Research Article

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Osteochondrosis-Related Gene Expression in Equine Leukocytes Differs among Affected Joints in Foals

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Abstract

Osteochondrosis (OC) is a developmental disease in horses with a significant impact on the horse's welfare and performance. Previously, differentially expressed genes in leukocytes of OC-affected have been identified and were differentially expressed in horses of different ages when compared to their age-matched controls.As the time course of the development of OC lesions seems to be joint dependent,the aim of this study is to compare in young OC-affected horses (between 8 to 12 months), the different expression of selected genes depending the joints involved.The expression of OC-related genes were analysed by rt-PCR and subsequent statistical analysis (ΔΔCT) in the leukocytes of 30 Belgian Warmblood horses aged between 8 to 12 months divided in groups depending the affected joints (fetlock, hock and stifle).In the three groups, expression of ApoB-3G, MGAT4A, B4GALT6 and PRKCG genes were significantly higher in the OC-affected foals compared to the healthy foals. Based on the profiles of expression ofApoB-3G, Dsh1/Dvl1, Foxl1, Hp, ISG15, Mark2, PPR2A, RUSC2 and WASH1 genes,the localization of the disease can be determined: expression levels of ApoB3G, WASH1 and FOXl1 to identify fetlock, ApoB3G, PPR2A to identify OC-development in the hock and ApoB3G, Dsh1/Dvl1, WASH1, PPP2R1A and Mark2 geneto identify OC-development in the stifle. However at this moment, the rt-PCR analysis of the identified genes as biomarkers gives only diagnostic information. For the future, the profile of expression of these genes could give also some predictive information on the evolution of the disease such as remission or permanent OC-lesions.

Keywords: Horse; Developmental orthopedic disease; Osteochondrosis; Endochondral ossification; Growth

Introduction

The developmental orthopaedic disease osteochondrosis (OC) affects growing horses and severely compromise their athletic careers [1-4]. OC is considered a major cause of considerable economic losses in the horse breeding industry [5,6] with reported incidences ranging from 10% to 44% [6-9].

A common mechanism of dyschondroplasia may affect different joints, particularly the metacarpo- and metatarso-phalangeal joints, the lateral ridge of the talus or the tibial cochlea in the tibio-tarsal joint, and the lateral ridge of the trochlea in femoro-patellar joints. The disease appears to be multifactorial in origin, including skeletal growth rates, nutrition, endocrinologic factors, and exercise of the horse, biomechanics, and genetic effects [10]. High energy diet is known to induce OC lesions in foals [11,12]. Furthermore, it appears that mares fed with concentrates during gestation are more likely to produce foals that are subsequently affected by OC compared with other mares [13].

Several studies focused on the identification of target genes and biomarkers. Studies on signal transduction and gene expression in equine cartilage metabolism tried to elucidate the underlying molecular mechanisms of OC [12,14] and to identify candidate genes [15,16].

In a previous study, we analyzed the transcript profile of leukocytes from horses affected with OC using the high throughput sequencing method digital gene expression analysis (DGE) [17]. Metabolic pathways analysis showed an obvious dysregulation of several signaling pathways related to cartilage formation and cartilage repair like e.g. Wnt-, Indian hedgehog- and TGF-beta signaling pathways. Other genes regulated may play a role in high carbohydrate diet, abnormal insulin metabolism or inflammation [17]. Based on this DGE analysis, a more recent study has demonstrated that OC-related genes are differentially expressed in horses of different ages when compared to their age-matched controls. Some of the genes are implicated in cell signaling and differentiation as well as carbohydrate and lipid metabolism and inflammation (data submitted).

However, the time course of the development of OC lesions seems to be joint dependent. It has been shown that OC lesions appear rapidly after birth but most of these lesions regress in the following months up to the age of 1 year. OC lesions develop in the hock joint in the first month of life. Followed by a period of regression they seem to be stationary after the fifth month of life. In the stifle, this process is more delayed and most OC lesions develop around the fifth month of age, thereafter they can decrease to become stationary at the age of eleven months [18]. Thus, the profile of expression of genes could be different depending to the localisation or the stage of the disease. The aim of this study is to compare in young OC-affected horses (between 8 to 12 months), the different expression of selected genes depending the joints involved.

Material and Methods

Animal

30 Belgian Warmblood horses with osteochondrosis and 11 Belgian Warmblood horses free of osteochondrosis were included in this study. All horses were between 8 to 12 months old.

