Age-dependent expression of osteochondrosis-related genes in equine leukocytes

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ABSTRACT

Introduction: Osteochondrosis (OC) is a developmental disease in horses which has a significant impact on the horse’s welfare and performance. The early disturbance in the process of endochondral ossification progresses to inflammatory and repair processes in older horses. Previously, differentially expressed genes in leukocytes of OC-affected horses have been identified. The aim of the present study is to detect age-related changes in these differentially expressed genes.

Materials and Methods: The expression of OC-related genes was analysed by real-time PCR and subsequent statistical analysis (ΔΔ CT) in the leukocytes of 135 Belgian Warmblood horses divided into three different age groups: <12 months (n=47), 18–24 months (n=50) >30 months (n=38).

Results: Relative expression of genes of horses less than 12 months of age showed significant induction of the genes MGAT4A, PRKCG, MHCII, ApoB, ApoB3G, B4GALT6 and a significantly lower expression of the genes OAS3. Horses of 18–24 months of age showed significant induction of the genes TBC1D9, MGAT4A, IFIH1, MHCIIa and MMP1. Horses of more than 30 months of age showed a significantly higher expression of the genes MGAT4A, HP, SECTM1 compared with their age-matched control groups.

Conclusions: The study demonstrates that OC-related genes are differentially expressed in horses of different ages compared with their age-matched controls. Some of the genes may be implicated in cell signalling and differentiation as well as carbohydrate and lipid metabolism and inflammation. However, the causal relationship between the differentially expressed genes and the development and progression of the OC lesions needs to be determined.

INTRODUCTION

Osteochondrosis (OC) is a developmental disease of growing horses. It is defined as a disturbance in the process of endochondral ossification (Rejnö and Strömberg 1978). The disturbance can lead to the formation of detached fragments (osteochondrosis dissicans (OCD)), fissures, or subchondral bone cysts at the epiphyseal growth cartilage (Rejnö and Strömberg 1978, Ytrehus and others 2007). OCD is a frequent cause of pain, lameness and reduced performance in young athletic horses (McIlwraith 1993, Verwilghen and others 2013).

The disease appears to be multifactorial in origin, including skeletal growth rates, nutrition, endocrinological factors, exercise, biomechanics and genetic effects (Jeffcott 1991). Several studies have demonstrated differentially expressed genes like, for example, Indian hedgehog (Ihh), transforming growth factor β 1 (TGF-β 1), Gli1 and insulin-like growth factor I (IGF-I) parathyroid hormone-related peptide (PTH-rP) in the cartilage of OC-affected horses (Semevolos and others 2001, 2002, 2005, Zabek and others 2002).

In a previous study, we analysed the transcript profile of leukocytes from horses affected with OC using a high throughput sequencing method (Serteyn and others 2010). Metabolic pathway analysis showed an obvious dysregulation of several signalling pathways related to cartilage formation and cartilage repair, such as Wnt, Indian hedgehog and TGF-β signalling pathways. Other genes that were differentially regulated may play a role in high-carbohydrate diet, abnormal insulin metabolism or inflammation (Serteyn and others 2010). Horses of this study had a mean age of 2.5 years and were relatively old regarding the origin of the disease. OC occurs early in the age of the horse (Lecoq and others 2008). The lesions undergo a variety of processes until they become visible as radiographic finding. Thereafter they may become modified by regenerative or degenerative processes (Jacket and others 2013). The global aim of our research is to find biomarkers of samples which can be easily obtained. The suitability of leukocytes for gene profiling is related to their active metabolism and accessibility by simple venipuncture (Liew and others...
The aim of the present study is to investigate the leukocyte gene expression of horses of different age groups with OC lesions.

MATERIALS AND METHODS

One hundred and thirty-five Belgian Warmblood horses were selected for this study, which followed a 'case control study' model. Selection was made on the base of the undoubtable results of the radiographies among the horses presented at the European Centre of the horse (Vielsalm, Belgium) by the breeders in the context of an orthopaedic diseases screening programme. OC-affected horses were classified into three different age groups: <12 months (AGE 1, n=35), 18–24 months (AGE 2, n=38), >30 months (AGE 3, n=26). Furthermore, 36 horses (AGE 1: n=12, AGE 2: n=12, AGE 3: n=12) without any evidence of OC lesions were selected and analysed as control groups.

