression of this gene was reduced [56]. The *VIT* downregulation detected in the present study may disturb the production of cell adhesion proteins, reducing the integrity of the umbilical ring making this tissue more susceptible to hernia occurrence.

Two other genes were clustered only in the cellular and biological adhesion processes: Cadherin 7 (*CDH7*) and Leucine Rich Repeat Containing 15 (*LRRC15*), being, respectively, 2.5 and 1.8 times upregulated in the affected group compared to the normal pigs (S1 Table). The *CDH7* gene is involved in the cell structural and functional organization in various tissues [57]. Cadherins have a very important role in the cell adhesion process promoting cell binding, and mutations in these genes were associated with delayed growth and development [58,59]. The *LRRC15* gene is involved with cellular interactions, acting mainly on cell adhesion and on cell-cell and extracellular matrix interactions [60]. In animals, the actions of *LRRC15* are still poorly understood, however, in humans, cancer-damaged tissues had higher expression of this gene than

normal tissues [61]. Possibly, the imbalance in the expression of the genes grouped in those BPs can interfere in tissue remodeling, causing disorders related to muscular fibers, weakening the umbilical ring tissue and favoring the occurrence of umbilical hernias in pigs.

Development of anatomical structure

Twenty-five DE genes were grouped in the development of anatomical structure BP (Fig 5, S2 Table). Furthermore, 11 of them participate in extracellular matrix CC and four enriched molecular functions related to calcium ion bonds (Fig 6). From this set of genes, we highlight *KRT14* (Keratin 14), *CCBE1*, *ACAN*, Desmoglein 2 (*DSG2*) and *EPYC*, which were more enriched in the anatomic development process in the gene network (Fig 7) and were upregulated in animals affected by umbilical hernia. The *KRT14* gene encodes proteins from the keratin family, which are structural proteins that provide skin resistance and elasticity [62,63]. Studies in humans have identified mutations in this gene being responsible for different skin diseases [62-65].

The *EPYC* gene, previously known as Dermatan Sulfate Proteoglycan 3 (*DSPG3*), is predominantly expressed in cartilaginous tissues [66,67]. *EPYC* has main functions in the fibrilogenesis, which is characterized by the development and regulation of collagen fibrils in the embryonic period [68]. Tajima et al. (1999) [69], demonstrated that deficiency in the *EPYC* gene could cause Ehlers-Danlos syndrome in Dutch breed calves. This hereditary syndrome causes a defect in the connective tissue, due to changes in collagen synthesis and/or assembly of the collagen structure [70-72]. Due to the problems in the fibrillar collagen, the skin becomes fragile and with high risk of rupture [72]. Several studies have associated this syndrome with defects in collagen production [73], however, the actual function of the *EPYC* is not yet well known in pigs.

Two other gene, *DSG2* and *CCBE1,* were enriched in the anatomical structure BP and grouped in the calcium ion binding MF. The *DSG2* gene belongs to the desmoglein family, which are important components of the cadherins that integrate the desmosomes [74]. Desmossomes are characterized as structures of cell-to-cell linkages, which provide mechanical stability [74,75] and are crucial for embryonic development and tissue integrity [76]. Moreover, *DSG2* is expressed in many tissues and participates in calcium binding and cell adhesion [75]. The *CCBE1* gene encodes proteins responsible for the remodeling and migration of the extracellular matrix and is directly related to the calcium and collagen [77,78]. In humans, studies have shown that

mutations in the *CCBE1* gene are associated with genetic problems, including the occurrence of umbilical and inguinal hernias [79-81]. Thus, the differential expression of these genes related to the development of the anatomical structure is a strong indication that alterations in this BP can cause deregulations or modifications in the structure of the tissue. These findings are reinforced by the histological changes observed in the umbilical ring tissue between the normal and UH-affected pigs. The umbilical ring of animals affected with umbilical hernia was thickened by an abundant proliferation of dense connective tissue, while a normal amount of collagen fibers of connective tissue interspersed with adipose tissue was found in the umbilical ring region of the normal pigs (Fig 1).

Immune system

In this study, several DE genes enriched BP related to the immune system, where most of them were downregulated in the affected animals (S2 Table). Also, the gene network indicated three significant BP: lymphocyte activation, leukocyte proliferation and immune system process (Fig 7). These three similar processes grouped 21 genes, of which 11 were grouped in membrane CC and six of them in molecular function of transduction activity (Fig 5 and 6). Here, the cluster of differentiation (CD) gene family were highly represented by the *CD2*, *CD3D*, *CD5*, *CD6*, *CD247* and *CD8A* genes, that were downregulated in UH-affected animals compared to the normal pigs. These CD genes are directly linked to other biological processes, such as regulation of response to stimuli, development of anatomical structure, cell adhesion, and developmental processes (Fig 7). For instance, the *CD3D* gene is responsible for encoding a T cell receptor protein and performing signal transduction [82]. Another gene of this family, *CD34*, was downregulated in humans affected by inguinal hernias [83]. However, information about these genes are scarce in the literature and here is the first time that these genes are being associated with umbilical hernia in pigs.

The *EDIL3* (EGF as repeats and discoid domains 3) is another immune-related gene and was 3 times upregulated in affected animals compared to normal pigs (Table S1). Studies have indicated that when there is injury, epithelial cells, macrophages and fibroblasts produce growth factors such as epidermal growth factor (EGF) and transforming growth factor (TGF) to prevent the problem [84]. When this occurs, there is an increase in the epithelial-mesenchymal transition, which is responsible for the healing, regeneration and fibrogenesis of the tissue [85-87].

However, when the animal has a disease that cannot be controlled, it becomes chronic and may result in an increase in the expression of EGF, fibronectin and proteoglycans [88]. In our study, the upregulation of genes responsible for the production of EGF (*EDIL3*) and proteoglycans (*ACAN* and *EPYC*) indicates that this expression profile is due to the advanced process of irreversible tissue degradation, where tissue repair genes are no longer active [89,90], and the *EDIL3*, *ACAN* and *EPYC* genes are over expressed producing high levels of proteoglycans and fibronectin. This may account for the accumulation and increased proliferation of dense connective tissue in pig tissue samples affected with umbilical hernia as observed in the histopathology (Fig 1). Thus, the downregulated genes clustered in the immune system BP are probably a consequence of the umbilical hernia, while the upregulated ones are possibly involved in the cause of the histological changes of the UH-affected tissue.

Genes located in QTL regions

Ding et al. (2009) [8] were the first to identify regions related to the occurrence of umbilical hernias in pigs. Afterwards, other studies also identified regions related to umbilical hernias in pigs [5,9,10]. Six of the genes DE in our study were mapped to QTL regions already described in the literature for umbilical hernias in pigs: *ACER2*, *SLC2A6*, *PTGS1*, *LGALS3*, *KANK3* and *FOS*. These genes have very distinct functions, such as cell proliferation and survival (*ACER2*) [91], regulation of prostaglandin (*PTGS1*) [92] and glucose transport (*SLC2A6*) [93].

The *LGALS3* gene is part of membrane CC and was enriched in the gene network due to its involvement in several BP (Fig 7). Besides harboring a QTL region for umbilical hernia, *LGALS3* differential expression profile was confirmed by the qPCR methodology. This gene encodes a protein located in the extracellular matrix that acts in cell growth, survival, migration and adhesion [94]. *LGALS3* is also involved in the cellular apoptosis and innate immunity [95,96]. Another gene found in a QTL region for umbilical hernia is the *FOS*, which was upregulated in affected pigs and also validated by qPCR. The *FOS* gene has a role in survival, proliferation, differentiation and cell death, organogenesis and stress response [97]. Studies with cancer patients have shown that *FOS* upregulation was correlated with the increase in cell death [98,99].

Furthermore, three other genes were located in QTL regions already identified for scrotal/inguinal hernias [8,100]: *ACAN*, *BCHE* and *KANK3*. The *ACAN* gene, which has been

previously mentioned, related to cell adhesion and extracellular matrix [46], has been pointed out as a potential gene involved in the occurrence of hernias. The localization of *ACAN* in the QTL region for scrotal/inguinal hernias suggests a pleiotropic effect of this gene, being also involved in the manifestation of umbilical hernia.

The *KANK3* gene was downregulated in the affected animals and has been mapped to a QTL region for umbilical hernia and for scrotal/inguinal hernia [8]. This indicates a possible pleiotropic effect of *KANK3* in the manifestation of various types of hernias in pigs. Genes from *KANK* family (*KANK1* and *KANK2*) have already been related to the polymerization of actin filaments, fiber formation and cell migration [101]. These genes drive many cellular processes [102], especially those of transport and muscle contraction. This action may be related to the problem of umbilical hernia, since actin filaments are an important part of the body and especially of the muscle [103]. Furthermore, actin polymerization together with that of calcium is a key part of the adhesion process of epithelial cells [104]. Therefore, *KANK3* become a strong functional candidate to the development of umbilical hernia in pigs.

Through the characterization of the umbilical ring transcriptome of normal and umbilical hernia-affected pigs, a set of DE genes was prospected. In the genetic ontology analysis of the DE genes, several biological processes and molecular functions possibly related to the herniation process were identified. The constructed gene network helped to understand the functional importance of some of these transcripts. Thus, according to our results, the main biological processes involved with umbilical hernia were related to extracellular matrix, immune system, anatomical development, cell adhesion, membrane components, receptor activation, calcium binding and immune synapse. Therefore, genes involved in these main groups, such as *ACAN*, *MMP's*, *COL's*, *EPYC*, *VIT*, *LRRC15*, *CCBE1* and *LGALS3* are strong candidates for the occurrence of umbilical hernia in pigs and in other mammals. Additionally, in this study, the stability of 10 endogenous candidate genes in the inguinal ring tissue was evaluated since no specific reference genes were reported for this tissue in pigs to date. Therefore, the *H3F3A* and *RPL32* were the most reliable reference genes under this experimental condition to obtain accurate gene expression profiles in this complex tissue.

Conclusions

We have generated the first transcriptome of the pig umbilical ring tissue, which allowed the identification of several genes that had not yet been related to umbilical hernias in pigs. The results pointed out *ACAN*, *MMPs*, *COLs*, *EPYC*, *VIT*, *CCBE1* and *LGALS3* genes as strong candidates to trigger umbilical hernias in pigs. Nevertheless, further studies are needed to identify the causal mutations, SNPs and CNVs in these genes to improve our understanding of the mechanisms of gene regulation. The knowledge of the genetic factors involved in the manifestation of umbilical hernia brings perspectives for elaborating strategies to identify animals with alleles related to this pathology, in order to reduce the occurrence of umbilical hernia in pig production systems.

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Author Contributions

Conceived and designed the experiment: JOP MCL AMGI.

Performed the experiment: MRS AMGI IRS JOP MAZM MCL

Data analysis and curation: MRS AMGI MEC

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Funding Acquisition and supervision of the research: MCL

Competing Interests

The authors declare that they have no competing interests.

References

1. Gerber PJ, Vellinga T V., Steinfeld H. Issues and options in addressing the environmental consequences of livestock sector's growth. Meat Sci. Elsevier; 2010;84: 244–247. doi:10.1016/j.meatsci.2009.10.016

2. Alexandratos N, Bruinsma J. World agriculture towards 2030/2050: the 2012 revision. Food and Agriculture Organization of the United Nations. 2012;03: 1-160. Disponivel em: http://www.fao.org/economic/esa

3. Petersen HH, Nielsen EO, Hassing AG, Ersbøll AK, Nielsen JP. Prevalence of clinical signs of disease in Danish finisher pigs. Vet Rec. 2008;162: 377–382. doi:10.1136/vr.162.12.377

4. Searcy-Bernal R, Gardner IA, Hird DW. Effects of and factors associated with 469 umbilical hernias in a swine herd. J Am Vet Med Assoc. 1994;204: 1660–1664.

5. Liao XJ, Li L, Zhang ZY, Long Y, Yang B, Ruan GR, et al. Susceptibility Loci for Umbilical Hernia in Swine Detected by Genome-Wide Association. Russ J Genet. 2015;51: 1163–1170. doi:10.7868/s0016675815100100

6. Thaller G, Dempfle L, Hoeschele I. Investigation of the inheritance of birth defects in swine by complex segregation analysis. J Anim Breed Genet. 1996;113: 77–92. doi:10.1111/j.1439-0388.1996.tb00593.x

7. Hayes HM. Congenital umbilical and inguinal hernias in cattle, horses, swine, dogs and cats: risk by breed and sex among hospital patients. Am J Vet Res. 1974;35: 839–42.

