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Expression of lactate transporters MCT1, MCT2, MCT4 and the ancillary protein CD147 in horse muscle and red blood cells

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ACADEMIC DISSERTATION

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Abstract

Monocarboxylate transporters (MCTs) transport lactate and protons across cell membranes. During intense exercise, lactate and protons accumulate in the exercising muscle and are transported to the plasma. In the horse, MCTs are responsible for the majority of lactate and proton removal from exercising muscle, and are therefore also the main mechanism to hinder the decline in pH in muscle cells. Two isoforms, MCT1 and MCT4, which need an ancillary protein CD147, are expressed in equine muscle. In the horse, as in other species, MCT1 is predominantly expressed in oxidative fibres, where its likely role is to transport lactate into the fibre to be used as a fuel at rest and during light work, and to remove lactate during intensive exercise when anaerobic energy production is needed. The expression of CD147 follows the fibre type distribution of MCT1. These proteins were detected in both the cytoplasm and sarcolemma of muscle cells in the horse breeds studied: Standardbred and Coldblood trotters. In humans, training increases the expression of both MCT1 and MCT4. In this study, the proportion of oxidative fibres in the muscle of Norwegian-Swedish Coldblood trotters increased with training. Simultaneously, the expression of MCT1 and CD147, measured immunohistochemically, seemed to increase more in the cytoplasm of oxidative fibres than in the fast fibre type *IIB*. Horse MCT4 antibody failed to work in immunohistochemistry. In the future, a quantitative method should be introduced to examine the effect of training on muscle MCT expression in the horse.

Lactate can be taken up from plasma by red blood cells (RBCs). In horses, two isoforms, MCT1 and MCT2, and the ancillary protein CD147 are expressed in RBC membranes. The horse is the only species studied in which RBCs have been found to express MCT2, and the physiological role of this protein in RBCs is unknown. The majority of horses express all three proteins, but 10-20% of horses express little or no MCT1 or CD147. This leads to large interindividual variation in the capacity to transport lactate into RBCs. Here, the expression level of MCT1 and CD147 was bimodally distributed in three studied horse breeds: Finnhorse, Standardbred and Thoroughbred. The level of MCT2 expression was distributed unimodally. The expression level of lactate transporters could not be linked to performance markers in Thoroughbred racehorses. In the future, better performance indexes should be developed to better enable the assessment of whether the level of MCT expression affects athletic performance.

In human subjects, several mutations in MCT1 have been shown to cause decreased lactate transport activity in muscle and signs of myopathy. In the horse, two amino acid sequence variations, one of which was novel, were detected in MCT1 (V432I and K457Q). The mutations found in horses were in different areas compared to mutations found in humans. One mutation (M125V) was detected in CD147. The mutations found could not be linked with exercise-induced myopathy. MCT4 cDNA was sequenced for the first time in the horse, but no mutations could be detected in this protein.

Keywords: horse, lactate, MCT1, MCT2, CD147, red blood cell, skeletal muscle, performance

Contents

List of original papers

This thesis is based on the following original publications, referred to in the text by their Roman numerals (I-IV):

I.   **Mykkänen, A. K.**, Pösö, A. R., McGowan, C. M., & McKane, S. A. (2010). Expression of lactate transporters MCT1, MCT2 and CD147 in the red blood cells of three horse breeds: Finnhorse, Standardbred and Thoroughbred. Equine Veterinary Journal, suppl 38, 161-166.

II.   **Mykkänen, A. K.**, Hyyppä, S., Pösö, A. R., Ronéus, N., & Essén-Gustavsson, B. (2010). Immunohistochemical analysis of MCT1 and CD147 in equine skeletal muscle fibres. Research in Veterinary Science, 89, 432-437.

III.   Revold, T., **Mykkänen, A. K.**, Karlström, K., Ihler, C. F., Pösö, A. R., & Essén-Gustavsson, B. (2010). Effects of training on equine muscle fibres and monocarboxylate transporters in young Coldblooded Trotters. Equine Veterinary Journal, suppl 38, 289-295.

IV.   **Mykkänen, A. K.**, Koho, N. M., Reeben, M., McGowan, C. M., & Pösö, A. R. MCT1, MCT4 and CD147 gene polymorphisms in horses. Research in Veterinary Science, in press.

Papers I-IV are included in this dissertation as reprints with the permission of their copyright holders. In addition, some unpublished material is presented.

Abbreviations

AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
cDNA	Complementary DNA
CHC	α-cyano-4-hydroxycinnamate
CK	Creatine kinase
COPD	Chronic obstructive pulmonary disease
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid
ELISA	Enzyme-linked immunosorbent assay
EMMPRIN	Extracellular matrix metalloproteinase inducer
FAD	Flavin adenine dinucleotide
FH	Finnhorse
FRET	Fluorescence energy transfer
Hb	Haemoglobin
HCT	Haematocrit
HT	High lactate transport activity
Ig	Immunoglobulin
IQR	Interquartile range
K_m	Michaelis constant, equal to the substrate concentration in which the reaction rate is half of maximum
LT	Low lactate transport activity
MCT	Monocarboxylate transporter
MEV	Mevalonate
mRNA	Messenger RNA
Mutation	Change in amino acid sequence compared to reference sequence
MyHC	Myosin heavy chain
NAD	Nicotinamide adenine dinucleotide
PAS	Periodic acid-Schiff
*p*CMBS	*p*-chloromercuribenzene sulphonate
PCR	Polymerase chain reaction
pK_a	Logarithmic measure of the acid dissociation constant
PSSM	Polysaccharide storage myopathy
RAO	Recurrent airway obstruction
RBC	Red blood cell
RT-PCR	Reverse transcription polymerase chain reaction
SB	Standardbred
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SLC16AX	The gene encoding MCT: solute carrier family 16, member X
TB	Thoroughbred
V_{max}	Maximal velocity, equal to the substrate concentration in which the reaction rate is maximal

1 Introduction

Despite its large body mass, the horse is an exceptional athlete. The respiratory, cardiovascular and musculoskeletal systems are highly adapted for exercise, and racehorses are capable of reaching a maximal speed of approximately 18 m/s. In a racehorse, more than 50% of the body weight consists of skeletal muscle (Gunn 1987). The efficient oxygenation of blood in the lungs, large splenic reserve pool of erythrocytes, and good capacity to deliver oxygen to exercising muscles result in a high maximal aerobic capacity. However, during intense exercise, aerobic ATP production is not sufficient to meet the energy demands of muscle. The metabolism shifts towards the anaerobic pathway of ATP production, which results in the accumulation of lactate and protons in the muscles and blood. The lactate concentration in the blood rapidly increases when the speed increases above 11-12 m/s. The onset of this rapid increase in lactate is termed the lactate threshold (Thornton et al. 1983; Eaton et al. 1995).

After a race, the blood lactate concentration can reach up to 30 mmol/L (Harris et al. 1987). However, this blood lactate concentration represents a balance between lactate production in the muscle, its removal into the blood and uptake into liver and other tissues. Lactate concentrations measured from muscle are far higher and can reach 52 mmol/L of muscle water (Harris et al. 1987). At the pH of exercising muscle, more than 99% of lactic acid is in a dissociated form and therefore needs a transporter to cross the muscle cell membrane into the plasma. Monocarboxylate transporters (MCTs) are responsible for the majority of lactate transport across the sarcolemma (Juel 2008). MCTs are symports, which transport a monocarboxylate anion, such as lactate, across the cell membrane together with a proton (Halestrap and Meredith 2004). At rest, the Na^+/H^+ antiporter is the main regulator of intracellular pH. However, during exercise, MCT function not only effectively removes lactate from the cell, but is also the main mechanism hindering the pH decline in exercising muscle by the removal of protons (Juel 2008).