All horses were sedated for the radiographic examination using detomidine (0.01 mg/kg intravenously) alone or combined with butorphanol (0.02 mg/kg intravenously). The following views were taken: lateromedial views of the four fetlocks, lateromedial and plantarolateral-dorsomedial oblique views of the hocks and a lateromedial view of the stifles. Horses were diagnosed on the basis of characteristic lesions of abnormal endochondral ossification located in the metacarpus and metatarso phalangeal tibiotarsal, and femoro-patellar joints (Denoix and others 2013). Concerning the fetlock joint, fragmentation and irregularity observed on the dorsal aspect of the sagittal ridge and the condyles of the metacarpus or metatarsus were considered as OC lesions. Horses with dorsal, palmar or plantar fragmentations at the margin of the proximal phalanx were not included in this study. Concerning the hock, OC lesions were identified as fragments still in place (intermediate ridge) or surface irregularities of the trochlear ridge(s) or malleolus. Concerning the stifle joint, variably sized irregularity or flattening of the lateral trochlear ridge of the femur with sometimes partial calcification of the tissue within the defect were considered as OC lesions.

Horses with osteoarthritis and other radiological abnormalities, which were not clearly OC lesions, were excluded from this study. 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 whole blood (2.5 ml) 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, California, USA). RNA quantity and purity were measured using a spectrophotometer NanoDrop ND-1000 (Thermo Scientific, Les Ulis, France).

cDNA was synthesised using ~300 ng of total RNA and performed, using the VILO reverse transcription Kit, according to the manufacturer’s protocol (Invitrogen, Cergy Pontoise, France).

Pre-amplification was performed with a 22 pooled (final 0.2× of each) SYBR Green assay. After pre-amplification PCR, the product was treated with 4 U of Exonuclease 1 (NEB) then diluted 1:5 with 1× TE Buffer and stored in −80°C until needed. Quantitative PCR was carried out using the 96.96 dynamic array (Fluidigm Corporation, California, USA) following the manufacturer’s protocol. Specifically, a 5 μl sample mixture was prepared for each sample containing 1× TaqMan Gene Expression Master Mix, 1× GE Sample Loading Reagent (Fluidigm PN 85000746), 1× EvaGreen (Interchim) and each of diluted pre-amplified cDNA. 5 μl of Assay mix was prepared with 1× each of SYBR Green assay and 1× Assay Loading Reagent (Fluidigm PN 85000736). An IFC controller was used to prime the fluids 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 two minutes, 70°C for 30 minutes and 25°C for 10 minutes, then UNG & HotStart step at 50°C for two minutes followed by 95°C for two minutes. PCR was performed by 35 cycles at 95°C for 15 seconds and 60°C for one minute. Finally, melting curve was performed by increasing temperature from 60 to 95°C. The data were analysed with Real-Time PCR Analysis Software in the BIOMARK instrument (Fluidigm Corporation, California, USA).

RNA level quantification was assessed by calculating 2−ΔΔCT (Schmittgen and Livak 2008).

In the previous study, we identified 2553 genes significantly upregulated or downregulated between the OC group and the control group (Serteyn and others 2010). For this study, we have chosen 34 genes following different criteria: a low P value (inferior to 0.1 per cent), fold induction superior to 5, quality of annotation (existence of a lot of predicted mRNA in the horse genome), and a mix of upregulated and downregulated genes membership of a known signalling pathway (Table 1). Four control genes: WARS (tryptophanyl-tRNA synthetase), RIGE (retinoic acid induced gene E protein), B2M (β2 microglobulin) and TUBB2C (tubulin, β 2C), referred to as housekeeping genes, were used to normalise mRNA levels between different samples. These four control genes were defined in a previous transcriptomic study (Serteyn and others 2010).