8. Ding NS, Mao HR, Guo YM, Ren J, Xiao SJ, Wu GZ, et al. A genome-wide scan reveals candidate susceptibility loci for pig hernias in an intercross between White Duroc and Erhualian. J Anim Sci. 2009;87: 2469–2474. doi:10.2527/jas.2008-1601

9. Long Y, Su Y, Ai H, Zhang Z, Yang B, Ruan G, et al. A genome-wide association study of copy number variations with umbilical hernia in swine. Anim Genet. 2016;47: 298–305. doi:10.1111/age.12402

10. Grindflek E, Hansen MHS, Lien S, van Son M. Genome-wide association study reveals a QTL and strong candidate genes for umbilical hernia in pigs on SSC14. BMC Genomics. BMC Genomics; 2018;19: 1–9. doi:10.1186/s12864-018-4812-9

11. Li X, Xu P, Zhang C, Sun C, Li X, Han X, et al. Genome-wide association study identifies variants in the CAPN9 gene associated with umbilical hernia in pigs. Anim Genet. 2019; 10–13. doi:10.1111/age.12760

12. Fernandes LT, Ono RK, Ibelli, AMG, Lagos EB, Morés MAZ, Cantão ME, et al. Novel putative candidate genes associated with umbilical hernia in pigs. In: World Congress on Genetics Applied to Livestock Production. Auckland, New Zealand. 2018. Available from: http://www.wcgalp.org/proceedings/2018/novel-putative-candidate-genes-associated-umbilical-hernia-pigs

13. Zhbannikov IY, Hunter SS, Foster JA, Settles ML. SeqyClean. 2017; 407–416. doi:10.1145/3107411.3107446

14. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29: 15–21. doi:10.1093/bioinformatics/bts635

15. Anders S, Pyl PT, Huber W. 2015. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31: 166–169. doi:10.1093/bioinformatics/btu638

16. Robinson MD, Mccarthy DJ, Smyth GK. EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26: 139–140, 1 January 2010. doi:10.1093/bioinformatics/btp616

17. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013.

18. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. 1995;57: 289-300.

19. Lorenzetti WR, Ibelli AMG, Peixoto JO, Mores MAZ, Savoldi IR,Carmo KB, et al. Identification of endogenous normalizing genes for expression studies in inguinal ring tissue for scrotal hernias in pigs. PLoS ONE. 2018;13: 9. doi:10.1371/journal.pone.0204348

20. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012;13: 134. doi:10.1186/1471-2105-13-134

21. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26: 509–515. doi:10.1023/B:BILE.0000019559.84305.47

22. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3: research0034.1–0034.11. doi:10.1186/gb-2002-3-7-research0034

23. Andersen CL, Jensen JL, Ørntoft TF. Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. Cancer Res. 2004;64: 5245–5250. doi:10.1158/0008-5472.CAN-04-0496

24. Pihur V, Datta S, Datta S. Weighted rank aggregation of cluster validation measures: a Monte Carlo cross-entropy approach. Bioinformatics. 2007;23: 1607–1615. doi:10.1093/bioinformatics/btm158

25. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13: 2498–2504. doi:10.1101/gr.1239303

26. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci. 2010;123: 4195–4200. doi:10.1242/jcs.023820

27. Daley WP, Peters SB, Larsen M. Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci. 2008;121: 255–264. doi:10.1242/jcs.006064

28. Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, et al. Molecular Biology of the Cell. 6th ed. Garland Science; 2014.

29. Jarvelainen H, Sainio A, Koulu M, Wight TN, Penttinen R. Extracellular matrix molecules: potential targets in pharmacotherapy. Pharmacol. Rev. 2009;61: 198-223. doi: 10.1124/pr.109.001289

30. Rozario T, DeSimone DW. The extracellular matrix in development and morphogenesis: A dynamic view. Dev Biol. Elsevier Inc.; 2010;341: 126–140. doi:10.1016/j.ydbio.2009.10.026

31. Dragos A, Kovács, AT. The Peculiar Functions of the Bacterial Extracellular Matrix. Trends In Microbiology. 2017;25: 257-266. doi:10.1016/j.tim.2016.12.010

32. Peacock J. Fascia and muscle. In: Peacock J, editor. Wound repair. 3rd edition. Philadelphia: W.B. Saunders; 1984. pp. 332–362.

33. Glorieux FH. Osteogenesis imperfecta. Best Pract Res Clin Rheumatol. 2008;22: 85 – 100. doi:10.1016/j.berh.2007.12.012

34. Vincenti MP, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: Integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res. 2002;4: 157–164. doi:10.1186/ar401

35. Zempo N, Kenagy RD, Au YPT, Bendeck M, Clowes MM, Reidy MA, et al. Matrix metalloproteinases of vascular wall cells are increased in balloon-injured rat carotid artery. J Vasc Surg. 1994;20: 209–217. doi:10.1016/0741-5214(94)90008-6

36. Antoniou SA, Antoniou GA, Granderath FA, Simopoulos C. The role of matrix metalloproteinases in the pathogenesis of abdominal wall hernias. Eur J Clin Invest. 2009;39: 953–959. doi:10.1111/j.1365-2362.2009.02199.x

37. Sabatelli P, Gara SK, Grumati P, Urciuolo A, Gualandi F, Curci R, et al. Expression of the collagen VI α5 and α6 Chains in normal human skin and in skin of patients with collagen VI-related myopathies. J Invest Dermatol. Elsevier Masson SAS; 2011;131: 99–107. doi:10.1038/jid.2010.284

38. Zheng H, Si Z, Kasperk R, Bhardwaj RS, Schumpelick V, Klinge U, et al. Recurrent inguinal hernia: Disease of the collagen matrix? World J Surg. 2002;26: 401–408. doi:10.1007/s00268-001-0239-5

39. Zhao X, Du Z, Vukasinovic N, Rodriguez F, Clutter AC, Max F. Association of HOXA10, ZFPM2, and MMP2 genes with scrotal hernias evaluated via biological candidate gene analyses in pigs. Am J Vet Res. 2009;70: 1006–1012. doi:doi.org/10.2460/ajvr.70.8.1006

40. Camacho Vanegas O, Bertini E, Zhang R-Z, Petrini S, Minosse C, Sabatelli P, et al. Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci. 2001;98: 7516–7521. doi:10.1073/pnas.121027598

41. Zhang RZ, Sabatelli P, Pan TC, Squarzoni S, Mattioli E, Bertini E, et al. Effects on collagen VI mRNA stability and microfibrillar assembly of three COL6A2 mutations in two families with Ullrich congenital muscular dystrophy. J Biol Chem. 2002; 277: 43557–43564. doi:10.1074/jbc.M207696200

42. Sabatelli P, Gualandi F, Gara SK, Grumati P, Zamparelli A, Martoni E, et al. Expression of collagen VI α5 and α6 chains in human muscle and in Duchenne muscular dystrophy-related muscle fibrosis. Matrix Biol. International Society of Matrix Biology; 2012; 31: 187–196. doi:10.1016/j.matbio.2011.12.003

43. Tagliavini F, Pellegrini C, Sardone F, Squarzoni S, Paulsson M, Wagener R, et al. Defective collagen VI α6 chain expression in the skeletal muscle of patients with collagen VI-related myopathies. Biochim Biophys Acta - Mol Basis Dis. Elsevier B.V.; 2014; 1842: 1604–1612. doi:10.1016/j.bbadis.2014.05.033

44. Huang S, Ingber DE. The structural and mechanical complexity of cell-growth control. Nature Cell Biology. 1999;1: 131-13. doi:10.1038/13043

45. Khalili AA, Ahmad MR. A Review of cell adhesion studies for biomedical and biological applications. Int J Mol Sci. 2015; 16: 18149–18184. doi:10.3390/ijms160818149

46. Heinegard D, Oldberg A. Structure and biology of cartilage and bone matrix non collagenous macromolecules. FASEB J. 1989;3: 2042–2051. doi:10.1096/fasebj.3.9.266358

47. Eser B, Cora T, Eser O, Kalkan E, Haktanir A, Erdogan MO, et al. Association of the Polymorphisms of Vitamin D Receptor and Aggrecan Genes with Degenerative Disc Disease. Genetic Testing and Molecular Biomarkers. 2010;14: 313-317. doi:10.1089/gtmb.2009.0202

48. Mayer JE, Iatridis JC, Chan D, Qureshi SA, Gottesman O, Hecht AC. Genetic polymorphisms associated with intervertebral disc degeneration. Spine J. Elsevier Inc; 2013;13: 299–317. Doi:10.1016/j.spinee.2013.01.041

49. Harrison B, Sanniec K, Janis J. Collagenopathies—Implications for Abdominal Wall Reconstruction: A Systematic Review. Plast Reconstr Surg Glob Open. 2016;10: e1036. doi:10.1097/GOX.0000000000001036

50. Perera RS, Dissanayake PH, Senarath U, Wijayaratne LS, Karunanayake AL, Dissanayake VHW. Variants of ACAN are associated with severity of lumbar disc herniation in patients with chronic low back pain. PLoS One. 2017;7: e0181580. doi:10.1371/journal.pone.0181580

51. Colombatti A, Bonaldo P, Doliana R. Type A modules: interacting domains found in several non-fibrillar collagens and in other extracellular matrix proteins. Matrix. 1993;13: 297–306. doi:10.1016/S0934-8832(11)80025-9

52. Whittaker CA, Hynes RO. Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol. Biol. Cell 2002;13: 3369–3387. doi:10.1016/S0934-8832(11)80025-9

53. Zhang J, Fei T, Li Z, Zhu G, Wang L, Chen YG. BMP induces cochlin expression to facilitate self-renewal and suppress neural differentiation of mouse embryonic stem cells. J. Biol. Chem. 2013;288: 8053–8060. doi:10.1074/jbc.M112.433995

54. Bae SH, Robertson NG, Cho HJ, Morton CC, Jung DJ, Baek JI, et al. Identification of pathogenic mechanisms of COCH mutations, abolished cochlin secretion, and intracellular aggregate formation: genotype-phenotype correlations in DFNA9 deafness and vestibular disorder. Hum. Mutat. 2014;35: 1506–1513. doi:10.1002/humu.22701

55. Abdulhaleem MF, Song X, Kawano R, Uezono N, Ito A, Ahmed G, et al. (2015). Akhirin regulates the proliferation and differentiation of neural stem cells inintact and injured mouse spinal cord. Dev. Neurobiol. 2015;75: 494–504. doi:10.1002/dneu.22238

56. Sato T, Konomi K, Yamasaki S, Aratani S, Tsuchimochi K, Yokouchi M, et al. Comparative analysis of gene expression profiles in intact and damaged regions of human osteoarthritic cartilage. Arthritis Rheum. 2006;54: 808–817. doi:10.1002/art.21638

57. Kremmidiotis G, Baker E, Crawford J, Eyre HJ, Nahmias J, Callen DF. Localization of human cadherin genes to chromosome regions exhibiting cancer-related loss of heterozygosity. Genomics. 1998;49: 467–471. doi:10.1006/geno.1998.5281

58. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science. 1991;251: 1451-1455. doi:10.1126/science.2006419

59. Gumbiner BM. Cell Adhesion: The Molecular Basis of Tissue Architecture and Morphogenesis. Cell. 1996;84: 345-357. doi:10.1016/S0092-8674(00)81279-9

60. Satoh K, Hata M, Yokota H. A novel member of the leucine-rich repeat superfamily induced in rat astrocytes by β-amyloid. Biochem Biophys Res Commun. 2002;290: 756–762. doi:10.1006/bbrc.2001.6272

61. Purcell JW, Tanlimco SG, Hickson J, Fox M, Sho M, Durkin L, et al. LRRC15 Is a Novel Mesenchymal Protein and Stromal Target for Antibody–Drug Conjugates. Cancer Research. 2018;78: 4059-4072. doi:10.1158/0008-5472.CAN-18-0327

62. Csikós M, Szalai Z, Becker K, Sebõk B, Schneider I, Horváth A, et al. Novel keratin 14 gene mutations in patients from Hungary with epidermolysis bullosa simplex. Exp Dermatol. 2004;13: 185–191. doi:10.1111/j.0906-6705.2004.0120.x

63. Müller FB, Küster W, Wodecki K, Almeida H, Bruckner-Tuderman L, Krieg T, et al. Novel and recurrent mutations in keratin KRT5 and KRT14 genes in epidermolysis bullosa simplex: implications for disease phenotype and keratin filament assembly. Hum Mutat. 2006;27: 719–720. doi:10.1002/humu.9437

64. Lugassy J, Itin P, Ishida-Yamamoto A, Holland K, Huson S, Geiger D, et al. Naegeli-Franceschetti-Jadassohn Syndrome and Dermatopathia Pigmentosa Reticularis: Two Allelic Ectodermal Dysplasias Caused by Dominant Mutations in KRT14. Am J Hum Genet. 2006;79: 724–730. doi:10.1086/507792

65. Schuilenga-Hut PHL, Vlies P v.d., Jonkman MF, Waanders E, Buys CHCM, Scheffer H. Mutation analysis of the entire keratin 5 and 14 genes in patients with epidermolysis bullosa simplex and identification of novel mutations. Hum Mutat. 2003;21: 447. doi:10.1002/humu.9124

66. Deere M, Dieguez JL, Yoon SJK, Hewett-Emmett D, De La Chapelle A, Hecht JT. Genomic characterization of human DSPG3. Genome Res. 1999;9: 449–456.

67. Kurita K, Shinomura T, Ujita M, Zako M, Kida D, Iwata H, et al. Occurrence of PG-Lb, a leucine-rich small chondroitin/dermatan sulphate proteoglycan in mammalian epiphyseal cartilage: molecular cloning and sequence analysis of the mouse cDNA. Biochem J. 2015;318: 909–914. doi:10.1042/bj3180909

68. Wang P, Li S, Xiao X, Guo X, Zhang Q. An evaluation of OPTC and EPYC as candidate genes for high myopia. Mol Vis. 2009;15: 2045–2049.