To date, 14 members of the MCT family have been identified in numerous species, but only isoforms 1- 4 are known to transport lactate (Halestrap and Meredith 2004). MCT1 is the most widely expressed isoform in the body and found in almost all tissues studied (Halestrap and Meredith 2004). In horse muscle, MCT1, MCT4 and their ancillary protein CD147 have been recognized (Koho et al. 2006). CD147 (EMMPRIN, basigin, neurothelin) is vital for the correct expression of both MCT1 and MCT4 in the cell membrane (Kirk et al. 2000). The distribution of MCTs in different muscle fibre types is not known in the horse. Studies in rats and humans have found MCT4 to be mostly expressed in glycolytic fibres, where it is suggested to be responsible for the removal of lactate from the muscle during intense exercise (Wilson et al. 1998; Pilegaard et al. 1999a). The lactate produced can be oxidized by other tissues, such as the heart, liver and resting or submaximally exercising muscle, or used for glycogen resynthesis mainly in muscle or gluconeogenesis in the liver (Johnson and Bagby 1988; Putman et al. 1999; Krssak et al. 2000). In human and rat muscle MCT1 is mostly expressed in oxidative fibres and is probably responsible for the uptake of lactate into these fibres (Wilson et al. 1998; Pilegaard et al. 1999a). In human, training is known to increase the expression of

both MCT1 and MCT4 in muscle (Juel 2008). However, little is known about the effect of training on horse muscle MCT expression (Kitaoka et al. 2010).

The variation in the expression of MCTs among species is quite significant, and several other isoforms, namely MCT2, MCT5, MCT6, MCT8, MCT10 and possibly MCT7, have been identified in the muscle of other species (Kim et al. 2002; Sepponen et al. 2003; Bonen et al. 2006; Mebis et al. 2009). While MCT2 is capable of transporting monocarboxylates, the other isoforms are orphan transporters with different substrate specificities, and the physiological role of these proteins in muscle is unknown (Halestrap and Meredith 2004). Since there are marked physiological differences among species, the results of studies performed on other animal species must be applied to horses with caution.

The significance of MCTs to muscle physiology during exercise becomes evident when their function is impaired. In humans, mutations in MCT1 have been shown to reduce lactate transport in muscle and result in exercise-induced myopathy (Merezhinskaya et al. 2000; Cupeiro et al. 2010). Among athletic horses, recurrent exercise-induced myopathy is one of the causes of poor performance. Up to 7% of racehorses have been reported to suffer from myopathy (McGowan et al. 2002a; Upjohn et al. 2005). Subclinical myopathy can result in economic losses, while severe cases of myopathy can be life-threatening and have a significant influence on the well-being of the animal. A preliminary study has sequenced horse MCT1 and partial CD147 cDNA in a small number of horses. Two mutations were reported, but they were not associated with myopathy (Reeben et al. 2006).

Two MCT isoforms, MCT1 and MCT2, as well as the ancillary protein CD147 are also expressed in horse red blood cells (RBCs; Koho et al. 2002). A significant amount of blood lactate, up to 50%, can be found in RBCs after a race (Pösö et al. 1995; Väihkönen et al. 1999). The influx of lactate from the plasma into RBCs sustains the gradient between muscle cells and the plasma, possibly enabling more lactate to be removed from the muscle. Pösö et al. (1995) suggested that in the horse, RBCs act as a lactate "sink", which might be beneficial to exercise. This theory found support in the work of Räsänen et al. (1995), who showed that horses with a higher lactate concentration in their RBCs after a race were better performers.

Interestingly, in the horse, the capacity to transport lactate across the RBC membrane shows marked interindividual variation. This is a unique feature among species and it allows horses to be divided into two distinct groups based on their lactate transport activity (Väihkönen and Pösö 1998; Väihkönen et al. 1999, 2001; Koho et al. 2002, 2006). The RBC lactate concentration is higher in horses with a high lactate transport activity (HT) than in horses with a low lactate transport activity (LT) after submaximal or maximal exercise (Väihkönen et al. 1999; Koho et al. 2002). Lactate transport activity is determined by the level of CD147 expression, which is distributed bimodally in horse RBCs (Koho et al. 2002). Previously, lactate transport activity has only been studied in Standardbred horses, in which approximately 70% of the population studied belonged to the HT group and the rest to the LT group (Väihkönen and Pösö 1998). In this breed, the LT trait is inherited in an autosomal recessive manner. The horses can be grouped as foals and remain in their group throughout life (Väihkönen et al. 2002).

2 Literature review

2.1 Lactate formation in muscle

At rest and during light work, muscle cells produce ATP through aerobic pathways to meet their energy demand. Equine muscle uses free fatty acids and glucose from blood as well as intramuscular glycogen as primary fuel substrates during aerobic exercise (Rivero and Piercy 2008). Oxidization of glucose and glycogen by glycolysis results in pyruvate formation. Pyruvate and fatty acids are converted to acetyl-CoA, which enters the tricarboxylic acid cycle where NADH and $FADH_2$ are formed. These then undergo oxidative phosphorylation within the inner mitochondrial membrane, forming ATP. However, this process is relatively slow and reliant on the delivery of oxygen. Furthermore, the number of mitochondria in the cell becomes a limiting factor in ATP production through oxidative phosphorylation. As the demand for ATP increases during intense exercise, aerobic energy production cannot provide enough energy to muscles and the anaerobic pathway of ATP production becomes increasingly important. Glycogenolysis in muscle increases and the pyruvate formed does not enter the tricarboxylic acid cycle, but is reduced to lactate instead (Nelson and Cox 2005).

2.2 Effect of lactate accumulation on muscle metabolism

The recruitment of the anaerobic pathway leads to the accumulation of lactate and protons in muscle cells. The accumulation of these two ions occurs at nearly the same rate in active muscle (Juel 2008). In horse muscle, type *IIB* fibres contain the most glycogen, and due to rapid glycogenolysis during intense exercise, the highest lactate concentrations are measured in these fibres (Valberg et al. 1985). At rest, the lactate content of horse muscles is approximately 4.5 mmol/L of muscle water, and after a 2000 m gallop, concentrations of up to 52 mmol/L have been reported (Harris et al. 1987).

At the onset of fatigue, muscle loses its ability to contract efficiently. The accumulation of lactate and protons has been considered as the main cause of muscle fatigue (Fitts 1994; Hogan et al. 1995). In humans, an increase in the lactate concentration in the legs following arm exercise leads to the more rapid development of fatigue (Bangsbo et al. 1996). Cell volume is one of the major regulators of cell function, and osmotic swelling of cells induced by lactate accumulation has been suggested as a cause of impaired force production in the muscle (Fitts 1994). However, the compensatory effects of intracellular H^+ changes on the number of osmotically active particles have been shown to balance the effect of an increasing intracellular lactate concentration on cell volume (Usher-Smith et al. 2006). Furthermore, there is controversial evidence of the intramuscular lactate concentration not correlating well with muscle fatigue in humans (Karlsson et al. 1975). Studies with skinned muscle fibres have shown that even at abnormally high concentrations, lactate has little effect on muscle contraction and does not

explain fatigue (Allen et al. 2008). Some controversy also exists concerning the actual mechanism of fatigue, and it is likely that several mechanisms, such as ionic changes affecting the membrane potential, the failure of Ca^{2+} release from the sarcoplasmic reticulum and effects of reactive oxygen species contribute to it (Allen et al. 2008).

The concurrent increase in free H^+ also has various consequences for the cell. A low pH has inhibitory effects on the activation of the contractile apparatus and Ca^{2+} release from the sarcoplasmic reticulum (Fitts 1994). However, recent evidence has shown that the effects are not as strong as believed, and the effect of low pH on the Ca^{2+} pump in the sarcoplasmic reticulum actually favours force development (Allen et al. 2008). In any case, effective regulation of both pH and ion concentrations in muscle has been shown to be beneficial for performance (Juel 2008). Current knowledge of the effects of lactate and proton accumulation on muscle is mostly based on rat and human studies. To my knowledge, similar studies have not been performed on horse muscle.