Statistical analysis was performed according to Yuan and others (2008) using the data of control groups as references values (∆∆CT) respectively for each AGE. Then, the significance analysis of microarrays (SAM) method was used to compare the levels of gene expression as a marker of OC, taking into account the age effect between control samples and study samples. We used the corrected P value based on the false discovery rate (FDR) method (Tusher and others 2001). The results are expressed for each gene as a ratio between the expression levels in the OC-affected group versus the control study.
healthy horses. Our statistical criterion for the SAM analysis was the following: only the genes with a level of expression strictly below 0.7 or superior to 1.5 and satisfying at least a FDR strictly lower than 5 per cent were considered as possible biomarkers.

RESULTS

The results of the SAM analysis were reported in Table 2 for each age group. We found several biomarkers satisfying our biomarker selection criterion.

Relative expression of genes of horses less than 12 months of age showed significant induction of the genes MGAT4A (mannosyl-glycol-protein acetylglucosaminyltransferase), PRKCG (protein kinase C, gamma), MHCI (MHC class I, α chain), ApoB (apolipoprotein B), ApoB3G (similar to apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G), B4GALT6 (β-1,4-galactosyltransferase 6), the three latter genes were above the golden threshold of two-fold change. Further, there was a significantly lower expression of the gene OAS3 (2’-5’-oligoadenylate synthetase 3) with a two-fold change.

Horses of 18–24 months of age, showed a significantly higher expression of the genes TBC1D9 (TBC1 domain family, member 9B) (with GRAM domain), MGAT4A, IFIH1 (interferon-induced helicase C domain-containing protein 1), MHCIla (similar to MHC class II antigen DQ α chain) and MMP1 (interstitial collagenase precursor), with IFIH1 and MMP1 above the two-fold change for upregulated genes.

Horses of more than 30 months of age showed a significantly higher expression of the genes MGAT4A, HP (haptoglobin), SECTM1 (secreted and transmembrane protein 1 precursor (protein K12)), MHCIla, PYGL (phosphorylase, glycogen, liver), CUL5 (cullin-5, CRKL Crk-like protein, and CSNK1E Casein kinase I isozyme epsilon, with CUL5 and CSNK1E above the two-fold change for downregulated genes.

TABLE 1: List of the 33 genes selected on several criteria from Serteyn et al. (2010): a low P value (inferior to 0.1 per cent), fold induction superior to 5, quality of annotation, a mix of upregulated and downregulated genes membership of a known signalling pathway

| Gene symbol | Name | Transcript ID (Ensembl or Genbank) |
|-------------|------|----------------------------------|
| ADAMTS4 | ADAMTS-like protein 4 precursor | ENSECAT00000020422 |
| ApoB | Apolipoprotein B | ENST00000233242 |
| ApoB3G | similar to ApoB mRNA editing enzyme catalytic polypeptide-like 3G | XM_001916520 |
| B4GALT6 | β-1,4-galactosyltransferase 6 | ENSECAT00000018745 |
| BMP5 | Bone morphogenetic protein 5 | ENSECAG0000007138 |
| CLK1 | Dual specificity protein kinase | ENSECAT00000016444 |
| CRKL | Crk-like protein | ENSECAT00000018670 |
| CSNK1E | Casein kinase I isozyme epsilon | ENSECAT00000023450 |
| CtBP1 | C-terminal-binding protein 1 | ENSECAT00000016987 |
| CUL5 | Cullin-5 (vasopressin-activated calcium-mobilising receptor) | ENSECAT00000026414 |
| DVL1 | Segment polarity protein dishevelled homologue DVL-1 | ENST00000378891 |
| DVL3 | Segment polarity protein dishevelled homologue DVL-3 | ENSECAT0000009739 |
| FOXL1 | Forkhead box L1 | ENSECAT000000320241 |
| FOXO1 | Forkhead box O1 | ENSECAT00000379561 |
| FZD1 | Frizzled family receptor 1 | ENSECAG00000013206 |
| GRB2 | Growth factor receptor-bound protein 2 | ENSECAG00000065240 |
| GSK3B | Glycogen synthase kinase 3 β | ENSECAT00000026913 |
| Hp | Haptoglobin | XM_001498710 |
| IFIH1 | Interferon induced with helicase C domain | ENSECAT00000026346 |
| IKKB | Inhibitor of nuclear factor κB kinase subunit β | ENSECAT00000005219 |
| ISG15 | Interferon-induced protein precursor | ENSECAT00000001183 |
| MGAT4A | Mannosyl glycoprotein acetylglucosaminyltransferase | ENSECAG00000015468 |
| MHCI | MHC class I heavy chain | ENSECAT00000021999 |
| MHCIla | Similar to MHC class II antigen DQ α chain | ENSECAT00000022398 |
| MMP1 | Interstitial collagenase precursor (Matrix metalloproteinase-1) | ENSECAT00000025715 |
| OAS3 | 2’-5’-oligoadenylate synthetase 3 | ENST00000549198 |
| PPP1CB | Protein phosphatase 1, catalytic subunit, β isoform | ENSECAG00000009359 |
| PPR2A | Protein phosphatase 2, regulatory subunit A, α isoform | ENSECAT00000019754 |
| PRKCG | Protein kinase C, γ | ENSECAT00000020980 |
| PYGL | Phosphorylase, glycogen, liver | ENSECAT00000013037 |
| RUSC2 | Iporin (interacting protein of Rab1) (RUN and SH3 domain-containing protein 2) | ENSECAT00000026463 |
| SECTM1 | Secreted and transmembrane protein 1 precursor | ENSECAT00000060309 |
| SMAD5 | SMAD family member 5 | ENSECAT0000000815 |
| TBC1D9 | TBC1 domain family member 9B | ENSECAT00000018936 |
| WAS1 | WAS protein family homologue 1 | XM_001493185.2 |
vasopressin-activated calcium mobilising receptor), GSK3β (glycogen synthase kinase 3 β) and MMP1. Again we found MMP1 above the golden two-fold change threshold. Two other genes are worth mentioning: SECTM1 and HP. For the downregulated genes a significant lower expression of OAS3 and WASH1 (WAS protein family homologue 1) were found by the SAM analysis.