69. Tajima M, Miyake S, Takehana K, Kobayashi A, Yamato O, Maede Y, et al. Gene Defect of Dermatan Sulfate Proteoglycan of Cattle Affected With a Variant Form of Ehlers-Danlos Syndrome. J Vet Intern Med. 1999;13: 202–205. doi:10.1111/j.1939-1676.1999.tb02179.x

70. Byers PH, Murray ML. Heritable Collagen Disorders: The Paradigm of the Ehlers—Danlos Syndrome. J Invest Dermatol. Elsevier Masson SAS; 2012;132: E6–E11. doi:10.1038/skinbio.2012.3

71. De Paepe A, Malfait F. The Ehlers-Danlos syndrome, a disorder with many faces. Clin Genet. 2012;82: 1–11. doi:10.1111/j.1399-0004.2012.01858.x

72. Spycher M, Bauer A, Jagannathan V, Frizzi M, De Lucia M, Leeb T. A frameshift variant in the COL5A1 gene in a cat with Ehlers-Danlos syndrome. Anim Genet. 2018;49: 641–644. doi:10.1111/age.12727

73. Neice AE, Stubblefield EE, Woodworth GE, Aziz MF. Peripheral nerve block in patients with Ehlers-Danlos syndrome, hypermobility type: A case series. J Clin Anesth. Elsevier Inc.; 2016;33: 26–30. doi:10.1016/j.jclinane.2016.01.005

74. Brooke MA, Nitoiu D, Kelsell DP. Cell-cell connectivity: Desmosomes and disease. J Pathol. 2012;226: 158–171. doi:10.1002/path.3027

75. Stephan S, Peter KWF. Identification of the Ubiquitous Human Desmoglein, DSG2, and the Expression Catalogue of the Desmoglein Subfamily of Desmosomal Cadherins. Exp Cell Res. 1994;211: 391–399. doi:10.1006/excr.1994.1103

76. Johnson JL, Najor NA, Green KJ. Desmosomes: Regulators of cellular signaling and adhesion in epidermal health and disease. Cold Spring Harb Perspect Med. 2014;4. doi:10.1101/cshperspect.a015297

77. Connell F, Kalidas K, Ostergaard P, Brice G, Homfray T, Roberts L, et al. Linkage and sequence analysis indicate that CCBE1 is mutated in recessively inherited generalised lymphatic dysplasia. Hum Genet. 2010;127: 231–241. doi:10.1007/s00439-009-0766-y

78. Hogan BM, Bos FL, Bussmann J, Witte M, Chi NC, Duckers HJ, et al. Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. Nat Genet. 2009;41: 396–398. doi:10.1038/ng.321

79. Ivanovski I, Akbaroghli S, Pollazzon M, Gelmini C, Caraffi SG, Mansouri M, et al. Van Maldergem syndrome and Hennekam syndrome: Further delineation of allelic phenotypes. Am J Med Genet Part A. 2018;176: 1166–1174. doi:10.1002/ajmg.a.38652

80. Jackson CC, Best L, Lorenzo L, Casanova JL, Wacker J, Bertz S, et al. A Multiplex Kindred with Hennekam Syndrome due to Homozygosity for a CCBE1 Mutation that does not Prevent Protein Expression. J Clin Immunol. 2016;36: 19–27. doi:10.1007/s10875-015-0225-6

81. Scheuerle AE, Sweed NT, Timmons CF, Smith ED, Alcaraz WA, Shinde DN. An additional case of Hennekam lymphangiectasia–lymphedema syndrome caused by loss-of-function mutation in ADAMTS3. Am J Med Genet Part A. 2018;176: 2858–2861. doi:10.1002/ajmg.a.40633

82. Benson M, Mobini R, Barrenäs F, Halldén C, Naluai ÅT, Säll T, et al. A haplotype in the inducible T-cell tyrosine kinase is a risk factor for seasonal allergic rhinitis. Allergy Eur J Allergy Clin Immunol. 2009;64: 1286–1291. doi:10.1111/j.1398-9995.2009.01991.x

892 83. Jiménez-Heffernan JA, Aguilera A, Aroeira LS, Lara-Pezzi E, Bajo MA, Del Peso G, et
893 al. Immunohistochemical characterization of fibroblast subpopulations in normal
894 peritoneal tissue and in peritoneal dialysis-induced fibrosis. Virchows Arch. 2004;444:
895 247–256. doi:10.1007/s00428-003-0963-3
896
897 84. Polosa R, Prosperini G, Tomaselli V, Howarth PH, Holgate ST, Davies DE. Expression of
898 c-erbB receptors and ligands in human nasal epithelium. J Allergy Clin Immunol.
899 2000;106: 1124–1131. doi:10.1067/mai.2000.110931
900
901 85. Boyer B, Vallés AM, Edme N. Induction and regulation of epithelial-mesenchymal
902 transitions. Biochem Pharmacol. 2000;60: 1091–1099. doi:10.1016/S0006-
903 2952(00)00427-5
904
905 86. Strutz F, Zeisberg M, Ziyadeh FN, Yang CQ, Kalluri R, Müller GA, et al. Role of basic
906 fibroblast growth factor-2 in epithelial-mesenchymal transformation. Kidney Int. 2002;61:
907 1714–1728. doi:10.1046/j.1523-1755.2002.00333.x
908
909 87. Zavadil J, Bitzer M, Liang D, Yang Y-C, Massimi A, Kneitz S, et al. Genetic programs of
910 epithelial cell plasticity directed by transforming growth factor- . Proc Natl Acad Sci.
911 2002;98: 6686–6691. doi:10.1073/pnas.111614398
912
913 88. Okuda S, Languino LR, Ruoslahti E, Border WA. Elevated expression of transforming
914 growth factor-beta and proteoglycan production in experimental glomerulonephritis. J
915 Clin Invest. 1990;86: 453–462. doi:10.1172/JCI114731
916
917 89. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008;214: 199 – 210.
918 doi:10.1002/path.2277
919
920 90. Eming SA, Krieg T, Davidson JM. Gene therapy and wound healing. Clin Dermatol.
921 2007;25: 79-92. doi:10.1016 / j.clindermatol.2006.09.011
922

91. Xu R, Jin J, Hu W, Sun W, Bielawski J, Szulc Z, et al. Golgi alkaline ceramidase regulates cell proliferation and survival by controlling levels of sphingosine and S1P. FASEB J. 2006;20: 1813–1825. doi:10.1096/fj.05-5689com

92. Helmersson J, Ärnlöv J, Axelsson T, Basu S. A polymorphism in the cyclooxygenase 1 gene is associated with decreased inflammatory prostaglandin F2α formation and lower risk of cardiovascular disease. Prostaglandins Leukot Essent Fat Acids. 2009;80: 51–56. doi:10.1016/j.plefa.2008.11.001

93. Doege H, Bocianski A, Joost HG, Schürmann A. Activity and genomic organization of human glucose transporter 9 (GLUT9), a novel member of the family of sugar-transport facilitators predominantly expressed in brain and leucocytes. Biochemical Journal. 2000;350: 771-776.

94. Liu FT. Regulatory roles of galectins in the immune response. Int Arch Allergy Immunol. 2005;136: 385–400. doi:10.1159/000084545

95. Leffler H, Carlsson S, Hedlund M. Qian Y, Poirier F. Introduction to galectins. Glycoconj J. 2004;19: 433–440. doi:10.1023/b:glyc.0000014072.34840.04

96. Rabinovich GA, Liu FT, Hirashima M, Anderson A. An emerging role for galectins in tuning the immune response: Lessons from experimental models of inflammatory disease, autoimmunity and cancer. Scand J Immunol. 2007;66: 143–158. doi:10.1111/j.1365-3083.2007.01986.x

97. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol. 2002;4: 131–136. doi:10.1038/ncb0502-e131

98. Appierto V, Villani MG, Cavadini E, Lotan R, Vinson C, Formelli F. Involvement of c-Fos in fenretinide-induced apoptosis in human ovarian carcinoma cells. Cell Death Differ. 2004;11: 270–279. doi:10.1038/sj.cdd.4401349

99. Mahner S, Baasch C, Schwarz J, Hein S, Wölber L, Jänicke F, et al. C-Fos expression is a molecular predictor of progression and survival in epithelial ovarian carcinoma. Br J Cancer. 2008;99: 1269–1275. doi:10.1038/sj.bjc.6604650

100. Sevillano CA, Lopes MS, Harlizius B, Hanenberg EH, Knol EF, Bastiaansen JW. Genome-wide association study using deregressed breeding values for cryptorchidism and scrotal/inguinal hernia in two pig lines. Genet Sel Evol. 2015;47: 1–8. doi:10.1186/s12711-015-0096-6

101. Gee HY, Zhang F, Ashraf S, Kohl S, Sadowski CE, Warner VV, et al. KANK deficiency leads to podocyte dysfunction and nephrotic syndrome. J Clin Invest. 2015;125: 2375–2384. doi:10.1172/jci79504

102. Mogilner A, Oster G. Cell motility driven by actin polymerization. Biophys J. Elsevier; 1996;71: 3030–3045. doi:10.1016/S0006-3495(96)79496-1

103. Korn E, Carlier M, Pantaloni D. Actin polymerization and ATP hydrolysis. Science. 1987;238: 638-644. doi:10.1126/science.3672117

104. Vasioukhin V, Bauer C, Yin M, Fuchs E. Directed Actin Polymerization Is the Driving Force for Epithelial Cell-Cell Adhesion-catenin associates with several other actin-binding. AJs Drubin Nelson. 2000;100: 209–219.

Supporting information

S1 Table: List of 230 differentially expressed genes between normal and umbilical hernia-affected piglets.

S1 Figure: Multi-Dimensional scaling (MDS) plot to visualize the separation between the five normal and the five umbilical hernia-affected piglets.

S2 Table: Main biological processes of genes differentially expressed between normal and umbilical hernia-affected piglets.

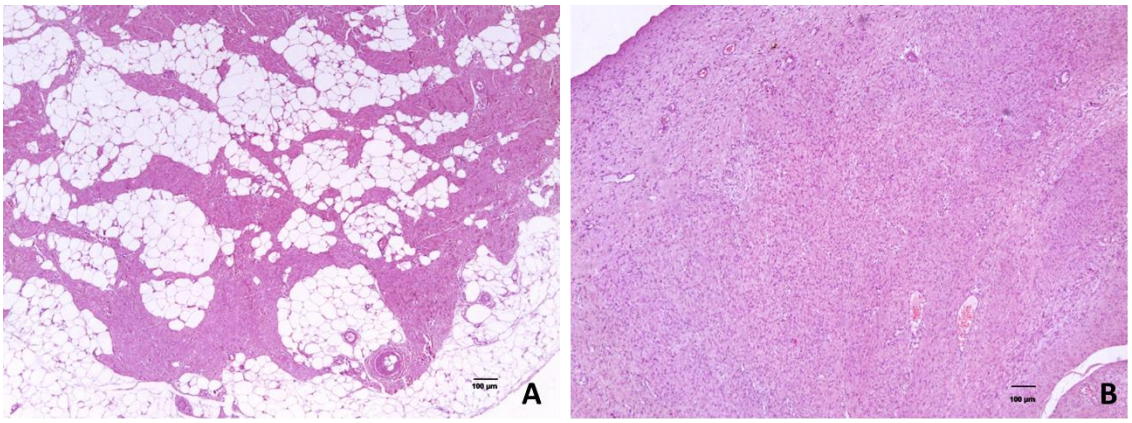


Fig 1. Hematoxylin & eosin stain histological section of the umbilical ring tissue sample from a normal (A) and a umbilical hernia-affected (B) piglet.