Cells have adapted to the physiological stress caused by exercise by having several mechanisms to prevent the cell from becoming too acidotic. In horse muscle, several buffers, including proteins, bicarbonate, inorganic phosphate and carnosine dipeptide, reduce the fluctuations in cell pH during muscle activity (Hyyppä and Pösö 1998). The buffering capacity of horse muscle exceeds that of human muscle (Harris et al. 1990). The high carnosine content, especially in type *IIB* fibres, has been suggested as a cause (Sewell et al. 1992). In addition to buffers, lactate and protons can also be transported from the cell by several mechanisms to hinder the pH decline (Juel 2008).

2.3 Transport of lactate from muscle

Lactate is a monocarboxylic acid and can move across phospholipid membranes by free diffusion of the undissociated acid (Walter and Gutknecht 1984). However, due to the low pK_a value of lactate (3.86), more than 99.9% of lactate in muscle is in a dissociated form at the physiological pH (7.4). During intense exercise, the pH in horse muscle can drop to as low as 6.5, increasing the amount of undissociated acid (McCutheon et al. 1992). However, still only less than 1% of lactic acid is in an undissociated form and can freely diffuse across the cell membrane. Therefore, at a physiological pH in concentrations of <10 mmol/L of lactate, free diffusion accounts for less than 5% of the total lactate transport across the membrane (Deuticke et al. 1982).

The first known transport protein for lactate was the band 3 protein, also known as the inorganic anion transporter, which is an antiport carrier that exchanges lactate for Cl^- or HCO_3^-. In the early work of Halestrap (1976), this transporter was shown to be the main transporter of lactate into human erythrocytes at high (20 mmol/L) concentrations. However, blocking of the band 3 protein did not stop lactate transport, indicating a third mechanism of lactate transport. This third transport mechanism was shown to be active at a pH closer to the physiological range (Halestrap 1976). Earlier on, Halestrap and Denton (1974) had suggested a lactate and pyruvate carrier in the human erythrocyte membrane on the basis of transport inhibition by an aromatic analogue of these monocarboxylates, α-cyano-4-hydroxycinnamate (CHC). Later studies revealed that in addition to CHC,

organomercurials, such as *p*-chloromercuribenzene sulphonate (*p*CMBS), also inhibit lactate transport to RBCs, which provided further evidence of a third mechanism of lactate transport in the erythrocyte membrane (Halestrap and Denton 1974; Halestrap 1976; Deuticke et al. 1978). Dubinsky and Racker (1978) demonstrated that at the physiological pH and lactate concentrations resembling those of submaximal exercise, carrier-mediated transport was the most important form of transport. It was concluded that the transport of lactate across mammalian erythrocyte membranes has three separate mechanisms: non-ionic diffusion, the band 3 protein and most importantly of all, a specific lactate transporter. The lactate transporter was later named as the monocarboxylate transporter (Poole and Halestrap 1993).

2.4 MCT transporter family

2.4.1 Discovery of MCT1

The first studies on monocarboxylate transporters in the 1970s were performed on human red blood cells using various substrates, such as pyruvate, acetate and lactate (Halestrap and Denton 1974; Halestrap 1976; Dubinsky and Racker 1978). The functional properties of the lactate transporter were described well before the actual transporter protein was identified. The erythrocyte monocarboxylate transporter was shown to be electroneutral, as one proton was transported together with a lactate ion across the membrane (Dubinsky and Racker 1978; Deuticke 1982). The direction of transport was reversible and governed by the proton concentrations on either side of the membrane (Deuticke 1982). Furthermore, lactate transport was shown to be stereoselective. L-lactate was transported several-fold faster than D-lactate (Deuticke et al. 1978).

The first protein in the MCT family was identified by labelling studies in rat and rabbit erythrocytes, followed by sequencing of the N-terminus of the erythrocyte lactate transporter (Poole and Halestrap 1992, 1994). The sequence was identical to a putative 12-transmembrane domain transporter (MEV) of unknown function, which was earlier cloned by Kim et al. (1992) from a Chinese hamster ovary cell line that exhibited advanced mevalonate uptake. The wild type protein was shown to catalyse proton-linked lactate and pyruvate transport and was named monocarboxylate transporter 1 (Garcia et al. 1994a). The full length cDNA of human MCT1 was published soon after (Garcia et al. 1994b). In several species, the molecular weight of the MCT1 protein is about 43 kDa (Poole and Halestrap 1992; Garcia et al. 1994a). MCT1 has a higher affinity for pyruvate than for lactate. The K_m for L-lactate and pyruvate has been reported to range between 3.5-8.3 mmol/L and 1.0-3.1 mmol/L, respectively, among several species studied (Garcia et al. 1995; Lin et al. 1998; Bröer et al. 1999).

MCT1 is widely distributed and in the species studied it has been found to be expressed in almost all tissues (Halestrap and Meredith 2004). In the horse, MCT1 is at least present in muscle and erythrocytes (Koho et al. 2002, 2006). Horse MCT1

(*SLC16A1*) cDNA was first sequenced by Dayly and Chirazi-Beezhey (GenBank accession no. AY457175.1) and is 91% homologous to the putative MCT1 sequence of a dog and wild boar, and 90% homologous to cattle. The respective similarities compared to the horse MCT1 protein sequence (GenBank accession no. NP_001075260) are 93%, 92% and 89%. The discovery of monocarboxylate transporters with slightly different properties in several tissues, i.e. erythrocytes, hepatocytes and cardiac myocytes, led to the proposal that a whole family of MCTs might exist (Poole and Halestrap 1993).

2.4.2 Other family members

2.4.2.1 MCT2

A protein that was 60% homologous to hamster MCT1 was first found in a hamster liver cDNA library and was named MCT2 (Garcia et al. 1995). The tissue distribution of MCT2 was found to be strikingly different from MCT1 in humans (Garcia et al. 1995). MCT2 was found in human skeletal muscle, but not in erythrocyte membranes (Garcia et al. 1995). Jackson et al. (1997) detected MCT2 mRNA with northern blotting in hamster, but not in rat or mouse skeletal muscle. Later on, studies reporting the amount of MCT2 mRNA in different human tissues proved conflicting. Price et al. (1998) reported only little if any MCT2 mRNA in human skeletal muscle or any other tissue examined. On the other hand, Lin et al. (1998) detected an abundance of MCT2 mRNA in human skeletal muscle. Despite MCT2 mRNA being found in hamster skeletal muscle, no protein expression was detected by Jackson et al. (1997) in hamster muscle with Western blotting. However, Bonen et al. (2006) reported the expression of MCT2 in rat skeletal muscle. Some of the controversy could be due to the fact that some commercial antibodies have shown poor specificity to MCT2 (Bergersen et al. 2001). There have also been reports of human and swine skeletal muscle expressing this isoform (Sepponen et al. 2003; Bonen et al. 2006). Koho et al. (2006) studied the expression of MCT2 in horse skeletal muscle, but did not detect any MCT2 expression apart from very weak traces of MCT2. However, MCT2 is abundantly expressed in horse erythrocytes (Koho et al. 2002, 2006).

MCT2, like MCT1, transports monocarboxylates, but the kinetics of MCT2 differ (Garcia et al. 1995). MCT2 has a higher affinity for several substrates, including lactate and pyruvate, compared to MCT1 (Lin et al. 1998; Bröer et al. 1999). Like MCT1, MCT2 has a higher affinity for pyruvate than for lactate. The K_m values for L-lactate and pyruvate have been reported to range between 0.74 -8.7 mmol/L and 0.025-0.8 mmol/L, respectively, in several species studied (Garcia et al. 1995; Lin et al. 1998; Bröer et al. 1999). However, the capacity of MCT2 to transport lactate is much lower than that of MCT1. When rat MCT isoforms are expressed in *Xenopus* oocytes, MCT1 has an over 20 times greater V_{max} for lactate than MCT2 (Bröer et al. 1999).