The horses were checked for osteochondrosis-specific lesions using radiography. All horses were sedated for the radiographic examination using detomidine (0.01 mg/kg IV) alone or combined with butorphanol (0.02 mg/kg IV). The following views were taken: dorso 60° proximo-palmarodistal oblique and weight bearing lateromedial views of the front feet, lateromedial views of the 4 fetlocks, lateromedial and plantarolateral-dorsomedial oblique views of the hocks and a lateromedial view of the stifles. Horses with palmar or plantar fragmentations of the proximal phalanx were not included in this study. Horses were diagnosed on the basis of characteristic lesions of abnormal endochondral ossification located in the fetlock (n=12), hock (n=6), and stifle joints (n=12). Horses with multiple lesions were excluded from the study. Eleven horses without any evidence of osteochondrosis-specific lesions were analyzed as a control group.

Rt-PCR analysis

Blood was collected from each horse with PAXgene blood RNA tubes (BD Diagnostics, Le Pont de Claix, France) and frozen at -80°C for later analysis. Total RNA was extracted from total blood (2.5ml) using the PAXgene blood RNA kit (Qiagen, Courtaboeuf, France). The integrity of total RNA extracted using the PAXgene method was

checked by capillary electrophoresis analysis on Agilent BioAnalyser 2100 (Agilent, Palo Alto, CA, USA). RNA quantity was measured using a spectrophotometer NanoDrop ND-1000 (Thermo Scientific, Les Ulis, France).

cDNA were synthesized using ~300ng of total RNA and performed, using the VILO reverse transcription Kit, according to the manufacturer's protocol (Invitrogen, CergyPontoise, France).

Pre-amplification was performed with a 22 pooled (final 0.2X of each) SYBR Green assay. After pre-amplification PCR, the product was treated with 4U of Exonuclease I (NEB) then diluted 1:5 with 1X TE Buffer and stored in -80°C until needed. qPCR was carried out using the 96.96 dynamic array (Fluidigm Corporation, CA, USA) following the manufacturer's protocol. Specifically, a 5 µl sample mixture was prepared for each sample containing $1 \times$ TaqMan Gene Expression Master Mix, $1 \times$ GE Sample Loading Reagent (Fluidigm PN 85000746), 1 x EvaGreen (Interchim) and each of diluted pre-amplified cDNA. 5 µl of Assay mix was prepared with $1 \times$ each of SYBR Green assay and $1 \times$ Assay Loading Reagent (Fluidigm PN 85000736). An IFC controller was used to prime the fluidics array (chip) with control line fluid and then with samples and assay mixes in the appropriate inlets. After loading, the chip was placed in the BioMark Instrument for a first step of thermal mixing at 50°C for 2 min, 70°C for 30 min and 25°C for 10 min, then UNG & HotStart step at 50°C for 2 min followed by 95°C for 2 min. The PCR was performed by 35 cycles at 95°C for 15 sec and 60°C for 1 min. Finally, melting curve was performed by increasing temperature from 60°C to 95°C. The data was analyzed with Real-Time PCR Analysis Software in the BIOMARK instrument (Fluidigm Corporation, CA, USA).

Gene symbol	Name	Trancrypt ID (ensembl or Genbank)
ADAMTLS4	ADAMTS-likeprotein 4 precursor	ENSECAT00000020422
ApoB	Apolipoprotein B	ENST00000233242
ApoB3G	similar to ApoBmRNAediting enzyme catalytic polypeptide-like 3G	XM_001916520
B4GALT6	Beta-1,4-galactosyltransferase 6	ENSECAT00000018745
BMP5	Bonemorphogeneticprotein 5	ENSECAG00000007138
CLK1	Dual specificityprotein kinase	ENSECAT00000016444
CRKL	Crk-likeprotein	ENSECAT00000018670
CSNK1E	Casein kinase I isoform epsilon	ENSECAT00000023450
CtBP1	C-terminal-binding protein 1	ENSECAT00000016987
CUL5	Cullin-5 (Vasopressin-activated calcium-mobilizingreceptor)	ENSECAT00000026414
DVL1	Segment polarityproteindishevelledhomolog DVL-1	ENST00000378891
DVL3	Segment polarityprotein disheveled homolog DVL-3	ENSECAT00000009739
FOXL1	Forkhead box L1	ENST00000320241
FOXO1	Forkhead box O1	ENST00000379561
FZD1	Frizzledfamilyreceptor 1	ENSECAG00000001326
GRB2	growth factor receptor-boundprotein 2	ENSECAG00000006524
GSK3B	glycogen synthase kinase 3 beta	ENSECAT00000026913