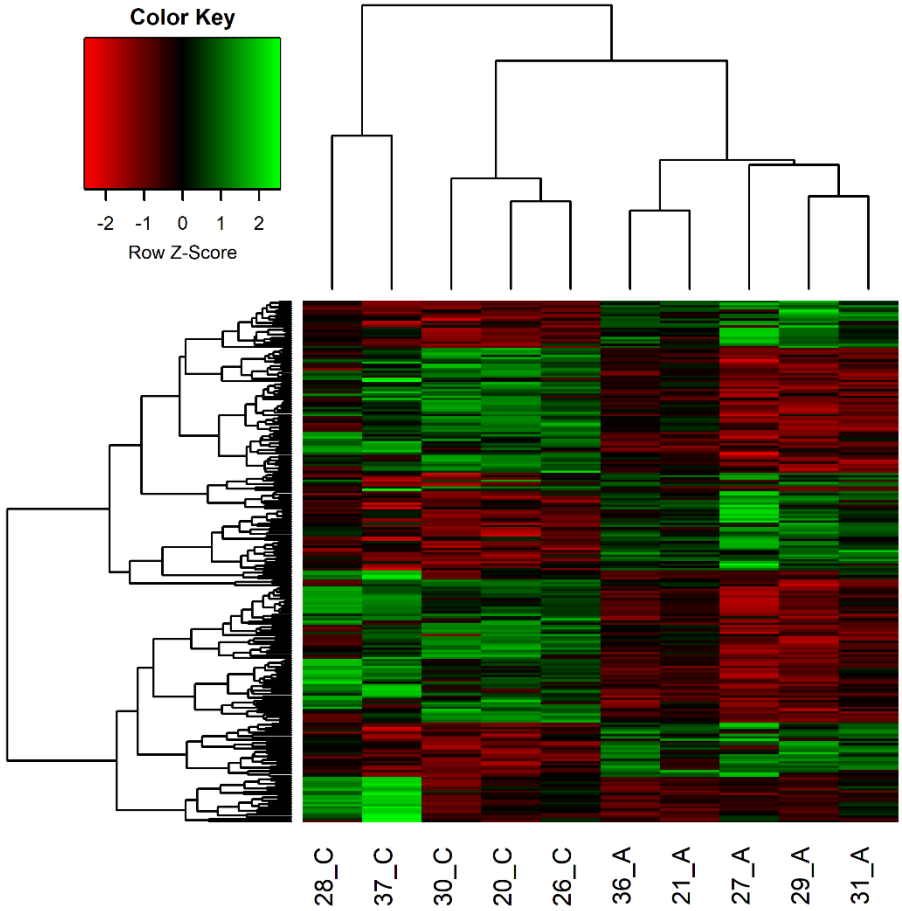


Fig 2. Heatmap with 230 differentially expressed genes between animals affected with umbilical hernia (21A, 27A, 27A, 29A, 31A and 36A) and normal piglets (20C, 26C, 28C, 30C and 37C). The expression for each gene is shown in the rows and samples are visualized in the columns, showing a hierarchical clustering of genes and samples. Genes are upregulated (in green) and downregulated (in red) in the affected samples.

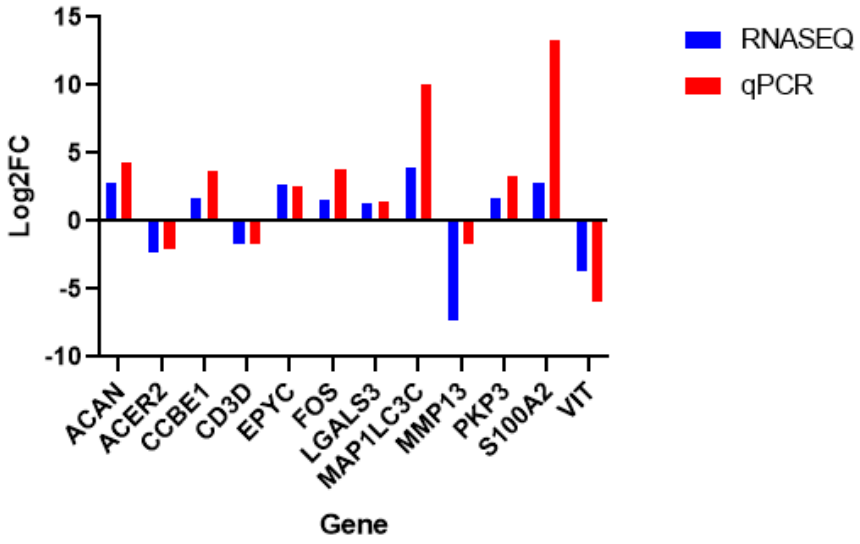


Fig 3. Comparison of Log2FC expressed values between the RNA-Seq and qPCR methodologies for the 12 target genes chosen for validation.

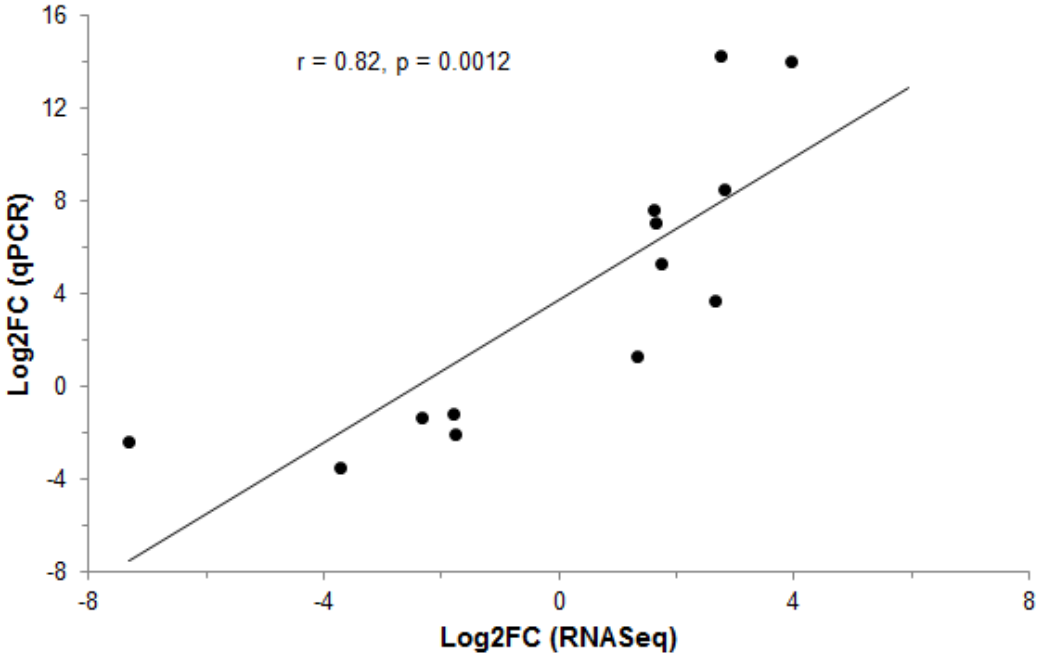


Fig 4. Pearson`s correlation (r) between Log2FC values of RNA-Seq and qPCR analyses for the 12 target genes selected for validation.

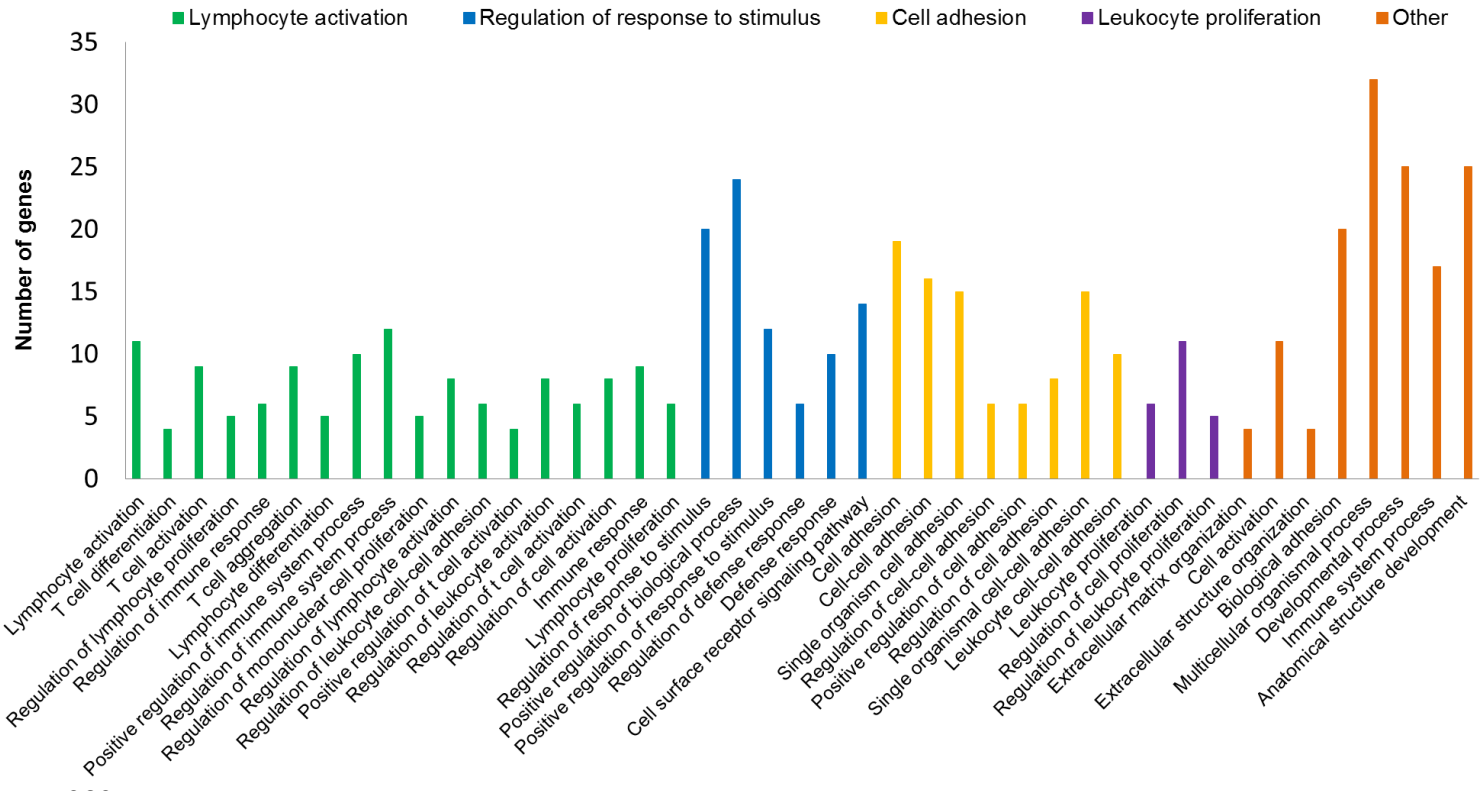


Fig 5. Significant biological processes of differentially expressed (DE) genes related to umbilical hernia in pigs. The X-axis shows the total number of genes that were DE in each biological process, based on the genetic ontology using the David 6.8.

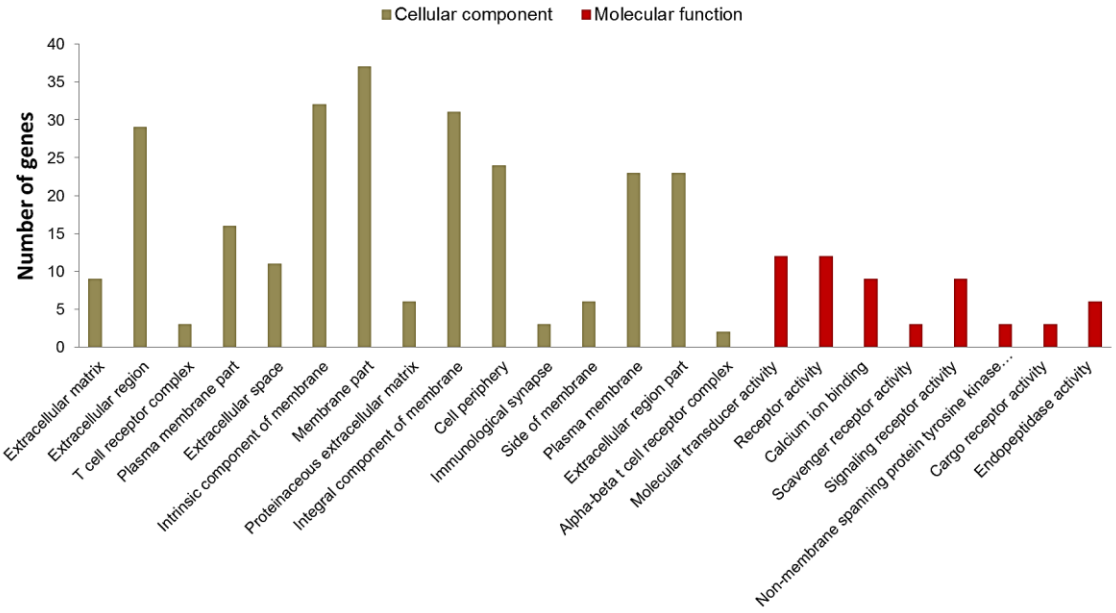


Fig 6. **Significant cell components and molecular functions of differentially expressed (DE) genes related to umbilical hernia in pigs.** The X-axis shows the total number of genes that were DE in each of them, based on the genetic ontology using the David 6.8 database.