MCT2 appears to have somewhat more interspecies differences in its amino acid sequence than MCT1 (Jackson et al. 1997). Putative horse MCT2 (*SLC16A7)* cDNA from the horse transcriptome (GenBank accession no. XM_001490658.1) is 86% homologous

to the predicted dog MCT2 cDNA and 85-87% homologous to the cDNA of several primates, including humans. The putative dog MCT2 protein most resembles horse MCT2 (XP_001490708.1) and shares 85% of the amino acid sequence. Several sizes of transcripts have been shown in rats, mice and humans (Jackson et al. 1997). However, there is no evidence of splice variants of the actual protein in any species. When MCT1 and MCT2 are expressed by the same tissue, they are located in different cell types, indicating a different function for the two proteins (Garcia et al. 1995; Bergersen et al. 2001). Interestingly, MCT2 mRNA is expressed more abundantly in cancer cell lines than in normal cells, suggesting that this isoform might be essential for cancerous cell metabolism (Lin et al. 1998).

2.4.2.2 MCT4

MCT4 has been found in the muscle of several species, including the horse, human, rat and swine (Sepponen et al. 2003; Bonen et al. 2006; Koho et al. 2006). Studies on rat and human MCT4 expressed in *X. laevis* oocytes have revealed MCT4 to have a much lower affinity for MCT substrates than MCT1. Studies report a K_m of 28-33 mmol/L and 25-153 mmol/L for L-lactate and pyruvate, respectively (Dimmer et al. 2000; Manning Fox et al. 2000). To my knowledge, MCT4 is not expressed in the erythrocytes of any species, including the horse (Koho et al. 2002). The sequence found in the horse transcriptome resembling the MCT4 (*SLC16A3)* cDNA sequence in other species is, respectively, 89% and 87% homologous to cattle and putative dog MCT4 cDNA sequences. Similarly, the candidate for horse MCT4 protein is 92% and 91% homologous to the cow and putative dog sequences, respectively.

2.4.2.3 Other members of the MCT family

In several species studied, 14 MCT isoforms have been identified, but only isoforms MCT1-4 have been experimentally demonstrated to be capable of transporting monocarboxylates, such as lactate and pyruvate (Halestrap and Meredith 2004). Of the other MCT family members, at least isoforms MCT5, MCT6, MCT8, MCT10 and possibly MCT7 have been identified in the muscle of other species (Kim et al. 2002; Bonen et al. 2006; Mebis et al. 2009). However, none of these isoforms have been found in equine muscle (Koho et al. 2006; Koho et al. unpublished). In a recent study by Koho et al. (2008), an abundance of MCT7 was detected in dog erythrocytes, which show a high lactate transport activity (Väihkönen et al. 2001). This finding led to the conclusion that MCT7 might also transport lactate.

2.4.3 The structure of the transporter complex

Unlike the vast majority of membrane proteins, MCTs are not glycosylated (Carpenter et al. 1996). Therefore, both MCT1 and MCT4 need an ancillary protein, CD147 (EMMPRIN, basigin, neurothelin), for both translocation to the cell membrane and to form an active transporter complex on the membrane (Kirk et al. 2000; Gallagher et al. 2007). In the absence of CD147, they accumulate in the endoplasmic reticulum or Golgi apparatus (Kirk et al. 2000). MCT1 has 12 membrane spanning domains and intracellular N- and C-termini (Figure 1; Poole et al. 1996). Topology predictions suggest a similar structure for MCT4 (Juel and Halestrap 1999). Wilson et al. (2002) provided evidence that an active transporter complex consists of two MCT1 molecules and two CD147 molecules (Figure 1). Fluorescence energy transfer (FRET) could be demonstrated between MCT1 and CD147, when both proteins were tagged on their intracellular domains. This indicated that the C terminus of CD147 in the cytosol was close to the C-terminus of the other CD147, as well as the N- and C-termini of MCT1 (Figure 1).

Several studies with mutated proteins have highlighted essential areas in the amino acid sequence for the normal function of the transporter complex. Helix 8 is important for successful lactate binding and the coupling of lactate transport with proton translocation (Rahman et al. 1999). In order to gain substrate specificity, the structure of helix 10 and the intracellular loop between helixes 4 and 5 are essential (Rahman et al. 1999; Galic et al. 2003). The three-dimensional molecular model of MCT1 was first based on the glycerol-3-phosphate transporter structure in *E.coli* (Manoharan et al. 2006). More recently, the same group suggested that MCT1 has an outward facing conformation. In this model, the transporter first accepts a proton, then a lactate ion, and the substrates are then passed through an ion channel across the membrane (Wilson et al. 2009).

While other species studied have CD147 as an ancillary protein for MCT1, the rat is an exception. Poole and Halestrap (1997) discovered that a stillbene disulfonate caused crosslinking of MCT1 with another protein on the rat erythrocyte membrane. This protein was identified as gp70 (Poole and Halestrap 1997). At least in the rat, gp70 is also the ancillary protein for MCT2 (Wilson et al. 2005).
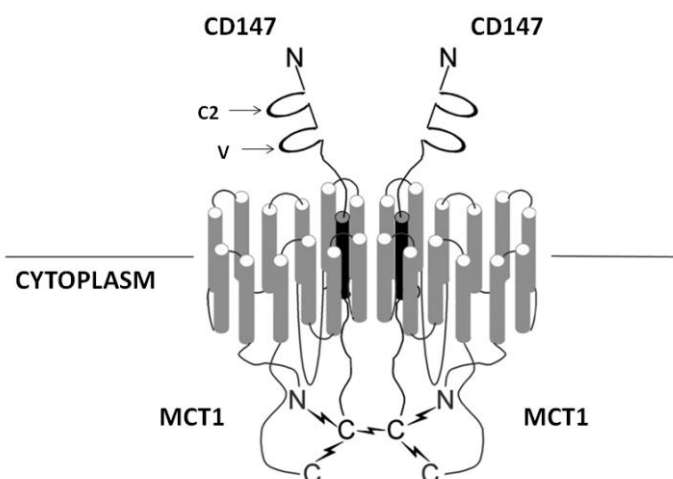
Figure 1. The hypothetical structure of the MCT1/CD147 complex on the cell membrane (modified from Wilson et al. 2002, with permission)

2.4.4 Ancillary protein CD147

The ancillary protein CD147 is also known as basigin, M6, extracellular matrix metalloproteinase inducer (EMMPRIN) and neurothelin. Human CD147 is a 54 kDa plasma membrane glycoprotein that has one extracellular immunoglobulin (Ig)-like C2-type domain and one V-type domain, a transmembrane domain, and a small intracellular domain that contains the C-terminus (Figure 1; Kasinrerk et al. 1992; Biswas et al. 1995). Among others, horse CD147 cDNA has been sequenced, and there appear to be quite substantial differences in the nucleotide sequence among species (GenBank accession no. EF564280: Reeben et al. 2006). The protein sequence shows approximately 80% homology among the species studied to date (GenBank accession no. ABQ53583.1; Reeben et al. 2006).

The three-dimensional molecular model of MCT1 has suggested that the transmembrane domain of CD147 is situated alongside the third transmembrane helix of MCT1 on the transporter complex (Manoharan et al. 2006). More precisely, the hydrophobic amino acid residues at the N- and C-termini of the transmembrane segment of CD147 are essential for the interaction of the two proteins (Finch et al. 2009). In addition to mutations in MCT1, changes in the extracellular part of CD147 have also been shown affect MCT1 transporter function. For instance, the MCT inhibitor *p*CMBS functions by attacking CD147 (Wilson et al. 2005).

In addition to enabling lactate transport by the MCTs, CD147 also has several other functions. It is widely expressed in metabolically active tissues in the body and is known to interact with various proteins on the cell membrane (Nehme et al. 1995). To date, information has been reported on CD147 interacting with at least integrins, calveolin-1 and cyclophilins (Berditchevski et al. 1997; Yurchenko et al. 2001, 2002; Tang and Hemler 2004). A complete inability to produce CD147 has multiple severe consequences to the individual. CD147 knockout mice are usually unable to undergo implantation and

are small and sterile if they survive (Igakura et al. 1998). In mice red blood cells, the lacking surface expression of CD147 not only affects lactate transport, but also leads to trapping of erythrocytes in the spleen, inducing anaemia (Coste et al. 2001).