Based on our very low FDR rates we computed Fig 1 which combines in a Venn diagram the common markers between the groups. We found several common markers such as MGAT4A which is upregulated at all ages and could be seen as our ‘supermarker’, OAS3 as a common disease marker for groups of horses less than 12 months of age and horses of more than 30 months of age, and two common markers MHCIIa and MMP1 for horses of 18–24 months of age and horses of more than 30 months of age.

**DISCUSSION**

We previously demonstrated that leukocytes of affected horses showed differential expression of genes related to several metabolic pathways, for example, Wnt signalling, insulin signalling, TGF-β signalling and Ihh signalling (Serteyn and others 2010). Mature leukocytes have been shown to express a variety of mRNAs and they are able to respond to external stimuli by rapid and complex changes in gene expression (Bertrand and others 2004). Likewise, Liew and others (2006) considered peripheral blood leukocytes as a surrogate tissue to substitute for traditional tissue specimens that are not easily accessible. The suitability of leukocytes for gene profiling is related to their active metabolism and accessibility by simple venipuncture, which may allow a repeated time-series analysis of changes in gene expression in response to changing environmental or disease factors (Liew and others 2006). In horses, Kamm and others (2013) used a similar method to study the transcript profile of leukocytes from horses affected by osteoarthritis.

Histology studies (Laverty and Girard 2013) and cartilage-derived transcriptome studies have demonstrated timely changes in the initiation and the progression of the cartilage lesions. The initial step in the pathogenesis is one or several of the following events: formation of a fragile cartilage, failure of chondrocyte differentiation, subchondral bone necrosis, and failure of blood supply to the growth cartilage (Ytrehus and others 2007, Olstad and others 2013). In the more chronic stages of the disease, secondary degenerative and regenerative processes overshadow the initial events (Bertone and others 2005). The timely fashion of the events is also reflected in the transcriptome changes of leukocytes of OC-affected horses compared with age-matched OC-free controls. However, the transcript level of the MGAT4A gene remains significantly higher in the OC-affected horses of all ages. This gene is implicated in the intracellular transport of glucose via the membrane localisation of GLUT2 (Johswich and others 2014). The MGAT4A transcript level is significantly higher in white blood cells of peripheral blood of human patients affected by type 2 diabetes (T2D) (Lopez-Orduña and others 2007). They suggest that high levels of glucose and triglycerides could induce the high MGAT4A transcript levels, especially in subjects with a particular T2D genetic background. A similar explanation could be proposed for the OC-affected foals. In an animal model, transient hyperglycaemia causes persistent epigenetic changes and altered gene expression during subsequent normoglycaemia. This mechanism implicates a mitochondrial pathway with a high superoxide anion production