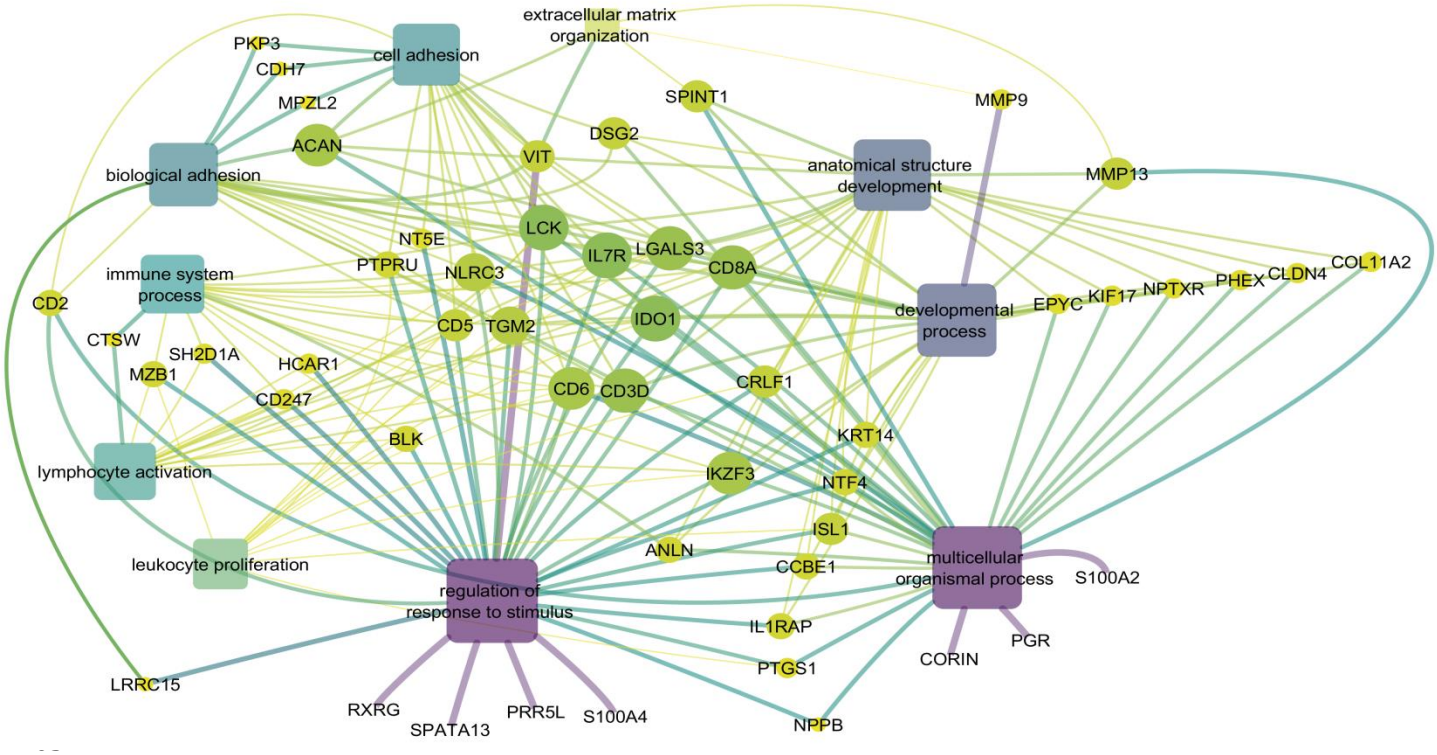


Fig 7. Gene network constructed with differentially expressed genes and main biological processes related to umbilical hernia using Cytoscape. Differentially expressed genes are visualized in the circles and biological processes in the rectangles. Node sizes indicate the number of predicted gene interactions. The edge colors indicate the betweenness of the edges (low values are in small size and in bright colors).

1111 **S1 Table:** List of 230 differentially expressed genes between normal and umbilical hernia-
1112 affected piglets.

Gene_stable_ID	logFC	FDR	Name	Gene_Description
ENSSSCG00000037358	-7,40	0,011	*SAA3*	Serum Amyloid A-3 Protein
ENSSSCG00000014988	-7,31	0,012	*MMP13*	Matrix Metallopeptidase 13
ENSSSCG00000037009	-5,95	0,001		
ENSSSCG00000036318	-5,76	0,003		
ENSSSCG00000036203	-5,64	0,003		
ENSSSCG00000004195	-5,53	0,014	*ARG1*	Arginase 1
ENSSSCG00000036127	-5,24	0,002		
ENSSSCG00000040651	-5,21	0,010		
ENSSSCG00000037141	-4,94	0,001		
ENSSSCG00000036445	-4,85	0,002	*CXCL13*	C-X-C Motif Chemokine Ligand 13
ENSSSCG00000034766	-4,83	0,002		
ENSSSCG00000037981	-4,51	0,002	*CNTNAP2*	Contactin Associated Protein Like 2
ENSSSCG00000037214	-4,49	0,037		
ENSSSCG00000021006	-4,39	0,002		
ENSSSCG00000011186	-4,38	0,002	*COL6A5*	Collagen Type VI Alpha 5 Chain
ENSSSCG00000036224	-4,33	0,013		
ENSSSCG00000039102	-4,22	0,036		
ENSSSCG00000031292	-4,19	0,002		
ENSSSCG00000039146	-4,15	0,007		
ENSSSCG00000031037	-4,00	0,010		
ENSSSCG00000006719	-4,00	0,002	*HSD3B1*	Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 1
ENSSSCG00000007436	-3,95	0,003	*MMP9*	Matrix Metallopeptidase 9
ENSSSCG00000036990	-3,89	0,000	*DIO3*	Iodothyronine Deiodinase 3
ENSSSCG00000032582	-3,86	0,027		
ENSSSCG00000039111	-3,77	0,042		
ENSSSCG00000008501	-3,73	0,043	*VIT*	Vitrin
ENSSSCG00000001473	-3,72	0,002	*COL11A2*	Collagen Type XI Alpha 2 Chain
ENSSSCG00000033417	-3,56	0,042		
ENSSSCG00000030775	-3,54	0,045		
ENSSSCG00000039569	-3,51	0,044		
ENSSSCG00000031106	-3,47	0,002	*PLA2G2D*	Phospholipase A2 Group IID
ENSSSCG00000029239	-3,43	0,012	*MZB1*	Marginal Zone B And B1 Cell Specific Protein
ENSSSCG00000025523	-3,41	0,002	*COL2A1*	Collagen Type II Alpha 1 Chain
ENSSSCG00000035443	-3,38	0,044		

ENSSSCG00000014977	-3,33	0,015	*PGR*	Progesterone Receptor
ENSSSCG00000040885	-3,31	0,029	*LY6G6E*	Lymphocyte Antigen 6 Family Member G6E
ENSSSCG00000007007	-3,26	0,002	*IDO1*	Indoleamine 2,3-Dioxygenase 1
ENSSSCG00000014834	-3,25	0,017	*UCP3*	Uncoupling Protein 3
ENSSSCG00000036983	-3,04	0,045		
ENSSSCG00000023746	-2,96	0,021		
ENSSSCG00000004270	-2,93	0,006	*ADGRB3*	Adhesion G Protein-Coupled Receptor B3
ENSSSCG00000025042	-2,80	0,015	*ICOS*	Inducible T Cell Costimulator
ENSSSCG00000015332	-2,76	0,010	*PON1*	Paraoxonase 1
ENSSSCG00000009239	-2,75	0,022		Placenta-Specific Gene 8 Protein
ENSSSCG00000036438	-2,75	0,006	*GPX3*	Glutathione Peroxidase 3
ENSSSCG00000000419	-2,73	0,003	*RDH16*	Retinol Dehydrogenase 16 (All-Trans)
ENSSSCG00000006328	-2,71	0,048	*RXRG*	Retinoid X Receptor Gamma
ENSSSCG00000009789	-2,51	0,048	*HCAR1*	Hydroxycarboxylic Acid Receptor 1
ENSSSCG00000007718	-2,48	0,039	*CLDN4*	Claudin 4
ENSSSCG00000012167	-2,48	0,032	*PHEX*	Phosphate Regulating Endopeptidase Homolog X-Linked
ENSSSCG00000013111	-2,42	0,007	*CD6*	CD6 Molecule
ENSSSCG00000023522	-2,41	0,015	*TGM2*	Transglutaminase 2
ENSSSCG00000027568	-2,34	0,044	*BLK*	BLK Proto-Oncogene, Src Family Tyrosine Kinase
ENSSSCG00000039627	-2,33	0,043	*TMEM200C*	Transmembrane Protein 200C
ENSSSCG00000003882	-2,33	0,026	*SLC5A9*	Solute Carrier Family 5 Member 9
ENSSSCG00000034213	-2,32	0,006	*ACER2*	Alkaline Ceramidase 2
ENSSSCG00000016832	-2,28	0,020	*IL7R*	Interleukin 7 Receptor
ENSSSCG00000028567	-2,25	0,019	*BTNL9*	Butyrophilin Like 9
ENSSSCG00000039581	-2,20	0,017		
ENSSSCG00000024166	-2,14	0,005	*SLC2A6*	Solute Carrier Family 2 Member 6
ENSSSCG00000013115	-2,13	0,027	*CD5*	CD5 Molecule
ENSSSCG00000036892	-2,12	0,034	*GPR132*	G Protein-Coupled Receptor 132
ENSSSCG00000000773	-2,11	0,024	*TUBA8*	Tubulin Alpha 8
ENSSSCG00000034335	-2,09	0,031		
ENSSSCG00000021734	-2,03	0,015	*GPR174*	G Protein-Coupled Receptor 174
ENSSSCG00000037885	-2,02	0,009		
ENSSSCG00000006736	-2,01	0,002	*CD2*	CD2 Molecule
ENSSSCG00000031905	-1,98	0,033	*KCNS3*	Potassium Voltage-Gated

				Channel Modifier Subfamily S Member 3
ENSSSCG00000034689	-1,98	0,027	*GPIHBP1*	Glycosylphosphatidylinositol Anchored High Density Lipoprotein Binding Protein 1
ENSSSCG00000026217	-1,96	0,044		Natural Killer Cells Antigen CD94
ENSSSCG00000038842	-1,92	0,016	*PCDH9*	Protocadherin 9
ENSSSCG00000012643	-1,91	0,036	*SH2D1A*	SH2 Domain Containing 1A
ENSSSCG00000007800	-1,90	0,003	*SEPT1*	Septin 1
ENSSSCG00000013788	-1,90	0,002		Adhesion G Protein-Coupled Receptor E2-Like
ENSSSCG00000007552	-1,85	0,034		Uncharacterized LOC102162791
ENSSSCG00000038003	-1,84	0,015	*ITK*	IL2 Inducible T Cell Kinase
ENSSSCG00000034390	-1,82	0,004	*CARD11*	Caspase Recruitment Domain Family Member 11
ENSSSCG00000003608	-1,79	0,004	*LCK*	LCK Proto-Oncogene, Src Family Tyrosine Kinase
ENSSSCG00000040581	-1,78	0,045	*CISH*	Cytokine Inducible SH2 Containing Protein
ENSSSCG00000022512	-1,77	0,044	*TRDC*	T Cell Receptor Delta Constant
ENSSSCG00000003590	-1,76	0,042	*PTPRU*	Protein Tyrosine Phosphatase, Receptor Type U
ENSSSCG00000017494	-1,76	0,002	*IKZF3*	IKAROS Family Zinc Finger 3
ENSSSCG00000007956	-1,75	0,023	*NLRC3*	NLR Family CARD Domain Containing 3
ENSSSCG00000015093	-1,75	0,024	*CD3D*	CD3d Molecule
ENSSSCG00000015766	-1,74	0,025	*WDR17*	WD Repeat Domain 17
ENSSSCG00000017926	-1,74	0,016	*SLC16A13*	Solute Carrier Family 16 Member 13
ENSSSCG00000040140	-1,72	0,026	*CD3E*	CD3e Molecule
ENSSSCG00000006309	-1,69	0,017	*CD247*	CD247 Molecule
ENSSSCG00000012970	-1,69	0,002	*CTSW*	Cathepsin W
ENSSSCG00000005533	-1,69	0,037	*PTGS1*	Prostaglandin-Endoperoxide Synthase 1
ENSSSCG00000013292	-1,68	0,042	*PRR5L*	Proline Rich 5 Like
ENSSSCG00000023320	-1,68	0,034	*CYP3A39*	Sus Scrofa Cytochrome P450 3A39 (CYP3A39), Mrna.
ENSSSCG00000013839	-1,66	0,005	*RASAL3*	RAS Protein Activator Like 3
ENSSSCG00000008193	-1,64	0,042	*ZAP70*	Zeta Chain Of T Cell Receptor Associated Protein Kinase 70
ENSSSCG00000021206	-1,64	0,005	*IL1RAP*	Interleukin 1 Receptor Accessory Protein
ENSSSCG00000008217	-1,63	0,015	*CD8A*	CD8a Molecule