Hp	Haptoglobin	XM_001497810
IFIH1	Interferoninducedwithhelicase C domain	ENST00000263642
IKBKB	Inhibitor of nuclear factor kappa-B kinase subunit beta	ENSECAT00000005219
ISG15	Interferon-inducedproteinprecursor,	ENSECAT00000001183
MGAT4A	Mannosylglycoproteinacetylglucosaminyltransferase	ENSECAG00000015468
MHCI	MHC class I heavychain	ENSECAT00000021999
MHCIIa	Similar to MHC class II antigen DQ alpha chain	ENSECAT00000022398
MMP1	Interstitialcollagenaseprecursor(Matrix metalloproteinase-1)	ENSECAT00000025715
OAS3	2'-5'-oligoadenylate synthetase 3	ENST00000549918
PPP1CB	protein phosphatase 1, catalyticsubunit, beta isoform	ENSECAG00000009359
PPR2A	protein phosphatase 2, regulatorysubunit A, alpha isoform	ENSECAT00000019754
PRKCG	protein kinase C, gamma	ENSECAT00000020980
PYGL	phosphorylase, glycogen, liver	ENSECAT00000013037
RUSC2	Iporin (Interactingprotein of Rab1) (RUN and SH3 domain-containingprotein 2)	ENSECAT00000026463
SECTM1	Secreted and transmembraneprotein 1 precursor	ENSECAT00000006039
SMAD5	SMAD familymember 5	ENSECAG00000000815
TBC1D9	TBC1 domainfamilymember 9B	ENSECAT00000018936
WASH1	WAS proteinfamilyhomolog 1	XM_001493185.2

Table 1: 35 selected genes based on the study of Serteyn et al. [17]: a low p-value (inferior to 0.1%), fold induction superior to 5, quality of annotation (existence of a lot of predicted mRNA in the horse genome), a mix of up- and down-regulated genes involved in known signaling pathways

In the previous study, we have identified 2,553 genes significantlyup or down regulated between the OC-group and the control group [17].For the present study, we chosen35genes following different criteria: a low p-value (inferior to 0.1%), fold induction superior to 5, quality of annotation (existence of a lot of predicted mRNA in the horse genome), a mix of up- and down-regulated genes involved in known signalling pathways (Table 1).Four control genes: WARS (Tryptophanyl-tRNAsynthetase), RIGE (Retinoic acid-induced gene E protein), B2M(Beta-2-microglobulin) and TUBB2C (Tubulin, beta 2C), referred to as housekeeping genes, were used to normalise mRNA levels between different samples.Real-time PCR analysis was performed for each of the markers and housekeeping genes, for each measured marker or housekeeping gene, a cycle threshold value (Ct-value) was obtained.

The expression levels of the markers in horses affected by osteochondrosis and in the control group were compared by calculating 2-ΔΔCt (SchmittgenetLivatk, 2008). Here, ΔCt=CtMarker - CtHousekeeping gene, and ΔΔCt=ΔCtSubject - ΔCtControl group.

Statistical analysis

For comparison of the quantitative variables the normality was tested using Shapiro-Wilk, the normality was rejected and a non-parametrical method was used to assert of significant differential gene expression. A Kruskall-Wallis test and a Nemenyi test were used to identify the difference. All statistical tests were performed on the Delta Cycle Threshold (dCt) matrix and using R software v2.14.0. All tests performed were performed with 5% type I (alpha) error.

Results

12 genes amongst the 35 genes studied showed a significant difference of expression between the OC-affected foals and the healthy foals are reported in Table 2.