(El-Osta and others 2008). Recently, abnormal mitochondria and endoplasmic reticulum were observed in the deep zone of OC cartilage (Desjardin and others 2014). The mitochondrial dysfunction could be partially explained by the altered Wnt signalling pathway observed in the OC-affected horses because this pathway is known as a key regulator of mitochondrial function (Serteyn and others 2010, Yoon and others 2010). Other genes identified as possible biomarkers in this study are related to the carbohydrate and lipid metabolism (APOB, TBC1D9, B4GALT6, PYGL, GSK3) or gene expression, cell growth, apoptosis and differentiation (APOB3G, PRKCG, CUL5, SECTM1, WASH1) or related to inflammatory and immune mechanisms (OAS3, MMP1, HP, IFIH1, MHCI, MHCIIa).

Their role in the pathogenesis of equine OC remains to be determined, but a high-energy diet is known to induce OC lesions in foals (Savage and others 1993, Mirams and others 2009). These observations were illustrated recently by Vander Heyden and others (2013). 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.

Even though the real-time PCR analysis of the 135 horses is conclusive, the potential markers should be subjected to individual analysis in order to validate their clinical potential. Limitations of this study include the carbohydrate and lipid metabolisms detected in 392 foals using a field radiographic protocol. (Veterinary Journal 197, 44–51)

Bertone A. L., Bramlage L. R., McIlwraith C. W., Malemud C. J. (2005) Comparison of proteoglycan and collagen in articular cartilage of horses with naturally developing osteochondrosis and healing osteochondral fragments of experimentally induced fractures. American Journal of Veterinary Research 67(1), 1881–1890.

Bertrand G., Coste J., Segarra C., Schved J. F., Commes T., Marti J. (2004) Use of serial analysis of gene expression (SAGE) technology reveals new granulocytic markers. Journal of Immunological Methods 292, 43–58.

Denoix J. M., Jacquet S., Lepeule J., Crevier-Denoix N., Valette J. P., Robert C. (2013) Radiographic findings of juvenile osteochondral conditions detected in 392 foals using a field radiographic protocol. Veterinary Journal 197, 44–51

El-Osta A., Brascacchio D., Yao D., Pocai A., Jones P., Roeder R., Cooper M., Brownlee M. (2008) Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. The Journal of Experimental Medicine 205, 2409–2417.

Jacket S., Robert C., Valette J. P., Denoix J. M. (2013) Evaluation of radiological findings detected in the limbs of 321 young horses between the ages of 6 and 18 months. Veterinary Journal 197, 58–64.

Jeffcott L. B. (1991) Osteochondrosis in the horse—searching for the key to pathogenesis. Equine Veterinary Journal 23, 331–338.

Johnswich A., Longuet C., Pawling J., Abdel Rahman A., Ryckmoen J., Drucker D. J., Dennis J. W. (2014) N-glycan remodeling on glucagon receptor is an effector of nutrient-sensing by the hexosamine biosynthesis pathway. The Journal of Biological Chemistry 289, 15927–41.

Kamm J. L., Frisbie D. D., McIlwraith C. W., Orr K. E. (2013) Gene biomarkers in peripheral white blood cells of horses with experimentally induced osteoarthritis. American Journal of Veterinary Research 74(1), 115–21.

Laverty S., Girard C. (2013) Pathogenesis of epiphyseal osteochondrosis. Veterinary Journal 197, 3–12.

Laverty S., Girard C. A., Fogarty U., Beauchamp G., Richard H., Laverty S. (2008) Cartilage matrix changes in the developing epiphyseal: early events on the pathway to equine osteochondrosis? Equine Veterinary Journal 40, 442–454.