ENSSSCG00000018063	-1,62	0,014		
ENSSSCG00000029596	-1,62	0,037		
ENSSSCG00000034763	-1,60	0,048	*IRS2*	Insulin Receptor Substrate 2
ENSSSCG00000037318	-1,54	0,026	*TRABD2B*	Trab Domain Containing 2B
ENSSSCG00000009293	-1,53	0,030	*SPATA13*	Spermatogenesis Associated 13
ENSSSCG00000033721	-1,51	0,038		
ENSSSCG00000035256	-1,45	0,002	*SPN*	Sialophorin
ENSSSCG00000000672	-1,45	0,002	*CLSTN3*	Calsyntenin 3
ENSSSCG00000015290	-1,43	0,038	*CDK18*	Cyclin Dependent Kinase 18
ENSSSCG00000011133	-1,43	0,010	*PFKFB3*	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3
ENSSSCG00000001472	-1,43	0,040	*SLA-DOA*	Major Histocompatibility Complex, Class II, DO Alpha
ENSSSCG00000000257	-1,42	0,011	*ITGB7*	Integrin Subunit Beta 7
ENSSSCG00000006452	-1,42	0,041	*CD1D*	CD1d Molecule
ENSSSCG00000021588	-1,41	0,019	*DAPK2*	Death Associated Protein Kinase 2
ENSSSCG00000036396	-1,40	0,040	*RF01956*	
ENSSSCG00000017705	-1,39	0,002	*CCL5*	C-C Motif Chemokine Ligand 5
ENSSSCG00000016892	-1,37	0,037	*FST*	Follistatin
ENSSSCG00000007470	-1,37	0,047	*RIPOR3*	RIPOR Family Member 3
ENSSSCG00000014894	-1,35	0,014	*TENM4*	Teneurin Transmembrane Protein 4
ENSSSCG00000006919	-1,33	0,042		Guanylate-Binding Protein 7
ENSSSCG00000011524	-1,32	0,008	*CHL1*	Cell Adhesion Molecule L1 Like
ENSSSCG00000008228	-1,32	0,039	*GNLY*	Granulysin
ENSSSCG00000033592	-1,32	0,048	*TMC8*	Transmembrane Channel Like 8
ENSSSCG00000032841	-1,31	0,042	*RF01955*	
ENSSSCG00000033020	-1,30	0,042	*SLA2*	Src Like Adaptor 2
ENSSSCG00000038643	-1,27	0,049	*KLF11*	Kruppel Like Factor 11
ENSSSCG00000023014	-1,26	0,037	*CD300LG*	CD300 Molecule Like Family Member G
ENSSSCG00000012126	-1,25	0,022	*GPM6B*	Glycoprotein M6B
ENSSSCG00000011297	-1,21	0,040	*ABHD5*	Abhydrolase Domain Containing 5
ENSSSCG00000029304	-1,20	0,025	*STEAP3*	STEAP3 Metalloreductase
ENSSSCG00000011849	-1,20	0,010	*TNK2*	Tyrosine Kinase Non Receptor 2
ENSSSCG00000018061	-1,19	0,027		
ENSSSCG00000032709	-1,18	0,034	*ARL4A*	ADP Ribosylation Factor Like Gtpase 4A
ENSSSCG00000029668	-1,17	0,031	*IL2RB*	Interleukin 2 Receptor Subunit Beta

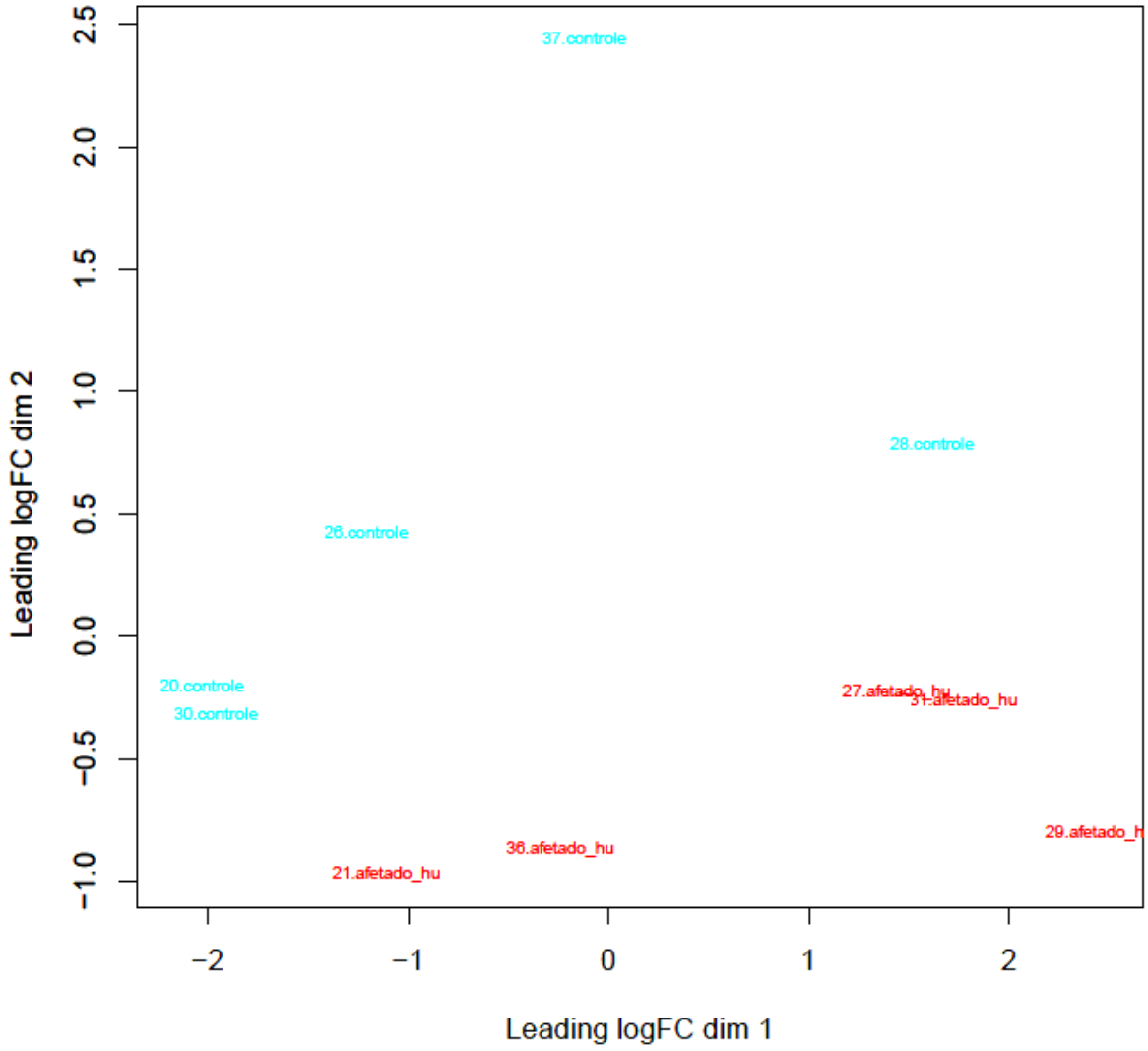
ENSSSCG00000001455	-1,15	0,023	*SLA-DRB1*	MHC Class II Histocompatibility Antigen SLA-DRB1
ENSSSCG00000001725	-1,14	0,041	*ADGRF5*	Adhesion G Protein-Coupled Receptor F5
ENSSSCG00000039751	-1,08	0,007	*NLRC5*	NLR Family CARD Domain Containing 5
ENSSSCG00000026506	-1,06	0,042	*RALGAPA2*	Ral Gtpase Activating Protein Catalytic Alpha Subunit 2
ENSSSCG00000013598	-1,05	0,045	*KANK3*	KN Motif And Ankyrin Repeat Domains 3
ENSSSCG00000012399	-1,03	0,042	*FOXO4*	Forkhead Box O4
ENSSSCG00000024071	-1,03	0,023	*SCARF1*	Scavenger Receptor Class F Member 1
ENSSSCG00000009370	-1,02	0,021	*FOXO1*	Forkhead Box O1
ENSSSCG00000000665	-0,99	0,034	*RIMKLB*	Ribosomal Modification Protein Rimk Like Family Member B
ENSSSCG00000036223	-0,99	0,042	*ACKR1*	Atypical Chemokine Receptor 1 (Duffy Blood Group)
ENSSSCG00000011295	-0,97	0,040	*SNRK*	SNF Related Kinase
ENSSSCG00000023569	-0,91	0,041		
ENSSSCG00000015203	-0,90	0,020	*ROBO4*	Roundabout Guidance Receptor 4
ENSSSCG00000016976	-0,86	0,042	*ZNF366*	Zinc Finger Protein 366
ENSSSCG00000011330	-0,82	0,043	*NBEAL2*	Neurobeachin Like 2
ENSSSCG00000006087	0,91	0,018	*CPQ*	Carboxypeptidase Q
ENSSSCG00000034441	1,01	0,018	*MRGPRF*	MAS Related GPR Family Member F
ENSSSCG00000004705	1,04	0,034	*MAP1A*	Microtubule Associated Protein 1A
ENSSSCG00000013380	1,09	0,012	*NUCB2*	Nucleobindin 2
ENSSSCG00000010312	1,12	0,042	*PLAU*	Plasminogen Activator, Urokinase
ENSSSCG00000022592	1,15	0,032	*FIBIN*	Fin Bud Initiation Factor Homolog
ENSSSCG00000002245	1,16	0,002	*KATNBL1*	Katanin Regulatory Subunit B1 Like 1
ENSSSCG00000029949	1,17	0,044	*CD248*	CD248 Molecule
ENSSSCG00000017306	1,17	0,044	*ITGB3*	Integrin Subunit Beta 3
ENSSSCG00000020963	1,18	0,034	*EPDR1*	Ependymin Related 1
ENSSSCG00000037142	1,19	0,030		Cysteine-Rich Protein 1
ENSSSCG00000028076	1,20	0,017	*ZBTB7C*	Zinc Finger And BTB Domain Containing 7C
ENSSSCG00000039488	1,22	0,045	*SPON2*	Spondin 2
ENSSSCG00000006335	1,23	0,037	*RGS4*	Regulator Of G Protein Signaling 4

ENSSSCG00000003876	1,24	0,002	*CDKN2C*	Cyclin Dependent Kinase Inhibitor 2C
ENSSSCG00000004027	1,28	0,012	*PDE10A*	Phosphodiesterase 10A
ENSSSCG00000011563	1,31	0,045	*FANCD2*	FA Complementation Group D2
ENSSSCG00000005055	1,33	0,033	*LGALS3*	Galectin 3
ENSSSCG00000009378	1,34	0,050	*CKAP2*	Cytoskeleton Associated Protein 2
ENSSSCG00000021899	1,35	0,038		
ENSSSCG00000037307	1,37	0,028	*PRC1*	Protein Regulator Of Cytokinesis 1
ENSSSCG00000008259	1,42	0,045	*LRRTM4*	Leucine Rich Repeat Transmembrane Neuronal 4
ENSSSCG00000000874	1,42	0,030	*GAS2L3*	Growth Arrest Specific 2 Like 3
ENSSSCG00000013517	1,44	0,040	*UHRF1*	Ubiquitin Like With PHD And Ring Finger Domains 1
ENSSSCG00000034765	1,44	0,018		
ENSSSCG00000014326	1,45	0,039	*KIF20A*	Kinesin Family Member 20A
ENSSSCG00000037120	1,49	0,044	*TK1*	Thymidine Kinase 1
ENSSSCG00000029756	1,50	0,003	*ADGRG2*	Adhesion G Protein-Coupled Receptor G2
ENSSSCG00000040332	1,51	0,012	*LBH*	Limb Bud And Heart Development
ENSSSCG00000033444	1,51	0,047	*SPC24*	SPC24, NDC80 Kinetochore Complex Component
ENSSSCG00000002847	1,54	0,030	*GPT2*	Glutamic--Pyruvic Transaminase 2
ENSSSCG00000033350	1,57	0,007	*BCHE*	Butyrylcholinesterase
ENSSSCG00000015091	1,59	0,022	*MPZL2*	Myelin Protein Zero Like 2
ENSSSCG00000002383	1,61	0,023	*FOS*	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
ENSSSCG00000004291	1,61	0,004	*NT5E*	5'-Nucleotidase Ecto
ENSSSCG00000036801	1,62	0,030	*C6orf132*	Chromosome 6 Open Reading Frame 132
ENSSSCG00000008555	1,62	0,004	*CGREF1*	Cell Growth Regulator With EF-Hand Domain 1
ENSSSCG00000017010	1,63	0,025	*INSYN2B*	Inhibitory Synaptic Factor Family Member 2B
ENSSSCG00000010325	1,63	0,001	*KCNMA1*	Potassium Calcium-Activated Channel Subfamily M Alpha 1
ENSSSCG00000016658	1,64	0,028	*ANLN*	Anillin Actin Binding Protein
ENSSSCG00000004907	1,65	0,006	*CCBE1*	Collagen And Calcium Binding EGF Domains 1
ENSSSCG00000002662	1,67	0,007	*C16orf74*	Chromosome 16 Open Reading Frame 74