2.4.5 Effect of inhibitors

The function of MCTs has been studied by using various inhibitors. Several of these inhibitors function by affecting the ancillary proteins of MCTs. *p*-chloromercuribenzene sulphonate (*p*CMBS) inhibits both MCT1 and MCT4 function by attacking the disulfide bridge in the Ig-like C2 domain of CD147 (Wilson et al. 2005). Therefore, *p*CMBS inhibits lactate transport in rabbit erythrocytes, where MCT1 interacts with CD147, but not in rat erythrocytes, where the ancillary protein for MCT1 is gp70 (Wilson et al. 2005). *p*CMBS also does not inhibit MCT2 function in sf9 insect cells transfected with hamster MCT2, because the ancillary protein for MCT2 is not CD147 (Garcia et al. 1995).

Another MCT inhibitor, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), causes rapid reversible MCT1 inhibition, which is competitive with respect to L-lactate (Poole and Halestrap 1991). This is followed by slowly developing irreversible inhibition, which is supposedly caused by one of the isothiocyanate groups of DIDS attacking a lysine residue in MCT1 (Poole and Halestrap 1991). Poole and Halestrap (1997) discovered that prolonged incubation with DIDS crosslinks MCT1 and gp70 in rat erythrocytes. The crosslinking was more rapid in alkaline buffer, which led to the conclusion that DIDS binds to MCT1 on its outward facing binding site (Poole and Halestrap 1997). Wilson et al. (2009) subsequently showed that DIDS can crosslink MCT1 and gp70 in rat erythrocytes by attacking lysine residues in both MCT1 and gp70. However, rabbit erythrocytes are not sensitive to DIDS, because MCT1 uses CD147 as an ancillary protein (Wilson et al. 2009). DIDS also inhibits the band 3 protein at <10 µmol/L concentrations, and >40 µmol/L is required for MCT inhibition (Poole and Halestrap 1993).

2.5 Expression of MCTs in different fibre types

2.5.1 Fibre type distribution of equine muscle

The fibres in skeletal muscle have different contractile and metabolic properties. Brooke and Kaiser (1970) introduced a method to identify different fibre types with histochemical staining for myofibrillar ATPase. Numerous studies have identified three fibre types in equine muscle, namely slow-contracting type *I* fibres and fast contracting type *IIA* and *IIB* fibres, of which type *IIB* is the fastest fibre (Lindholm and Piehl 1974; Essén-Gustavsson and Lindholm 1985; Valberg et al. 1985; Gottlieb et al. 1989; Ronéus et al. 1991, 1992). More recently, the myosin heavy chain (MyHC) type has been used to identify different

fibre types in the horse, resulting in the discovery of type *IIAB* hybrid fibres, which contain two types of myosin (Linnane et al. 1999; Quiroz-Rothe and Rivero 2001). The hybrid fibres represent a stable population in the horse muscle and are also found in untrained individuals (Linnane et al. 1999). When horse muscle is compared to rat muscle, the three main horse MyHCs correspond to the rat type *I*, *IIA* and *IIX* fibres (Rivero et al. 1999). The existence of true *IIB* fibres in horses has been questioned, and in recent publications, type *IIB* fibres have therefore often been referred to as *IIX* fibres and *IIAB* hybrid fibres *IIAX* fibres when immunohistochemical staining has been used to identify them (Rivero et al. 1997, 1999; Karlström and Essén-Gustavsson 2002).

The oxidative capacity of fibres can be evaluated by histochemical staining for mitochondrial enzymes, such as NADH dehydrogenase, which is the first enzyme of the mitochondrial electron transport chain. In the horse, the highest oxidative capacity is seen among fibre types *I* and *IIA*. Most *IIB* fibres have a low oxidative capacity, especially in young horses. However, well-trained horses can also have a high oxidative capacity in type *IIB* fibres (Essén et al. 1980; Hodgson et al. 1986; Karlström and Essén-Gustavsson 2002). In horse muscle, oxidative fibres are often found in areas with a rich vascular supply. Conversely, non-oxidative *IIB* fibres are found in areas with a poorer blood supply and they show a high glycolytic capacity (Armstrong et al. 1992).

There is substantial variation in the fibre type composition of different horse muscles (Snow and Guy 1980; van den Hoven et al. 1985; Karlström et al. 1994; Gellman et al. 2002; Grotmol et al. 2002; Kawai et al. 2009). Furthermore, within the muscle of a horse, the fibre type composition varies depending on the sample depth (Lopez-Rivero et al. 1992; Sewell et al. 1992; Rivero et al. 1993; Karlström et al. 1994; Serrano et al. 1996; Grotmol et al. 2002). In addition, there are differences in the fibre type composition of more and less athletic horse breeds, with sprinters having more type *IIB* glycolytic fibres in their gluteal muscles (Snow and Guy 1980; Bump et al. 1990). Training has a marked influence on the fibre type composition in several mammalian species studied, and both strength and endurance training induce changes in muscle, where the fibres tend to change from fast glycolytic towards slower, more oxidative fibres. As training progresses, this fibre type change can be observed in a graded sequential manner in the order: *IIB→IIAB→IIA→I* (Pette and Staron 1997).

The middle gluteal muscle (*m. gluteus medius*) is a locomotory muscle that has been subject to numerous studies in equine exercise physiology (Lindholm and Piehl 1974). In this muscle, all three fibre types, as well as hybrid fibres can be recognised (Rivero et al. 1996; 1997, 1999; Serrano et al. 2000; Karlström and Essén-Gustavsson 2002; Kawai et al. 2009). In the deep parts of this muscle, type *I* and *IIA* fibres dominate. The proportion of type *IIB* fibres increases towards the more superficial parts of the muscle, and they are the most common fibre type found in the outermost layer of the muscle (van den Hoven et al. 1985; Lopez-Rivero et al. 1992; Sewell et al. 1992; Serrano et al. 1996; Grotmol et al. 2002).

2.5.2 Distribution of MCTs in muscle

In skeletal muscle, MCT1 was first detected in oxidative mitochondria-rich hamster muscle fibres by Garcia et al. (1994a). Later, McCullagh et al. (1996) compared several rat hindlimb muscles, finding a positive correlation between the number of oxidative fibres and MCT1 content. In the same study, it was shown that muscles rich in oxidative fibres, such as the red gastrognemius and red tibialis anterior, can also take up more lactate from the circulation. This indicated that the likely role of MCT1 on the cell membrane is to transport lactate into the muscle cell, where it can be used in oxidation or glycogen resynthesis (Putman et al. 1999; Krssak et al. 2000). Several subsequent studies have confirmed that MCT1 expression correlates with the number of oxidative fibres in rat and human muscle (Wilson et al. 1998; Pilegaard et al. 1999b; Bonen et al. 2000). MCT4, on the other hand, was found in almost equal amounts in different muscles, indicating that it might serve a different function in the cell membrane compared to MCT1 (Wilson et al. 1998; Pilegaard et al. 1999b).

A more specific picture of the fibre type distribution of MCTs has been obtained from the immunohistochemistry of human and rat muscle sections (Wilson et al. 1998; Pilegaard et al. 1999b; Fishbein et al. 2002; Hashimoto et al. 2005). These studies have confirmed at the cellular level that MCT1 is highly expressed in the sarcolemma of oxidative muscle cells, especially in type *I* and *IIA* fibres, compared to non-oxidative type *IIB* fibres. On the other hand, immunohistochemical studies have shown that MCT4 is mainly expressed in non-oxidative type *IIB* fibres as well as type *IIA* fibres (Pilegaard et al. 1999b; Fishbein et al. 2002; Hashimoto et al. 2005). These results contradict earlier studies that reported little variation in the total amount of MCT4 between muscles (Wilson et al. 1998; Pilegaard et al. 1999b). However, the previous studies were performed on muscles that contained several fibre types. Furthermore, some of the inconsistency in the results might be due to greater interindividual variation among humans in the expression of MCT4 compared to MCT1 (Pilegaard et al. 1999b).