	Fetlock	Hock	Stifle
ApoB-3G	5.33 ± 1.95	5.76 ± 1.68	5.50 ± 2.08
MGAT4A	2.04 ± 0.14	2.14 ± 0.23	1.82 ± 0.12
B4GALT6	2.21 ± 0.24	2.36 ± 0.28	2.21 ± 0.18
PRKCG	1.59 ± 0.11	1.88 ± 0.09	1.94 ± 0.13
Dsh1/dvl1	0.73 ± 0.10	0.74 ± 0.12	0.34 ± 0.06
Foxl1	0.49 ± 0.10	1.04 ± 0.20	0.48 ± 0.14
Hp	0.77 ± 0.23	0.01 ± 0.01	0.63 ± 0.15
ISG15	0.88 ± 0.29	0.21 ± 0.07	0.23 ± 0.12
Mark2	0.73 ± 0.13	0.78 ± 0.14	0.46 ± 0.08

PPR2A	1.16 ± 0.17	0.57 ± 0.12	0.19 ± 0.04
RUSC2	0.84 ± 0.10	0.31 ± 0.05	0.25 ± 0.04
WASH1	1.414 ± 0.18	0.77 ± 0.08	0.46 ± 0.06

Table 2: Significant (p<0.05) fold induction differences (mean values ± standard deviations) between the OC-affected foals and the healthy foals

Whatever the joint affected, expression of ApoB-3G, MGAT4A, B4GALT6 and PRKCG genes were significantly higher in the OC-affected foals compared to the healthy foals. Fold induction of these genes are illustrated in Figure 1.
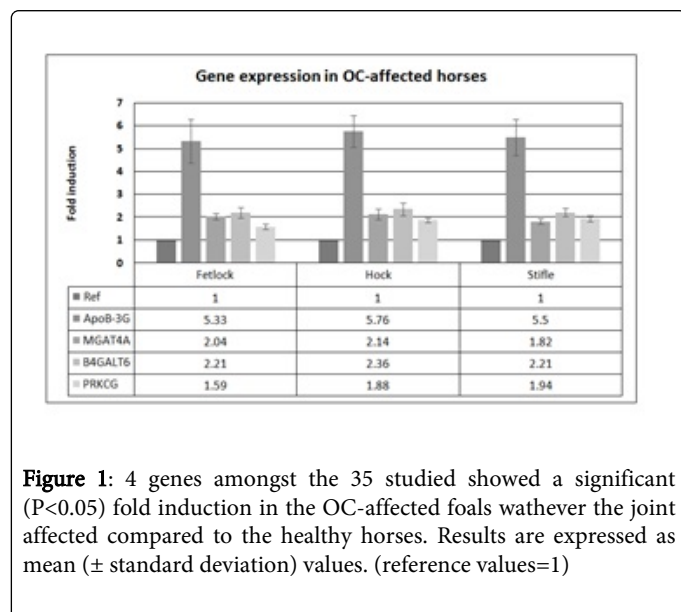

Figure 1: 4 genes amongst the 35 studied showed a significant (P<0.05) fold induction in the OC-affected foals wathever the joint affected compared to the healthy horses. Results are expressed as mean (± standard deviation) values. (reference values=1)
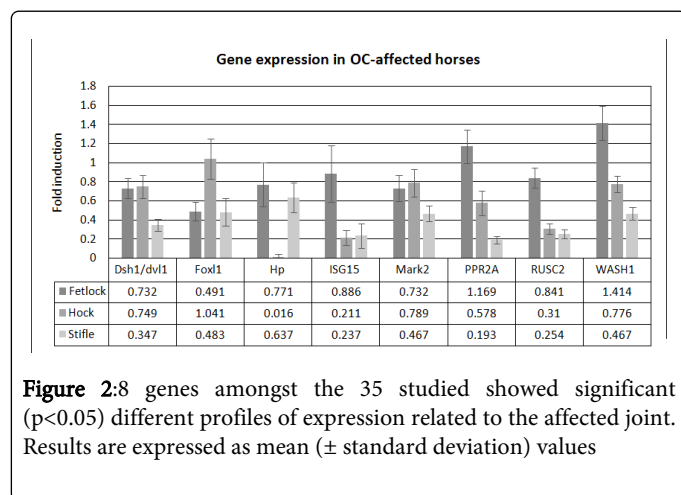

Figure 2: 8 genes amongst the 35 studied showed significant (p<0.05) different profiles of expression related to the affected joint. Results are expressed as mean (± standard deviation) values

Furthermore, the data show that the expression pattern of 8 genes (Dsh1/dvl1, Foxl1, HP, ISG15, Mark2, PPR2A, RUSC2, WASH1) show significant variations compared to the healthy foals but also between the affected joints as illustrated in Figure 2.