ENSSSCG00000002709	1,71	0,041	*CHST6*	Carbohydrate Sulfotransferase 6
ENSSSCG00000007072	1,73	0,033	*SPTLC3*	Serine Palmitoyltransferase Long Chain Base Subunit 3
ENSSSCG00000031741	1,73	0,033	*RRM2*	Ribonucleotide Reductase Regulatory Subunit M2
ENSSSCG00000025578	1,73	0,002	*ALDH1A2*	Aldehyde Dehydrogenase 1 Family Member A2
ENSSSCG00000027302	1,74	0,036	*PKP3*	Plakophilin 3
ENSSSCG00000010370	1,74	0,004	*ANXA8*	Annexin A8
ENSSSCG00000013714	1,81	0,023		
ENSSSCG00000022739	1,81	0,042	*DSG2*	Desmoglein 2
ENSSSCG00000009448	1,83	0,027	*DIAPH3*	Diaphanous Related Formin 3
ENSSSCG00000004886	1,84	0,036	*CDH7*	Cadherin 7
ENSSSCG00000038966	1,85	0,042	*KRT7*	Keratin 7
ENSSSCG00000008449	1,86	0,027	*SLC3A1*	Solute Carrier Family 3 Member 1
ENSSSCG00000006578	1,89	0,029	*S100A4*	S100 Calcium Binding Protein A4
ENSSSCG00000009138	1,89	0,030	*CFI*	Complement Factor I
ENSSSCG00000007528	2,01	0,026	*PHACTR3*	Phosphatase And Actin Regulator 3
ENSSSCG00000017448	2,07	0,023	*KRT14*	Keratin 14
ENSSSCG00000000092	2,10	0,045	*NPTXR*	Neuronal Pentraxin Receptor
ENSSSCG00000013909	2,11	0,037	*CRLF1*	Cytokine Receptor Like Factor 1
ENSSSCG00000035392	2,12	0,000	*IGFBP2*	Insulin Like Growth Factor Binding Protein 2
ENSSSCG00000033941	2,22	0,005	*C2orf88*	Chromosome 2 Open Reading Frame 88
ENSSSCG00000003508	2,23	0,017	*KIF17*	Kinesin Family Member 17
ENSSSCG00000035124	2,25	0,014	*ILDR2*	Immunoglobulin Like Domain Containing Receptor 2
ENSSSCG00000003160	2,25	0,028	*NTF4*	Neurotrophin 4
ENSSSCG00000008813	2,26	0,025	*CORIN*	Corin, Serine Peptidase
ENSSSCG00000013911	2,30	0,005	*TMEM59L*	Transmembrane Protein 59 Like
ENSSSCG00000021126	2,44	0,014	*SOSTDC1*	Sclerostin Domain Containing 1
ENSSSCG00000033958	2,46	0,002	*RYR3*	Ryanodine Receptor 3
ENSSSCG00000026868	2,54	0,031	*LRRC15*	Leucine Rich Repeat Containing 15
ENSSSCG00000004759	2,55	0,019	*SPINT1*	Serine Peptidase Inhibitor, Kunitz Type 1
ENSSSCG00000004191	2,56	0,038	*MOXD1*	Monooxygenase DBH Like 1
ENSSSCG00000000918	2,67	0,040	*EPYC*	Epiphycan
ENSSSCG00000002883	2,69	0,020		

ENSSSCG00000006580	2,76	0,000	*S100A2*	S100 Calcium Binding Protein A2
ENSSSCG00000003509	2,77	0,012	*SH2D5*	SH2 Domain Containing 5
ENSSSCG00000016883	2,78	0,037	*ISL1*	ISL LIM Homeobox 1
ENSSSCG00000001832	2,81	0,038	*ACAN*	Aggrecan
ENSSSCG00000006021	3,00	0,045	*KCNV1*	Potassium Voltage-Gated Channel Modifier Subfamily V Member 1 [Source:HGNC Symbol;Acc:HGNC:18861
ENSSSCG00000026780	3,01	0,005	*EDIL3*	EGF Like Repeats And Discoidin Domains 3
ENSSSCG00000003431	3,06	0,005	*NPPB*	Natriuretic Peptide B
ENSSSCG00000036566	3,39	0,002	*LY6G6C*	Lymphocyte Antigen 6 Family Member G6C
ENSSSCG00000038121	3,76	0,003	*TCHH*	Trichohyalin
ENSSSCG00000034838	3,95	0,002	*MAP1LC3C*	Microtubule Associated Protein 1 Light Chain 3 Gamma
ENSSSCG00000033927	4,26	0,001		

S1 Figure: Multi-Dimensional scaling (MDS) plot to visualize the separation between the five normal and the five umbilical hernia-affected piglets.



1142 **S2 Table:** Main biological processes of genes differentially expressed between normal and
1143 umbilical hernia-affected piglets. Genes in bold upregulated in the affected group.

Term_ID	David Bioprocesses	Enriched genes
GO:0046649	Lymphocyte activation	*NLRC3, IDO1, CD6, CD3D, IKZF3, LCK, IL7R, CD5, CD8A, MZB1,* ***LGALS3***
GO:0030217	T cell differentiation	*CD3D, LCK, IL7R, CD8A*
GO:0042110	T cell activation	*NLRC3, IDO1, CD6, CD3D, LCK, IL7R, CD5, CD8A,* ***LGALS3***
GO:0050670	Regulation of lymphocyte proliferation	*IDO1, CD6, IKZF3, MZB1,* ***LGALS3***
GO:0050776	Regulation of immune response	*IDO1, LCK, BLK, IL7R, CD247,* ***SH2D1A***
GO:0070489	T cell aggregation	*NLRC3, IDO1, CD6, CD3D, LCK, IL7R, CD5, CD8A,* ***LGALS3***
GO:0071593	Lymphocyte aggregation	*NLRC3, IDO1, CD6, CD3D, LCK, IL7R, CD5, CD8A,* ***LGALS3***
GO:0030098	Lymphocyte differentiation	*CD3D, IKZF3, LCK, IL7R, CD8A*
GO:0070486	Leukocyte aggregation	*NLRC3, IDO1, CD6, CD3D, LCK, IL7R, CD5, CD8A,* ***LGALS3***
GO:0002684	Positive regulation of immune system process	*IDO1, CD6, LCK, BLK, IL7R, CD5, CD247, MZB1, SH2D1A,* ***LGALS3***
GO:0022409	Positive regulation of cell-cell adhesion	*CD6, LCK, IL7R, CD5*
GO:0002682	Regulation of immune system process	*IDO1, CD6, IKZF3, LCK, BLK, HCAR1, IL7R, CD5, CD247, MZB1, SH2D1A,* ***LGALS3***
GO:0032943	Mononuclear cell proliferation	*IDO1, CD6, IKZF3, IL7R, MZB1,* ***LGALS3***
GO:0032944	Regulation of mononuclear cell proliferation	*IDO1, CD6, IKZF3, MZB1,* ***LGALS3***
GO:0051249	Regulation of lymphocyte activation	*IDO1, CD6, IKZF3, LCK, IL7R, CD5, MZB1,* ***LGALS3***
GO:0051251	Positive regulation of lymphocyte activation	*CD6, LCK, IL7R, CD5*

GO:1903039	Positive regulation of leukocyte cell-cell adhesion	*CD6, LCK, IL7R, CD5*
GO:1903037	Regulation of leukocyte cell-cell adhesion	*IDO1, CD6, LCK, IL7R, CD5,* ***LGALS3***
GO:0050870	Positive regulation of t cell activation	*CD6, LCK, IL7R, CD5*
GO:0002694	Regulation of leukocyte activation	*IDO1, CD6, IKZF3, LCK, IL7R, CD5, MZB1,* ***LGALS3***
GO:0050863	Regulation of t cell activation	*IDO1, CD6, LCK, IL7R, CD5,* ***LGALS3***
GO:0050865	Regulation of cell activation	*IDO1, CD6, IKZF3, LCK, IL7R, CD5, MZB1,* ***LGALS3***
GO:0006955	Immune response	*IDO1, CD6, LCK, BLK, IL7R, CTSW, CD247, CD8A, SH2D1A*
GO:0050851	Antigen receptor-mediated signaling pathway	*LCK, BLK, CD247*
GO:0046651	Lymphocyte proliferation	*IDO1, CD6, IKZF3, IL7R, MZB1,* ***LGALS3***
GO:0045321	Leukocyte activation	*NLRC3, IDO1, CD6, CD3D, IKZF3, LCK, IL7R, CD5, CD8A, MZB1,* ***LGALS3***
GO:0070663	Regulation of leukocyte proliferation	*IDO1, CD6, IKZF3, MZB1,* ***LGALS3***
GO:0030198	Extracellular matrix organization	*VIT, MMP13****, SPINT1, ACAN***
GO:0001775	Cell activation	*NLRC3, IDO1, CD6, CD3D, IKZF3, LCK, IL7R, CD5, CD8A, MZB1,* ***LGALS3***
GO:0043062	Extracellular structure organization	*VIT, MMP13****, SPINT1, ACAN***
GO:0048583	Regulation of response to stimulus	*NLRC3, IDO1, CD6, BLK, IL7R, CD247, CD8A, MZB1, SH2D1A, LCK, HCAR1, PRR5L, SPATA13****, CCBE1, NT5E, ISL1, CRLF1, LGALS3, S100A4, LRRC15***
GO:0048518	Positive regulation of biological process	*CD2, IDO1, CD3D, CD6, IKZF3, BLK, IL7R, CD5, CD247, IL1RAP, RXRG, CD8A, MZB1, SH2D1A, VIT, LCK, TGM2, PRR5L****, LGALS3, CCBE1, ISL1, CRLF1, S100A4, LRRC15***
GO:0048584	Positive regulation of response to stimulus	*IDO1, CD6, LCK, BLK, PRR5L, CD247, CD8A, SH2D1A****, CRLF1, CCBE1, ISL1,***

		S100A4
GO:0031347	Regulation of defense response	*IDO1, CD6, HCAR1, SH2D1A,* ***ISL1, NT5E***
GO:0006952	Defense response	*IDO1, CD6, PTGS1, LCK, BLK, HCAR1, CD8A, SH2D1A****,* ***ISL1, NT5E***
GO:0007166	Cell surface receptor signaling pathway	*CD3D, CD6, BLK, CD247, CD8A, LCK, PTPRU****,* ***LGALS3, LRRC15, KRT14, CCBE1, NTF4, ISL1, NPPB***
GO:0007155	Cell adhesion	*CD2, NLRC3, IDO1, CD3D, CD6, IL7R, CD5, CD8A, MPZL2, VIT, LCK, TGM2, PTPRU****,* ***CDH7, LGALS3, DSG2, PKP3, NT5E, ACAN***
GO:0098609	Cell-cell adhesion	*CD2, NLRC3, IDO1, CD3D, CD6, IL7R, CD5, CD8A, MPZL2, LCK, PTPRU,* ***CDH7, LGALS3, DSG2, PKP3, NT5E***
GO:0098602	Single organism cell adhesion	*CD2, NLRC3, IDO1, CD3D, CD6, IL7R, CD5, CD8A, MPZL2, LCK, PTPRU,* ***LGALS3, DSG2, PKP3, NT5E***
GO:0022407	Regulation of cell-cell adhesion	*IDO1, CD6, LCK, IL7R, CD5,* ***LGALS3***
GO:0045785	Positive regulation of cell adhesion	*VIT, CD6, LCK, IL7R, TGM2, CD5*
GO:0030155	Regulation of cell adhesion	*VIT, IDO1, CD6, LCK, IL7R, TGM2, CD5,* ***LGALS3***
GO:0016337	Single organismal cell-cell adhesion	*CD2, NLRC3, IDO1, CD3D, CD6, IL7R, CD5, CD8A, MPZL2, LCK, PTPRU,* ***DSG2, PKP3, NT5E, LGALS3***
GO:0007159	Leukocyte cell-cell adhesion	*NLRC3, IDO1, CD6, CD3D, LCK, IL7R, CD5, CD8A,* ***LGALS3, NT5E***
GO:0022610	Biological adhesion	*CD2, NLRC3, IDO1, CD3D, CD6, IL7R, CD5, CD8A, MPZL2, VIT, LCK, TGM2, PTPRU****,* ***CDH7, LGALS3, LRRC15, DSG2, PKP3, NT5E, ACAN***
GO:0032501	Multicellular organismal process	*CD2, CD6, CD3D, PTGS1, IKZF3, IL7R, PGR, TGM2, NLRC3, IDO1, PHEX, IL1RAP, CD8A, COL11A2, MMP13, LCK, CLDN4****, ANLN, S100A2, CCBE1, NPTXR, ISL1, ACAN, KIF17, DSG2, EPYC, KRT14, SPINT1, CORIN, NTF4, NPPB, CRLF1***

GO:0032502	Developmental process	*IDO1, CD3D, IKZF3, IL7R, IL1RAP, PHEX, CD8A, COL11A2, MMP13, LCK, TGM2, CLDN4****, LGALS3, CRLF1, KIF17, ANLN, DSG2, EPYC, KRT14, SPINT1, CCBE1, NPTXR, NTF4, ISL1, ACAN***
GO:0002376	Immune system process	*NLRC3, IDO1, CD3D, CD6, IKZF3, BLK, IL7R, CD5, CTSW, CD247, CD8A, MZB1, SH2D1A, LCK, HCAR1****, LGALS3, ANLN***
GO:0048856	Anatomical structure development	*IDO1, CD3D, IKZF3, IL7R, IL1RAP, PHEX, CD8A, COL11A2, MMP13, LCK, TGM2, CLDN4****, LGALS3, CRLF1, KIF17, ANLN, DSG2, EPYC, KRT14, SPINT1, CCBE1, NPTXR, NTF4, ISL1, ACAN***
GO:0018108	Peptidyl-tyrosine phosphorylation	*LCK, BLK,* ***CRLF1, ISL1***

3 CONSIDERAÇOES FINAIS

Um estudo do transcriptoma do anel umbilical de suínos normais e afetados com hérnia umbilical foi conduzido para identificar genes associados a ocorrência deste defeito. Um total de 230 genes diferencialmente expressos (DE) entre animais normais e afetados foram identificados, possibilitando um maior conhecimento sobre os fatores genéticos envolvidos no desenvolvimento da hérnia umbilical, principalmente devido a identificação dos processos biológicos, componentes celulares e funções moleculares que estes genes estão associados. Ainda, para confirmar e garantir os resultados encontrados no RNA-Seq, dos 230 genes DE, 12 foram escolhidos para avaliar a expressão gênica através da análise de qPCR. Os resultados de ambas análises foram consistentes, confirmando que os resultados encontrados com o RNA-Seq são confiáveis.

