8. ANEXO

Tabla S1: *Características de los 17 marcadores microsatélites estandarizados y recomendados por la ISAG en equino.*

Locus	Localización en cromosoma	Cebadores (Directo y Reverso)	Lomgitud amplicón (pb)
AHT4	24q14	D: AACCGCCTGAGCAAGGAAGT	144–164
		R: CCCAGAGAGTTTACCCT	
AHT5	8	D: ACGGACACATCCCTGCCTGC	126–144
		R: GCAGGCTAAGGAGGCTCAGC	
ASB2	15q21.3–q23	D: CCACTAAGTGTCGTTTCAGAAGG	216–250
		R: CACAACTGAGTTCTCTGATAGG	
ASB17	2p14–p15	F: ACCATTCAGGATCTCCACCG	87–129
		R: GAGGGCGGTACCTTTGTACC	
ASB23	3q22.1–q22.3	F: GAGGGCAGCAGGTTGGGAAGG	175–211
		R: ACATCCTGGTCAAATCACAGTCC	
CA425 UCDEQ425	28q18	F: AGCTGCCTCGTTAATTCA	226–246
		R: CTCATGTCCGCTTGTCTC	
HMS1	15	F: CATCACTCTTCATGTCTGCTTGG	170–186
		R: TTGACATAAATGCTTATCCTATGGC	
HMS2	10	F:CTTGCAGTCGAATGTGTATTAAATG	222–248
		R: ACGGTGGCAACTGCCAAGGAAG	
HMS3	9	F: CCATCCTCACTTTTTCACTTTGTT	148–170
		R: CCAACTCTTTGTCACATAACAAGA	
HMS6	4	F: GAAGCTGCCAGTATTCAACCATTG	151–169
		R: CTCCATCTTGTGAAGTGTAACTCA	
HMS7	1q25	F: TGTTGTTGAAACATACCTTGACTGT	165–185
		R: CAGGAAACTCATGTTGATACCATC	
HTG4	9	F: CTATCTCAGTCTTGATTGCAGGAC	127–139
		R: CTCCCTCCCTCCCTCTGTTCTC	
HTG6	15q26–q27	F: GTTCACTGAATGTCAAATTCTGCT	84–102
		R: CCTGCTTGGAGGCTGTGATAAGAT	
HTG7	4	F: CCTGAAGCAGAACATCCCTCCTTG	118–128
		R: ATAAAGTGTCTGGGCAGAGCTGCT	
HTG10	21	F: TTTTTATTCTGATCTGTCACATTT	95–115
		R: CAATTCCCGCCCCACCCCCGGCA	
LEX3	Xq	F: ACATCTAACCAGTGCTGAGACT	142–164
		R: GAAGGAAAAAAAGGAGGAAGAC	
VHL20	30	F: CAAGTCCTCTTACTTGAAGACTAG	87–105
		R: AACTCAGGGAGAATCTTCCTCAG	

***Tabla S2**: Componentes de la reacción de amplificación, PCR multiplex, M1*

Componentes	Volumen (µl)	Características
Quiagen Multiplex PCR Buffer,2X	3	Contiene iones $MgCl_2$, KCl, $(NH_4)_2SO_4$ dNTPs, y tampón para PCR multiplex.
Q-Solution, 5X	0.6	Contiene HotStartTaq DNA polimerasa
Mezcla de oligos M1	0.675	Microsatélites: AHT 4, HMS3, HMS6, HMS7, HTG4 y VHL20
H20 miliQ	0.725	Agua purificada
DNA	1	DNA purificado del apartado 4.1
Volumen final	6	

***Tabla S3**: Componentes de la reacción de amplificación, PCR multiplex, M2*

Componentes	Volumen (µl)	Características
Quiagen Multiplex PCR Buffer, X2	3	Contiene iones $MgCl_2$, KCl, $(NH_4)_2SO_4$ dNTPs, y tampón para PCR multiplex
Q-Solution, 5X	0.6	Contiene HotStartTaq DNA polimerasa
Mezcla de oligos M2	0.34	Microsatélites: ASB2, AHT5, HTG10 y HMS2
H20 miliQ	1.06	Agua purificada
DNA	1	DNA purificado del apartado 4.1
Volumen final	6	

***Tabla S4**: Componentes de la reacción de amplificación, PCR multiplex, M3*

Componentes	Volumen (µl)	Características
Quiagen Multiplex PCR Buffer, X2	3	Contiene iones $MgCl_2$, KCl, $(NH_4)_2SO_4$ dNTPs, y tampón para PCR multiplex
Q-Solution, 5X	0.6	Contiene HotStartTaq DNA polimerasa
Mezcla de oligos M3	0.12	Microsatélites: ASB17 y ASB23
H20 miliQ	1.28	Agua purificada
DNA	1	DNA purificado del apartado 4.1
Volumen final	6	