Dsh1/Dlv1 was found underexpressed in blood samples of horses affected in the three joints, but especially in the stifle joint. The expression of Foxl1 is normal in horse affected in the hock joint, but

this gene is found underexpressed in horses affected in the fetlock joint and the stifle joint. Hp is found underexpressed in fetlock joint and stifle joint affected horses, and is barely detectable in the hock joint affected horses. The expression of ISG15 is unchanged in fetlock joint-affected horses, but this gene is found underexpressed in hock joint and stifle joint-affected horses. The expression profile of Mark2 is basically the same than these of Dsh1/dvl1. PPR2A share the same expression profile. They are overexpressed in fetlock joint-affected horses, but they were found underexpressed in hock joint and stifle joint affected horses. RUSC2 is slightly underexpressed in fetlock joint-affected horses, and is much more underexpressed in hock joint and stifle joint-affected horses. Finally, Wash1 is found overexpressed in blood samples of fetlock joint-affected horses, but was found underexpressed in hock joint and stifle joint-affected horses.
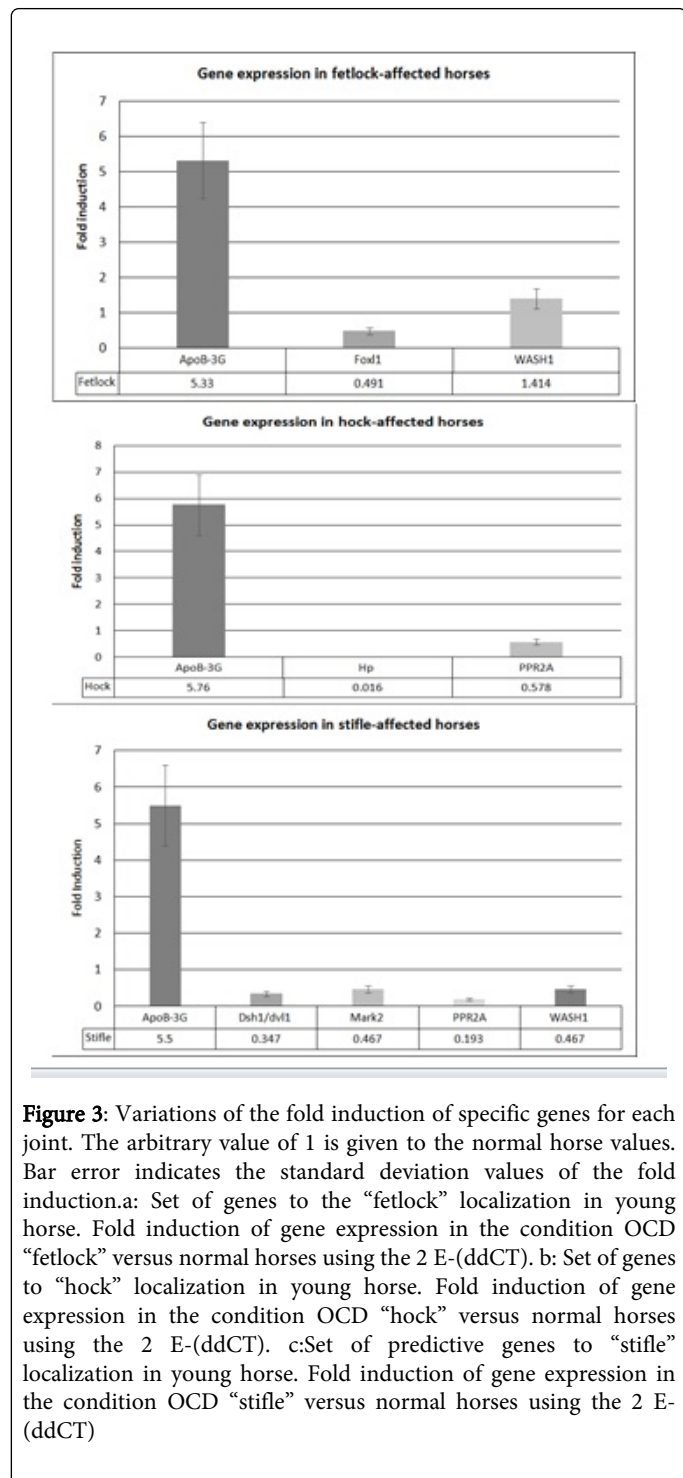
Based on the profiles of expression of ApoB-3G, Dsh1/Dvl1, Foxl1, Hp, ISG15, Mark2, PPR2A, RUSC2 and WASH1 genes, the localization of the disease can be determined: expression levels of ApoB-3G, WASH1 and FOXl1 to identify fetlock (Figure 3a), ApoB3G, Hp, PPR2A to identify OC-development in the hock (Figure 3b) and ApoB-3G, Dsh1/Dvl1, Mark2, PPP2R1A and WASH1 gene to identify OC-development in the stifle (Figure 3c).