Dos processos biológicos identificados no nosso estudo, os que possuem maior relação com as hérnias umbilicais são os de adesão celular, desenvolvimento da estrutura anatômica, matriz extracelular e sistema imune. Alguns genes envolvidos nesses processos são destacados como possíveis causadores das hérnias umbilicais, como o *ACAN, MMPs, COLs, EPYC, VIT* e *CCBE1*, pois atuam na remodelação da matriz extracelular, na produção, resistência e integridade do colágeno e também em ligações de cálcio. Acredita-se que essas funções específicas e o desbalanceamento da expressão desses genes são responsáveis por causar enfraquecimento e flacidez do tecido umbilical, levando ao aparecimento da hérnia umbilical

Com os resultados inovadores encontrados neste trabalho, afirma-se que essa anomalia é de característica poligênica, pois muitos genes foram identificados e atrelados ao aparecimento das hérnias umbilicais. Entretanto, ainda há necessidade de estudos mais aprofundados para identificar mutações funcionais, SNPs e CNVs, principalmente para aumentar a compreensão sobre os mecanismos genéticos envolvidos nesta anomalia.

Os resultados encontrados no nosso estudo confirmam que as hérnias umbilicais possuem caráter genético, não apenas ambiental e isto gera a perspectiva de que futuramente seja possível a identificação de animais portadores de alelos relacionados a ocorrência desta patologia. Com isso, seria possível uma seleção mais efetiva, buscando diminuir a ocorrência das hérnias umbilicais na produção de suínos.

REFERÊNCIAS

ABIPECS (Brasil). Associação Brasileira Da Industria Produtora e Exportadora De Carne Suína. **Relatório anual**. 2000. Disponível em: <http://abpabr.com.br/files/publicacoes/6c8d7f5aa4793dfca5577b2862fe0452.pdf>. Acesso em: 04 abr 2019.

ABPA (Brasil). Associação Brasileira de Proteína Animal. **Histórico da suinocultura no Brasil**. 2018. Disponível em: <http://abpa-br.com.br/setores/suinocultura/a-suinocultura-brasileira>. Acesso em: 04 abr 2019.

ADAMS, M. D. et al. Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned human infant brain cDNA library. **Nat. Genet**., v. 4, p. 373–380, 1993.

DING, N. S. et al. A genome-wide scan reveals candidate susceptibility loci for pig hernias in an intercross between White Duroc and Erhualian. **Journal of Animal Science**, v. 87, n. 8, p. 2469–2474, 2009.

DU, Z. Q. et al. Identification of species-specific novel transcripts in pig reproductive tissues using RNA-seq. **Animal Genetics**, v. 45, n. 2, p. 198–204, 2014.

EVANS, H. E.; SACK, W. O. Prenatal Development of Domestic and Laboratory Mammals: Growth Curves, External Features and Selected References. Anatomia, Histologia, **Embryologia**, v. 2, n. 1, p. 11–45, 1973.

GACHON C.; MINGAM A.; CHARRIER B. Real-time PCR: what relevance to plant studies?. **Journal Exp Bot**, v. 55, p. 1445–1454, 2004.

GRINDFLEK, E. et al. Genome-wide association study reveals a QTL and strong candidate genes for umbilical hernia in pigs on SSC14. **BMC Genomics**, v. 19, n. 1, p. 1–9, 2018.

GUO, X. et al. Identification of candidate genes of growth traits in pigs using RNA-sequencing. **Italian Journal of Animal Science**, v. 18, n. 1, p. 279–286, 2019.

HAAS, B. J.; ZODY, M. C. Advancing RNA-Seq analysis. **Nature Biotechnology**, v. 28, n. 5, p. 421–423, 2010.

HAIDER, S.; PAL, R. Integrated Analysis of Transcriptomic and Proteomic Data. **Current Genomics**, v. 14, n. 2, p. 91-110, 2013.

HECK, A. Fatores que influenciam o desenvolvimento dos leitões na recria e terminação. **Acta Scientiae Veterinariae**, v.37, p.s211‑s218, 2009.

HORODYSKA, J. et al. RNA-Seq of Liver From Pigs Divergent in Feed Efficiency Highlights Shifts in Macronutrient Metabolism, Hepatic Growth and Immune Response. **Frontiers in Genetics**, v. 10, n. February, 2019.

KEENLISIDE, J. Belly and scrotal ruptures (umbilical and inguinal hernias). 8th **Ann Swine Technol Workshop**, Red Deer, Alberta, Canada. 2006.

KOZERA, B.; RAPACZ, M. Reference genes in real-time PCR. **Journal of Applied Genetics**, v. 54, n. 4, p. 391–406, 2013.

LI, X. et al. Genome-wide association study identifies variants in the CAPN9 gene associated with umbilical hernia in pigs. **Animal Genetics**, v. 50, n. 2, p.162-165, 2019.

LIAO, X. J. et al. Susceptibility Loci for Umbilical Hernia in Swine Detected by Genome-Wide Association. **Russian Journal of Genetics**, v. 51, n. 10, p. 1163–1170, 2015.

LONG, Y. et al. A genome-wide association study of copy number variations with umbilical hernia in swine**. Animal Genetics**, v. 47, n. 3, p. 298–305, 2016.

LORENZETTI, W. R. et al. Identification of endogenous normalizing genes for expression studies in inguinal ring tissue for scrotal hernias in pigs. **PLoS ONE**, v. 13, n. 9, p.e0204348, 2018

MAFESSONI, E. L. Manual prático para produção de suínos. Guaíba: **AgroLivros**, 471 p. 2014.

MARGUERAT S.; BAHLER J. RNA-seq: from technology to biology. **Cellular and Molecular Life Sciences**, v.67, n.4, p.569–579. 2010.

MATTSSON, P. Prevalence of congenital defects in Swedish Hampshire, Landrace and Yorkshire pig breeds and opinions on their prevalence in Swedish commercial herds. 41 f. Tese (Mestrado) - Curso de Faculty Of Veterinary Medicine And Animal Science, Swedish University Of Agricultural Sciences, 2011.

MILLER, P. et al. Imaging of abdominal hernias. **Radiographics**. v.15, n.2, p.333–347. 1995.

MORTAZAVI A. et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq. **Nature Methods**, v. 5, n. 7, p.621–628. 2008.

MUSCHAWECK, U. Umbilical and epigastric hernia repair. **Surgical Clinics of North America**, v. 83, n. 5, p. 1207–1221, 2003.

NAGALAKSHMI U.; WAERN K.; SNYDER M. RNA-Seq: a method for comprehensive transcriptome analysis. **Current Protocols in Molecular Biology**, v. 4, n. 11, p.1-13. 2010.

PEREIRA, J. C. C. Melhoramento Genético Aplicado à Produção Animal: **Melhoramento genético dos suínos**. Belo Horizonte: Fepmvz, 617 p. 2008.

PERROTT, C. A. Inguinal Hernias: Room for a Better Understanding. **The American Journal Of Emergency Medicine,** v. 22, n. 1, p.48-50, 2004.

PETERSEN, H.H. et al. Prevalence of clinical signs of disease in Danish finisher pigs. **The Veterinary Record**, v.162, No. 12, p. 377–382. 2008.

POMMEREHN, L.; TAKEUTI, K.; NEIS, L. Z., BARCELLOS, D.E.S.N.B. Hérnias : patogenia e causas em leitões. **A Hora Veterinária**, v. 34, p. 62–65, 2014.

RAZ T., et al. Protocol dependence of sequencing-based gene expression measurements. **Plos One**, v. 6, n. 5, p.e19287. 2011.

REBOUÇAS, E. DE L. et al. Real time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. **Brazilian Archives of Biology and Technology**, v. 56, n. 1, p. 143–154, 2013.

ROPKA-MOLIK, K. et al. Comprehensive analysis of the whole transcriptomes from two different pig breeds using RNA-Seq method. **Animal Genetics**, v. 45, n. 5, p. 674–684, 2014.

SCHMITTGEN, T. D.; LIVAK, K. J. Analyzing real-time PCR data by the comparative CT method. **Nature Protocols**, v. 3, n. 6, p. 1101–1108, 2008. Disponível em: <https://www-nature.ez103.periodicos.capes.gov.br/articles/nprot.2008.73>. Acesso em: 14 mai 2019.

SEARCY-BERNAL, R.; GARDNER, I. A.; HIRD, D. W. Effects of and factors associated with umbilical hernias in a swine herd. Journal of the American **Veterinary Medical Association**, v. 204, p. 1660–1664, 1994.

SEVILLANO, C. A. et al. Genome-wide association study using deregressed breeding values for cryptorchidism and scrotal/inguinal hernia in two pig lines. **Genetics Selection Evolution**, v. 47, n. 1, p. 1–8, 2015.

SOBESTIANSKY J, CARVALHO L. F. O. S. Malformações. In: Sobestiansky, J. e Barcellos, D. **Doenças dos Suínos**. Goiânia: Cânone Editorial. p. 527-538. 2007.

SOBESTIANSKY, J.; CARVALHO, L. F. O. S.; BARCELLOS, D. Hérnias. In: Sobestiansky, J. e Barcellos, D. **Doenças dos Suínos**. 2ªed. Goiânia: Cânone Editorial. p. 641-642. 2012.

STRAW, B.; BATES, R.; MAY, G. Anatomical abnormalities in a group of finishing pigs: prevalence and pig performance. **J Swine Health Prod**, v. 17, n. 1, p.28–31, 2009.

SUTRADHAR, B. C. et al. Comparison between open and closed methods of herniorrhaphy in calves affected with umbilical hernia. **Journal of Veterinary Science**, v. 10, n. 4, p.343-347. 2009.

THALLER, G.; DEMPFLE, L.; HOESCHELE, I. Investigation of the inheritance of birth defects in swine by complex segregation analysis. **Journal of Animal Breeding and Genetics**, v. 113, n. 1–6, p. 77–92, 1996.

TIRANTI, N. et al. Morphological and karyotypic characterization of intersex pigs with hernia inguinalis. **Journal Agric Sci**., v. 138, p.333–340. 2002.

TRAPNELL, C.; PACHTER, L.; SALZBERG, S. L. TopHat: Discovering splice junctions with RNA-Seq. **Bioinformatics**, v. 25, n. 9, p. 1105–1111, 2009.

USDA (Estados Unidos). United States Departament Of Agriculture. Foreign Agricultural Service: **Pork Production - Selected Countries Summary**. 2018. Disponível em: <https://apps.fas.usda.gov/psdonline/app/index.html#/app/downloads>. Acesso em: 08 abr 2019.

WANG, Y. et al. Analyses of Long Non-Coding RNA and mRNA profiling using RNA sequencing during the pre-implantation phases in pig endometrium. **Scientific Reports**, v. 6, n. July 2015, p. 1–11, 2016.

WANG, Z.; GERSTEIN, M.; SNYDER, M. RNA-Seq: a revolutionary tool for transcriptomics. **Nature Reviews Genetics**, v. 10, n.1, p.57-63, jan. 2009.

YOUNG, G. B, ANGUS K. A note on the genetics of umbilical hernia. **The Veterinary Record**, v. 90, n. 9, p.245–247, 1972.

YUN, J. et al. The effects of amoxicillin treatment of newborn piglets on the prevalence of hernias and abscesses, growth and ampicillin resistance of intestinal coliform bacteria in weaned pigs. **PLoS ONE**, v. 12, n. 2, p. 1–16, 2017.

ANEXOS

Embrapa	Certificado de Conduta Ética	ETICA 1/1

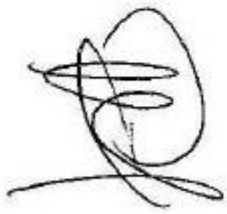
CERTIFICADO

Certificamos que o Protocolo nº**(000/AAAA): 011/2014**, sob título **"Identificação de genes e polimorfismos associados à formação de hérnias em suínos pela combinação do sequenciamento exômico total e do RNA",** sob responsabilidade de **Mônica Ledur** está de acordo com os Princípios Éticos na Experimentação Animal, adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), **TENDO SIDO CONSIDERADO APROVADO PELA** Comissão de Ética no Uso de Animais (CEUA/CNPSA) em reunião realizada em **07/ 11/ 2014**.

CERTIFICATE

We certify that the Protocol nº **(000/YYYY): 011/2014**, under the following title **"Identification of genes and polymorphisms associated with formation of hernias in swines combining RNA and whole exons sequencing."** is in agreement with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Embrapa Swines and Poultry Ethical Committee for Animals utilization in experimentation (CEUA/CNPSA) in **11/07/2014**.

Concórdia, **07/11/2014**.



Presidente CEUA/CNPSA

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