The fibre type-specific distribution of transporters has led to conclusions about their function on the membrane. Both MCT1 and MCT4 can catalyze both lactate efflux and influx, and the direction of transport follows the proton gradient (Deuticke 1982; Wilson et al. 1998). While MCT4 is probably responsible for the removal of lactate from glycolytic cells, during intensive exercise both carriers are likely to be responsible for lactate efflux from the muscle. In addition to the sarcolemma, several studies have shown MCTs to also be present in the mitochondrial membrane, where its function is probably associated with both pyruvate and lactate transport (Brooks et al. 1999; Benton et al. 2004; Butz et al. 2004). Butz et al. (2004) reported MCT1 to be present in both sub-sarcolemmal and interfibrillar mitochondria. However, according to Benton et al. (2004), the expression of MCT1 is limited to only sub-sarcolemmal mitochondria, where it is co-expressed with MCT2 and MCT4. To my knowledge, the expression of MCT isoforms on horse fibre types has not been investigated.

The expression of the ancillary protein CD147 has been extensively studied in myocardial cells, but little information is available on skeletal muscle (Huet et al. 2008). An immunohistochemical study has been performed on mouse skeletal muscle, in which

CD147 is found in the sarcolemma (Nakai et al. 2006). However, knowledge of the fibre type distribution of CD147 expression in the skeletal muscle of any species is lacking (Nakai et al. 2006).

2.5.3 Effect of training on MCT expression in muscle

In humans, training increases the rate of lactate and proton clearance from muscle (Juel et al. 2004a,b). There is also evidence of the same effect in the horse. McGowan et al. (2002b) measured increased plasma lactate concentrations in trained horses after a treadmill exercise, while the post-exercise lactate concentration in muscle did not increase. The enhanced lactate extrusion observed with training is likely to be at least partially due to an increase in MCT activity. In humans, various types of long-term training have been reported to increase MCT1 expression by 15-76% (Pilegaard et al. 1999b; Juel et al. 2004a,b). The effect seems to be quite rapid, since Bonen et al. (1998) reported an 18% increase in sarcolemmal MCT1 expression after a week of daily 2-hour bicycle training. The expression of MCT4 has also been reported to increase, but only by 11-32% (Pilegaard et al. 1999b; Juel et al. 2004a,b). There is indirect evidence, that training has an influence on MCT4 expression also in the horse. Koho et al. (2006) reported older and more trained animals to have more MCT4 but not MCT1 expression in their muscle compared to untrained young individuals (Koho et al. 2006). However, Kitaoka et al. (2010) failed to show an increase in either MCT1 or MCT4 expression in the *gluteus medius* of Thoroughbred racehorses after seven weeks of intensive training.

Interestingly, exercise also has an immediate effect on the expression level of MCTs. Bickham et al. (2006) reported an increase in MCT1 expression in humans 2 hours after a single exercise bout. The mRNA level did not increase, suggesting a post-transcriptional regulation of MCT expression in humans. However, when MCT1 and MCT4 were measured immediately after an acute period of high intensity exercise in human subjects, the membrane expression of these two proteins was found to have declined by approximately 20% (Bishop et al. 2007). There might be a species difference in the transcriptional and post-transcriptional regulation of MCT1 expression, since increased amount of both MCT1 mRNA and protein have been reported immediately after exercise in rats (Coles et al. 2004).

2.6 Genetic defects in MCT1 causing myopathy

Fishbein (1986) was the first to describe a lactate transporter defect in the muscle of a patient causing signs of myopathy. This individual was not otherwise sick, but following exercise had suffered from chest pain resembling the symptoms of coronary artery disease with angina. Despite rest, the patient had had repeated measurements of creatine kinase (CK) activities that were several-fold higher than normal. This individual produced lactate in a similar manner to control subjects, but the concentration did not decline as rapidly, indicating a failure to transport lactate from the muscle. Simultaneously, this patient was

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shown to have impaired lactate uptake into red blood cells (RBCs). Fishbein's conclusion was that both muscle and RBCs share a common lactate transporter that was deficient in this individual. The muscle sample was subsequently sequenced and a single nucleotide variation in the coding region of MCT1 was found (Merezhinskaya et al. 2000). This 610A>G variation caused a K204G mutation in a large intracytoplasmic loop between helixes 6 and 7 of MCT1. The patient was heterozygous for this allele. This mutation in MCT1 could not be found in 90 normal muscle samples studied and therefore provided a reasonable explanation for the clinical signs and abnormal plasma and RBC lactate measurements described earlier by Fishbein (1986). However, this mutation has more recently been associated with normal MCT function in *X. laevis* oocytes, and some controversy exists on the actual functional significance (Halestrap and Meredith 2004). In the same study, Merezhinskaya et al. (2000) also reported another heterozygous 1414G>A nucleotide sequence variation causing a G472R mutation near the intracytoplasmic C-terminus of the protein. This mutation was detected in two other individuals with high CK activities and signs of exercise intolerance but was not found in the control group.

Another sequence variation was found in people with myopathy that was also common in a healthy Caucasian population, with half of the people studied carrying it (Merezhinskaya et al. 2000). This 1470A>T nucleotide sequence variation caused a E490D mutation, which was located just 10 amino acids from the C-terminal of MCT1. In addition to Caucasians, this sequence variation was also found to be common in the Chinese population (Lean and Lee 2009). Interestingly, in a small study with 10 healthy humans, Cupeiro et al. (2010) observed that this 1470A>T sequence variation has physiological significance. People carrying the mutation showed higher lactate accumulation in a capillary blood sample after high intensity circuit weight training compared to non-carriers (Cupeiro et al. 2010).

Sequence variations in the lactate transporting proteins have also been described in the horse. In a small study involving 10 horses, 7 with signs of myopathy, variations in the coding sequence of MCT1 and partial coding sequence of CD147 were examined (Reeben et al. 2006). One 1573A>C nucleotide sequence variation causing a K457E mutation was found in the C-terminal area of MCT1. This mutation did not involve the same nucleotides in which mutations were found in the MCT1 C-terminus of human patients (Merezhinskaya et al. 2000). This sequence variation was recorded in a control horse, and therefore probably does not affect protein function. In addition, in several horses a 389T>C nucleotide sequence variation causing a M125V mutation was found in the extracellular domain of CD147. Although an intact extracellular structure of CD147 has been shown to be essential for the normal function of MCT1, this mutation in CD147 could not be linked with signs of myopathy or lactate transport activity (Wilson et al. 2005; Reeben et al. 2006). A larger number of horses should be studied in order to determine whether mutations in these proteins have a role in myopathy in this species.

2.7 Transport of lactate into red blood cells

2.7.1 Red blood cells as a lactate sink

From the plasma, lactate can be transported to red blood cells or other tissues, such as the heart, inactive skeletal muscle or liver, where it can be used as a fuel (Johnson and Bagby 1988; Putman et al. 1999). It has been speculated that the influx of lactate from the plasma into RBCs sustains the gradient between muscle cells and the plasma, enabling more lactate to be produced in the muscle cells (Pösö et al. 1995). The ability to use RBCs as a lactate sink might be beneficial in high intensity exercise, when an abundance of lactate is formed (Pösö et al. 1995; Juel et al. 2003; Bayly et al. 2006). The capacity to transport lactate into RBCs and the main pathway varies among species. Skelton et al. (1995) showed that horses and dogs have a several-fold greater lactate influx into RBCs compared to goats and cattle. Furthermore, it was demonstrated that in these species, monocarboxylate transporters are the primary pathway, accounting for about 90% of RBC lactate uptake. Ruminants, on the other hand, show little or no MCT or CD147 expression and the primary lactate transport mechanism is via the band 3 protein (Deuticke et al. 1978; Poole and Halestrap 1988; Skelton et al. 1995; Wilson et al. 2005). This also includes the reindeer, which is an athletic ruminant species also used in racing (Väihkönen et al. 2001).