Tabla S5: *Concentración y calidad del DNA obtenido en los Apartados 4.1.1 y 4.1.2.*

Sample ID	Concentración (ng/µl)	Ratio (A_{260nm}/A_{280nm})	Ratio (A_{260nm}/A_{230nm})
Sec. referencia	155.42	1.75	1.02
Potro afectado	117.94	1.56	1.19
Madre Juna	41.22	1.41	1.19
Abuela	36.11	1.43	1.85
Padre nas	25.44	1.35	1.33
Hermana madre	27.74	1.40	1.62
Potro hermano	30.26	1.48	2.25
Q-758	10.87	1.03	0.51
Q-759	23.65	1.47	1.17
Q-760	27.44	1.41	0.80
Q-761	23.32	1.44	1.19
Q-762	15.15	1.73	1.73
Q-763	17.62	1.24	0.72
Q-763	11.47	1.11	0.68
Q-764	14.44	1.36	0.68
Q-765	5.88	1.14	0.42
Q-766	16.00	1.39	0.78
Q-767	6.66	1.33	1.11
Q-768	12.02	1.39	1.50
Q-769	11.26	1.88	2.14
Q-770	8.14	0.96	0.30
Q-771	13.23	1.31	0.64
Q-772	12.64	1.29	0.60
Q-773	17.05	1.50	0.88
Q-774	12.45	1.23	0.68
Q-775	13.79	1.51	0.69
Q-776	12.84	1.58	0.98
Q-777	9.41	1.18	0.48
Q-778	11.26	1.31	0.40
Q-779	9.54	1.36	1.03
Q-780	2.66	1.13	1.54
Q-781	5.81	1.31	1.32
Q-782	11.84	1.15	0.49
Q-783	18.34	1.43	1.80
Q-784	25.95	1.12	1.32
Q-785	19.46	1.41	1.44
Q-786	13.37	1.44	1.68
Q-787	19.02	1.50	1.17
Q-788	14.53	1.37	0.93
Q-789	17.75	1.32	0.74
Q-790	79.98	1.76	2.31
Q-791	7.01	1.21	0.93
Q-792	82.37	1.67	1.57
Q-793	17.87	1.46	1.02
Q-795	16.19	1.48	1.26
Q-796	17.15	1.25	0.75
Q-797	8.81	1.17	0.51
Q-798	11.15	1.24	0.99

ESVP-ECVP ANNUAL MEETING 2015
2-5 September 2015 Helsinki, Finland

Acknowledgement: J. Asín has attended the meeting and presented this work thanks to a travel bursary granted by the ***Journal of Comparative Pathology Educational Trust.***

Congenital Liver Fibrosis in a Purebred Spanish Horse Foal

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Introduction

Congenital Liver Fibrosis (CLF) is a monogenic autosomal recessive inherited lethal disease described mainly in the Franches-Montagnes Horse (FMH), characterized by marked porto-portal bridging fibrosis and abundant dilated bile ducts often surrounded by inflammatory cells within the fibrotic tissue. CLF has been associated with two mutations in the Polycystic Kidney and Hepatic Disease 1 (*PKHD1*) gene[1,2]. Here we describe the first case of CLF in a Purebred Spanish Horse (PSH) foal and present the results of the genetic studies performed in this animal and others PSH horses.

Material and methods

A one-month-old PSH foal showed a clinical history of diarrhea with acholic faeces from birth and developed neurological signs and constipation leading to death.

Pathologic studies

Only the liver, kidney, spleen and a portion of small intestine were submitted for pathologic examination. Tissues were assessed grossly and fixed in 10 % formalin for histopathologic evaluation. Samples were stained with HE and Masson´s trichrome. Immunohistochemistry for cytokeratin (AE1/AE3) was performed.

Results

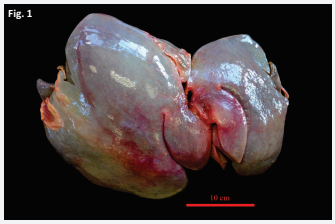


Fig. 1. Liver. Enlargement and gray discoloration. Increased consistency and weight.

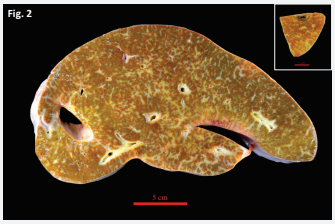
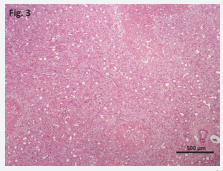
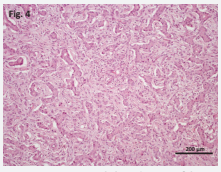
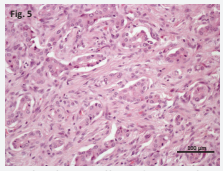


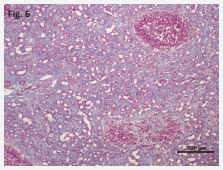
Fig. 2. Liver, cut surface. Diffuse reticular fibrosis. **Insert**: Presence of a small cyst.