Discussion

In this study, we identified localization-dependent differentially expressed genes in leukocytes of OC-affected horses. Gene expression analysis offers the opportunity to identify a particular environmental effect on biological pathways as well as how this regulation is potentially altered in the development of certain diseases [19]. Recently, peripheral blood leukocytes were suggested as a surrogate tissue to substitute for traditional tissue specimens that are not easily accessible [20,21]. The suitability of leukocytes for gene profiling is related to their active metabolism [22] and accessibility by simple venipuncture [21,23], which may allow a repeated time-series analysis of changes in gene expression in response to changing environmental or disease factors. Previous studies have demonstrated the potential of using gene expression levels of peripheral blood leukocytes as a novel biomarker of nutritional interventions [24], to predict weight loss regain in obese subjects [25,26], and to predict the inflammatory response to functional foods [27].

Amongst the environmental parameters influencing the onset of OC, a high energy diet is known to induce OC lesions in foals [11,12]. These observations were illustrated recently by Vanderheyden et al. [13]. Our previous study showed that MGAT4 gene remains overexpressed in OC-affected foals whatever the age [data submitted]. It was demonstrated that transient hyperglycemia causes in an animal model persistent epigenetic changes and altered gene expression during subsequent normoglycemia. This mechanism implicates a mitochondrial pathway with a high superoxide anion production [28]. Furthermore, TBC1D9 (GTPase activator activity, calcium) genes are up regulated in the OC-affected foals. This gene may act as a GTPase-activating protein for Rab family proteins and is also related to the insulin pathway and the glucose transporter 4. Recently, abnormal mitochondria and endoplasmic reticulum were observed in the deep zone of OC cartilage [29]. The mitochondrial dysfunction could be partially explained by the altered Wnt signaling pathway observed in the OC-affected horses because this pathway is known as a key regulator of mitochondrial function [17,30]. Furthermore, Power et al. [31] demonstrated by immunohistochemistry that sclerostin, a major inhibitor peptide of the canonical Wnt pathway signaling was

increased in naturally occurring lesions of equine osteochondrosis. Our study is limited to the mRNA expression without information on the proteins transcription.

Figure 3: Variations of the fold induction of specific genes for each joint. The arbitrary value of 1 is given to the normal horse values. Bar error indicates the standard deviation values of the fold induction.a: Set of genes to the "fetlock" localization in young horse. Fold induction of gene expression in the condition OCD "fetlock" versus normal horses using the 2 E-(ddCT). b: Set of genes to "hock" localization in young horse. Fold induction of gene expression in the condition OCD "hock" versus normal horses using the 2 E-(ddCT). c:Set of predictive genes to "stifle" localization in young horse. Fold induction of gene expression in the condition OCD "stifle" versus normal horses using the 2 E-(ddCT)

Our results showed clearly that in young horses (before one year)the profile expression of the selected genes was different in relation to the localization of the disease. We can assume that, at the time of the blood samples, the OC-affected foals were at different stages of the disease depending the affected joint. The time course of the development of OC lesions seems to be joint dependent.These observations confirm the results observed by the follow-up reported by van Weeren et al. [18]. For example, at the opposite to the fetlock and hock lesions, most OC lesions in the stifles develop around the fifth month of age, thereafter they can decrease to become stationary at the age of eleven months.

Based on these preliminary results, we can determine, by a Rt-PCR analysis of the selected genes identified as possible biomarkers, if the foal is affected by OC and which joint is concerned by the disease. However, the main limitations of this "case-contol study" is the few number of foals and the fact that the rt-PCR analysis of the identified genes as biomarkers give only diagnostic information. We plan to perform a "cohort study" on a more large population. For the future, the profile of expression of these genes could give also some predictive information on the evolution of the disease such as remission or permanent OC-lesions.

Acknowledgement

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