During exercise in the horse, cathecholamines cause the spleen to contract and a large pool of reserve erythrocytes is released into the circulation (Persson 1967). The total amount of RBCs in the blood stream increases significantly, as the haematocrit can reach 60-65% during strenuous exercise (Rose and Allen 1985). However, some of the increase is due to a loss of plasma volume (Kunugiyama et al. 1997). In any case, the capacity of horse RBCs to store lactate is not small, since up to 50% of blood lactate can be found in RBCs after exercise (Pösö et al. 1995; Väihkönen et al. 1999). The percentage is significantly higher than that in human athletes, in which around 20-30% of lactate can be found in RBCs after exercise (Juel et al. 1990; Lindinger et al. 1994; Smith et al. 1997).

In humans, MCT1 is responsible for the transport of lactate across the RBC membrane (Halestrap and Meredith 2004). Until recently, it was also considered to be the only MCT isoform present in the RBC membrane in other species. However, two isoforms have been found in the horse: MCT1 and MCT2, (Koho et al. 2002, 2006). A recent study on dog erythrocytes also revealed two isoforms, MCT1 and MCT7 (Koho et al. 2008). This is an interesting finding, since dogs are reported to have a high lactate transport activity in their RBCs (Skelton et al. 1995; Väihkönen et al. 2001; Koho et al. 2008).

2.7.2 Bimodal distribution of lactate transporter activity

Pösö et al. (1995) reported that in Standardbred trotters, the plasma/RBC lactate concentration ratio varies interindividually. Väihkönen and Pösö (1998) later found that this was due to interindividual variation in the rate of lactate influx into red blood cells.

The horses could be divided into two distinct groups based on their lactate transport activity (Figure 2). Roughly 30% of the 89 horses studied had a very low level of lactate transport (LT) into RBCs, and the rest had a high lactate transport activity (HT; Figure 2). These two groups were subsequently identified in several other studies (Väihkönen et al. 1999, 2001; Koho et al. 2002, 2006). After a larger group of horses had been investigated, the prevalence of low lactate transport activity seemed to be quite consistently 25% in the Standardbred (Väihkönen et al. 1999, 2001; Koho et al. 2002, 2006). This bimodal distribution of lactate transport activity seems to be unique quality of the horse, since it has not been shown in other species studied (Väihkönen et al. 2001).
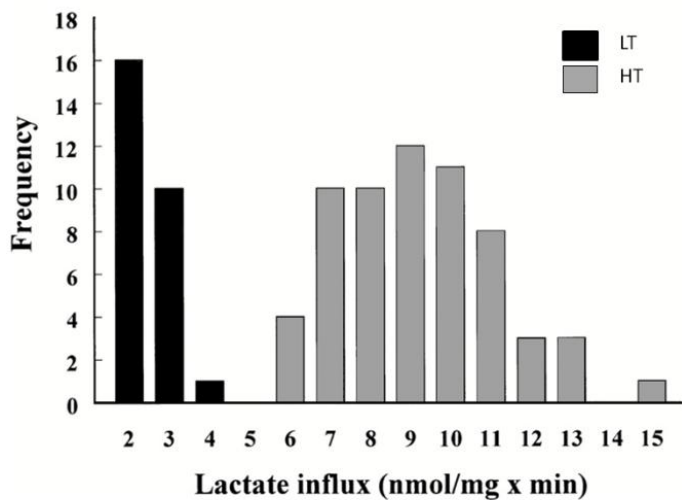

Figure 2. Frequency distribution of RBC total lactate influx in 89 Standardbred horses at 30 mM lactate concentration. LT = low lactate transport activity, HT = high lactate transport activity (modified from Väihkönen and Pösö 1998, with permission).

In vivo, it has been shown that after submaximal and maximal exercise, the lactate concentration in RBCs is higher in horses with a high lactate transport activity than in those with a low lactate transport activity (Väihkönen et al. 1999; Koho et al. 2002). Interestingly, an indirect connection between lactate transport activity and performance was demonstrated by Räsänen et al. (1995), who reported that horses with a higher lactate concentration in their RBCs were better performers. In this study, the 16 Standardbred trotters used as study animals consisted of a wide range of performers, including very good and very poor horses. However, Väihkönen et al. (1999) could not reproduce the results in a study with 55 trotters. In this case, no difference was found in the performance of horses with a high or low lactate transport activity. However, the range of performance indices in this group of horses varied much less than in the study of Räsänen et al. (1995).

Later on, Koho et al. (2002) observed that the horses in the LT and HT groups had similar levels of MCT1 and MCT2 expression in the RBC membrane, but the amount of the ancillary protein CD147 varied between the two groups (Koho et al. 2002, 2006). This led to the conclusion that the amount of CD147 determined the RBC lactate transport

activity of a horse. It was suggested that the role of MCT2 in RBCs is to transport lactate at low concentrations in horses, indicating that MCT1 is more important during exercise (Koho et al. 2002).

L-Lactate K_m values have been calculated separately for the LT and HT groups. LT horses have a somewhat lower K_m (0.63 mmol/L) compared to HT horses (0.88 mmol/L; Koho et al. 2002). The distribution of horses between two distinct lactate transport activity groups is not completely indisputable. From among more than 200 horses studied, one horse with intermediate lactate transport activity has been found (R. Pösö, personal communication). In addition, one Standardbred racehorse has shown both intermediate expression of CD147 on the RBC membrane and intermediate lactate transport activity (Koho et al. 2006).

Previously, lactate transport activity has only been examined in the Standardbred (Väihkönen and Pösö 1998; Väihkönen et al. 1999, 2001, 2002; Koho et al. 2002, 2006). Väihkönen et al. (2002) showed that horses could already be grouped into high and low lactate transport activity groups as 2-week-old foals. Although the individual lactate transport activity did change somewhat with age in the HT group, individuals remained in the same group in adulthood. In the same study, data from sires, dams and their offspring were used to demonstrate that low lactate transport activity was inherited as an autosomal recessive trait (Väihkönen et al. 2002). In the study of Väihkönen and Pösö (1998), the lactate transport activity in RBCs of Standardbred mares was higher than that of stallions. However, no difference was detected between sexes in later studies (Väihkönen et al. 1999, 2001, 2002). Since only one breed of horses has been studied, further research into the lactate transport activity in different breeds is warranted.

3 Aims of the study

-To examine the expression of MCT1, MCT2 and CD147 in the red blood cell membranes of racing Finnhorses, Standardbreds and Thoroughbreds (Study I).

-To determine whether the level of MCT1, MCT2 or CD147 expression in red blood cell membranes correlates with racing performance in Thoroughbred racehorses (Study I).

- To compare the expression of MCT1 and CD147 in different fibre types of the Standardbred horse gluteus medius muscle with immunohistochemistry (Study II).

-To investigate the effect of training on the expression of MCT1 and CD147 in different fibre types of horse gluteus medius muscle in Norwegian-Swedish Coldblood trotters (Study III).

- To study sequence variations in the cDNA of MCT1, MCT4 and CD147 in the muscle of healthy horses and horses with recurrent exercise-induced myopathy (Study IV).

4 Materials and methods

The study protocols were approved by the following authorities: the Ethics Committee for Animal Experiments of Agrifood Research, Finland (Finnhorse mare, Study II); the National Animal Research Authority, Norway (Study III), the National Animal Experiment Board, Finland (Standardbred horses, Studies I, IV) and the Helsinki University Ethics Committee for Animal Experimentation (Finnhorses, Studies I, IV).

For more detailed information on the materials and methods, see Studies I-IV.

4.1 Horses

Study I
Altogether, 118 Finnhorses (71 females and 47 males, aged from 1 to 22 years), 98 Thoroughbreds (54 females and 44 males, aged from 2 to 20 years) and 44 Standardbreds (24 females and 20 males, aged from 1 to 20 years) participated in this study. All horses were clinically healthy.

Study II
Twenty clinically healthy 2-year-old Standardbreds (14 fillies and 6 colts) stabled at the same yard in Sweden participated in this study. The horses were professionally trained and muscle biopsy samples were taken with the trainer's consent. Samples were collected in May during the training and racing season for 2-year-olds. Interval training on an uphill slope had been introduced into their training programme a few months earlier, but the horses were not yet race fit. The biopsy sample used for electron microscopy was taken from a healthy 8-year-old Finnhorse mare.