Liew C., Ma J., Tang H., Zheng R., Dempsey A. (2006) The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. The Journal of Laboratory and Clinical Medicine 147, 126–132.

Lopez-Orduña E., Cruz M., Garcia-Mena J. (2007) The transcription of MGMT4A4 glycosyl transferase is increased in white cells of peripheral blood of Type 2 Diabetes patients. BMC Genetics 8, 73–80.

McIlwraith C. W. (1993) Inferences from referred clinical cases of osteochondrosis dissecans. Equine Veterinary Journal. Supplement 16, 27–31.

Mirams M., Tatarczuch L., Ahmed Y. A., Pagel C. N., Jeffcott L. B., Davies H. M., Mackie E. J. (2009) Altered gene expression in early osteochondrosis lesions. Journal of Orthopaedic Research 27, 452–457.

Olstad K., Hendrickson E. H. S., Carlson C. S., Ekman S., Dolvik N. I. (2013) Transection of vessels in epiphyseal cartilage canals leads to osteoarthritis and osteochondrosis dissecans in the femoro–patellar joint of foals; a potential model of osteochondrosis dissecans. Osteoarthritis and Cartilage 21, 730–738.

Rejnø S., Strømberg B. (1976) Osteochondrosis in the horse. II. Pathology. Acta Radiologica. Supplimentum 358, 153–178.

Savage C. J., McCarthy R. N., Jeffcott L. B. (1993) Effects of dietary energy and protein on induction of dyschondroplasia in foals. Equine Veterinary Journal 25, 74–79.

Schmittgen T. D., Livak K. J. (2008) Analyzing real-time PCR data by the comparative C(T) method. Nature Protocols 3, 1101–1108.

Semevolos S. A., Brewer-Toland B. D., Bent S. J., Nixon A. J. (2002) Parathyroid hormone-related peptide and Indian hedgehog expression

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patterns in naturally acquired equine osteochondrosis. *Journal of Orthopaedic Research* 20, 1290–1297

Semevolos S. A., Nixon A. J., Brower-Toland B. D. (2001) Changes in molecular expression of aggrecan and collagen types I, II, and X, insulin-like growth factor-I, and transforming growth factor-beta1 in articular cartilage obtained from horses with naturally acquired osteochondrosis. *American Journal of Veterinary Research* 62, 1088–1094

Semevolos S. A., Strassheim M. L., Haupt J. L., Nixon A. J. (2005) Expression patterns of hedgehog signaling peptides in naturally acquired equine osteochondrosis. *Journal of Orthopaedic Research* 23, 1152–1159

Serteyn D., Piquemal D., Vander Heyden L., Lejeune J. P., Verwilghen D., Sandersen C. (2010) Gene expression profiling from leukocytes of horses affected by osteochondrosis. *Journal of Orthopaedic Research* 28, 965–970

Tusher V. G., Tibshirani R., Chu G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America* 98 (9), 5116–21

Vander Heyden L., Lejeune J. P., Caudron I., Detilleux J., Sandersen C., Chavatte P., Paris J., Delliègue B., Serteyn D. (2013) Association of breeding conditions with prevalence of osteochondrosis in foals. *The Veterinary Record* 172(3), 68

Verwilghen D. R., Janssens S., Busoni V., Pille F., Johnston C., Serteyn D. (2013) Do developmental orthopaedic disorders influence future jumping performances in Warmblood stallions? *Equine Veterinary Journal* 45, 578–581

Yoon J. C., Ng A., Kim B. H., Bianco A., Xavier R. J., Elledge S. J. (2010) Wnt signaling regulates mitochondrial physiology and insulin sensitivity. *Genes & Development* 24(14), 1507–18

Ytrehus B., Carlson C. S., Ekman S. (2007) Etiology and pathogenesis of osteochondrosis. *Veterinary Pathology* 44, 429–448

Yuan J. S., Wang D., Stewart C. N. Jr. (2008) Statistical methods for efficiency adjusted real-time PCR quantification. *Biotechnology Journal* 3, 112–123

Zabek T., Golonka P., Fomal A., Semik E. (2002) IHH gene polymorphism among three horse breeds and its application for association test in horses with osteochondrosis. *Hereditas* 150, 38–43