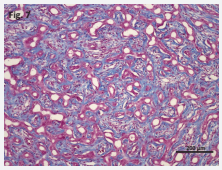


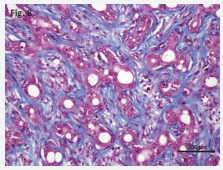




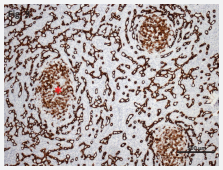
Figs. 3-5. Liver. HE stain. Diffuse porto-portal bridging fibrosis. Multiple small and irregular bile ducts, often dilated. The remaining hepatocytes show necrotic changes.

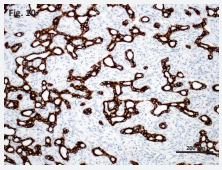


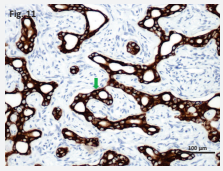




Figs. 6-8. Liver, Masson´s trichrome stain**.** Note the severely increased amount of fibrous tissue.







Figs. 9-11. Liver, immunohistochemistry for cytokeratin (AE1/AE3). Note the remaining hepatocytes (*) and the positive cuboidal epithelial cells that line the bile ducts (ı).

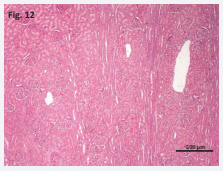


Fig. 12. Kidney, HE stain. Multifocal interstitial cysts in the cortex.

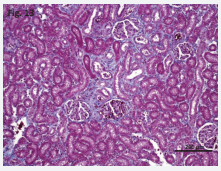


Fig. 13. Kidney, Masson´s trichrome stain. Moderate and multifocal interstitial fibrosis.

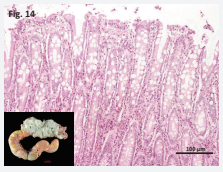


Fig. 14. Small intestine, HE stain. Goblet cell hyperplasia. **Insert**: Steatorrheic intestinal content

Genetic studies

1st The two mutations in the *PKDH1* were analyzed in the affected animal

-**Refence sequence**: NCBI Reference Sequence: NW_001867389.1 *Equus caballus* isolate Twilight breed thoroughbred chromosome 20 genomic scaffold, EquCab2.0 scaffold_7, whole genome shotgun sequence.

-**Two SNPs** were analysed by automatic sequentiation in ABI *Prism* 3130: **g,49,630,834G>A (*PKHD1*, exon 37: SNP c.6112C>T)** and **g,49,597,760A>T (*PKHD1*, exon 43: SNP c.6845T>A**) predicted to cause the non-conservative changes p.H2038Y and p.I2282N on the *PKHD1* protein[1].

Table 1. Details of the used primers (*Software Primer Express 2.0; Applied Biosystems*)

Primer name	Forward	Reverse	Tm (°C)
c.6112C>T	CTCTGCCACGGGAATTACAAC	TCCATCCTTGTTTCCCATGG	59
c.6845T>A	CACTGAAGCCTCCACTCCAAA	GGCCTGACAGCAGAATGTTAGAT	59

2nd A pedigree evaluation was performed

-5 relatives (**maternal grandmother, maternal aunt, mother, father and brother**) were analyzed for the two former SNPs.

-Relationship was established by the analysis of the standardized microsatellites recommended by the ISAG (International Society of Animal Genetics): AHT4, ASB2, ASB17, ASB23, HMS2, HMS3, HMS6, HMS7, HTG4 y VHL20.

3rd Both mutations were analyzed in a population of PSH horses

-**40 adult (5-10 years old) animals**, with no familiar relationship, were selected for these studies.

-Genotypic and genic frequencies were calculated and compared with a population of FMH[1].

Results

1st The foal was heterozygote for the two analyzed mutations (***CT; TA***)

2nd Pedigree of the affected foal

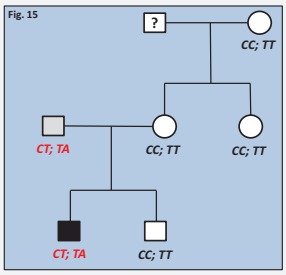


Fig. 15. Males are represented by squares and females by circles. Black square represents the case and the grey square the **father with the same genotypes for the two variants** PKHD1:c.6112C>T and PKHD1:c.6845T>A predicted to cause the non-conservative changes p.H2038Y and p.I2282N on the PKHD1 protein.

3rd Genotypic / genic frequencies of the mutations in a population of PSH and comparison with those obtained in a population of FMH[1].

Table 2. In the studied PSH population there are **approximately a 40% of heterozygotes and a 10 % of homozygotes for both mutations (circles).**

Analyzed SNP	SNP c.6112C>T g,49,630,834G>A (*PKHD1*, exon 37)		SNP c.6845T>A g,49,597,760A>T (*PKHD1*, exon 43)	
Breed	Franches-Montagnes Horse	Purebred Spanish Horse	Franches-Montagnes Horse	Purebred Spanish Horse
Genotype 1	CC	CC	TT	TT
Genotypic frequency	0,843	0,500	0,834	0,500
Genotype 2	CT	CT	TA	TA
Genotypic frequency	0,145	0,406	0,166	0,395
Genotype 3	TT	TT	AA	AA
Genotypic frequency	0,012	0,094	$3{,}93 \times 10^{-4}$	0,105
Alele 1	C	C	T	T
Genic frequency	0,916	0,703	0,917	0,697
Alele 2	T	T	A	A
Genic frequency	0,084	0,296	0,083	0,303

Conclusions

- **These findings are consistent with Congenital Liver Fibrosis. To the best of our knowledge this is the first report of this disease in the Purebred Spanish Horse.**
- **The affected animal is heterozygote for the two mutations that were strongly associated with the disease in the Franches-Montagnes Horse.**
- **The frequencies of heterozygotes and homozygotes in the Purebred Spanish Horse are higher than those found previously in the Franches-Montagnes Horse.**
- **This is the first study of the genic and genotypic frequencies for these two mutations in a Purebred Spanish Horse population.**

References:

1. Drögemüller et al., Congenital hepatic fibrosis in the Franches-Montagnes Horse Is associated with the Polycystic Kidney and Hepatic Disease 1 (*PKHD1*) gene. Plos One, PLoS One. 2014 Oct 8;9(10):e110125

2. Haechler S et al., Congenital hepatic fibrosis and cystic bile duct formation in Swiss Freiberger horses. Vet Pathol. 2000 Nov;37(6):669-71

CONGENITAL LIVER FIBROSIS IN A PUREBRED SPANISH FOAL

J. Asín[1], J. Molín[1], Vitoria A[1], Sánchez J[2], M. Gimeno[1], Romero A[1], Sanz A[2], P. Pinczowski[1], Pérez M[2], Vázquez FJ[1], Rodellar C[2], L. Luján[1]

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Introduction: Congenital liver fibrosis (CLF) is a monogenic autosomal recessive inherited lethal disease described in Swiss Freiberger breed horses, linked to a mutation in PKHD1 gene. We report the first case of CLS in a Purebred Spanish Horse (PSH) foal.

Materials and Methods: a 1-month-old PSH foal presented with a clinical history of diarrhea since birth followed by neurological signs. Due to bad prognosis, the animal was killed and samples from liver, kidney, small intestine and spleen were submitted for histopathological examination. Tissues were evaluated using HE, Masson's trichrome and PAS stains and also by IHC for cytokeratin. Genomic analyses are in progress to confirm both, the genetic etiology of the disorder and the defect in the PKHD1 gene.

Results: Gross examination showed an enlarged, pale and firm liver with marked reticular pattern on cut surface and an acholic intestinal content. Microscopically, the liver showed a severe diffuse porto-portal bridging fibrosis associated with intense multifocal irregular bile ducts proliferations, with occasional cyst formation. Remaining hepatic tissue was only observed around central veins. The kidney showed multifocal small cysts in the cortex. There was pronounced hyperplasia of goblet cells in the small intestine. The spleen showed follicular depletion and multifocal histiocytosis.

Conclusions: Histopathological lesions are consistent with CLF, the first case in a PSH foal and underline the importance of including this entity as a differential diagnosis in foals with clinical signs of progressive liver disease, independently of their breed.

ESVP-ECVP ANNUAL MEETING 2015
2-5 September 2015 Helsinki, Finland

THE EUROPEAN SOCIETY OF VETERINARY PATHOLOGY AWARD

This is to certificate that

J. Asin,
J. Molin,
Vitoria A,
Sánchez J,
M. Gimeno,
Romero A,
Sanz A,
P. Pinczowski,
Pérez M,
Vàzquez FJ,
Rodellar C
and L. Luján

was granted the poster award
at the joint 33rd Annual Meeting of ESVP
and the 26th Annual Meeting of ECVP

Prof. Carl Hård af Segerstad
ESVP President

Prof. C. Benazzi
Chairman of the Scientific Organising Committee