Study III
Nine Norwegian-Swedish Coldblood trotters (4 fillies and 5 colts) participated in this study. Muscle biopsy samples were taken on four occasions with six-month intervals during their training. The first sample was taken when the horses were approximately 2 years old and the last at 3.5 years old. The horses were trained by six separate trainers. The training protocol included 45- to 60-minute training sessions 4-5 times a week throughout the study period. By the final sampling occasion, the horses were race fit.

Study IV
Thirty Standardbreds (18 females and 12 males) and 12 Finnhorses (8 females and 4 males) aged from 2 to 20 years were included in this study. Of these, 16 horses (10 Finnhorses and 6 Standardbreds) were clinically healthy and had no reported history of myopathy. The remaining 26 horses (2 Finnhorses and 24 Standardbreds) had, according to the owner reports, suffered from repeated episodes of muscle stiffness post-exercise or

other signs of recurrent myopathy. All horses in the myopathy group had myopathy confirmed on at least one occasion by a veterinary surgeon and by serum biochemistry, which showed elevated activity of serum aspartate aminotransferase (AST) and creatine kinase (CK). During the study, all horses were subjected to light to intense training.

4.2 Collection of muscle and blood samples

4.2.1 Muscle samples (Studies II-IV)

Muscle biopsy samples were taken under local anaesthesia using a 5 mm modified Bergström biopsy needle from the middle gluteal muscle. The sample site was the midpoint of a line from tuber coxae to the root of the tail at a depth of 4 cm (Study III) or 6 cm (Studies II, IV; Lindholm and Piehl 1974). Biopsy samples were immediately frozen in liquid nitrogen and stored at -80 °C until analyzed using histochemical and immunohistochemical techniques. The sample for electron microscopy (Study II) was immediately cut into small pieces (1 mm^3) and fixed in 3% glutaraldehyde before further processing and embedding into resin blocks.

4.2.2 Blood samples (Studies I, IV)

Blood samples were collected from the jugular vein into serum and EDTA tubes and transported to the laboratory at room temperature. EDTA tubes were centrifuged and red blood cells (RBCs) and plasma separated. The RBCs were stored at -80 °C until analyzed (Studies I, IV). Serum tubes were centrifuged and serum analysed during the same day (Study IV).

4.3 Antibodies (Studies I-IV)

Horse MCT1 (GenBank accession No. AY457175.1), MCT2 (Reeben et al. unpublished), MCT4 (GenBank accession No. EF564279.2) and CD147 (GenBank accession No. EF564280.1) have been sequenced. Antibodies were raised in rabbits against the C-terminal peptides of horse MCT1 (CKGTEGDPKEESPL), MCT2 (CQSARTEDHPSERETNI), MCT4 (CEPEKNGEVVHTPETSV) and CD147 (CGHHVNDKDKNVRQRNAS). Antibodies were subsequently harvested and purified with affinity chromatography. Peptide synthesis, immunization and purification were carried out by Sigma Genosys (Cambridge, United Kingdom). The antibodies were tested in our laboratory and they gave a single band in Western blots, while preincubation of the antibody with the peptide that was used to immunize the rabbits blocked the staining. Commercial antibodies N2-261 (MHC *I + IIA*) and A4-74 (MHC *IIA*; Alexis

Biochemicals, Lausen, Switzerland) were used to stain the various myosin isoforms. These myosin antibodies have previously been used in the horse and the fibre typing results are consistent with the myosin ATPase stain (Karlström and Essén-Gustavsson 2002).

4.4 Analysis of muscle samples

4.4.1 Preparation of samples for histochemistry and immunohistochemistry (Studies II-IV)

Serial transverse sections (10 μm, Studies II, III; 20 μm, Study IV) of the muscle samples were cut in a cryostat (Reichert-Jung, Cambridge Instruments GmbH, Nussloch, Germany). For each staining, 2-3 sections were cut per horse.

4.4.2 Immunohistochemical staining (Studies II, III)

Immunohistochemical staining of myosin heavy chain isoforms was performed as described by Karlström and Essén-Gustavsson (2002). All slides included a negative control for primary antibody. The sections used for MCT1, MCT2, MCT4 and CD147 antibody staining were blocked with 5% goat serum (Dako, Glostrup, Denmark). The sections were then incubated with the primary antibody for 35 min at room temperature, after which they were stained with a DakoCytomation EnVision+ System-HRP (DAP) kit (Dako, Glostrup, Denmark) according to manufacturer's instructions. The slides were dehydrated, mounted in DPX and photomicrographed with automatic light exposure (Nikon Coolpix Microscope System, Tokyo, Japan). Neither MCT2 nor MCT4 antibody stained any of the muscle fibres. The amount of MCT1 and CD147 antibody in the cytoplasm of different fibre types was measured with an Olympus Cell^P imaging system (Olympus Biosystems GmbH, München, Germany) and in membranes using an AIDA image analyzer (Raytest isotopenmeßgeräte GmbH, Straubenhardt, Germany). The *IIB* cell cytoplasm was used as a baseline for the measurements. For a more detailed description of the measurement technique, see Study II.

4.4.3 Histochemical staining (Studies II-IV)

Muscle sections were also stained for myosin ATPase at pH 4.6 and NADH tetrazolium reductase according to Brooke and Kaiser (1970) and Novikoff et al. (1961). NADH staining intensities of different fibre types were measured with an Olympus Cell^P imaging system (Olympus Biosystems GmbH, München, Germany). The *IIB* cell cytoplasm was used as a baseline for the measurements.

Based on results from both the antibody stainings and the ATPase staining, fibres were classified into four types: *I, IIA*, *IIAB* and *IIB*. The fibres that stained as type *IIB* in the ATPase stain and also showed antibody staining for type *IIA* were identified as hybrid type *IIAB* fibres. In Study III, *IIB* and *IIAB* fibres are referred to as *IIX* and *IIAX* fibres, respectively.

In Study IV, muscle sections were first incubated for 30 min with amylase (1%), followed by periodic acid-Schiff (PAS) staining, to examine whether the samples contained abnormal glycogen indicative of polysaccharide storage myopathy (PSSM; Pearse 1960; Valberg et al. 1992).

4.4.4 Electron microscopy (Study II)

Samples were prepared as described by Gröhn and Lindberg (1982) and examined under a JEOL 100 S transmission electron microscope (JEOL Ltd., Tokyo, Japan).

4.4.5 Sequencing of MCT1, MCT4 and CD147 (Study IV)

Total RNA was extracted using QIAzol Lysis Reagent (QIAGEN, Valencia, CA, USA), after which mRNA was isolated using a Poly(A)Purist MAG-kit (Ambion, Inc; Austin, TX, USA) according to the manufacturer's instructions. RT-PCR was performed using PowerScript Reverse Transcriptase (Clontech Laboratories, Inc; Mountain View, CA, USA) with an oligo dT primer. Ribolock was used as an RNAse inhibitor during first-strand cDNA synthesis reaction (Fermentas GmbH, St. Leon-Rot, Germany). For a list of primers and PCR protocols, see Study IV. The PCR products were custom sequenced using both forward and reverse PCR amplification primers for the sequencing reactions (University of Helsinki, Biotechnical Institute, Finland).

4.5 Analysis of blood samples

4.5.1 Extraction of RBC membranes and Western blotting (Studies I, IV, unpublished data)

Plasma membranes of RBCs were isolated from frozen RBCs as described by Koho et al. (2002), and the protein concentration was measured with the BCA method (Uptima BC Assay, Interchim, Montlucon, France). Membranes were stored at -80 °C until analyzed. The amount of MCT1, MCT2 and CD147 on RBC membranes was detected by Western blotting according to Koho et al. (2002). For a detailed description of the quantitation of blots and validation of the antibodies used, see Study I.

4.5.2 Haematological and muscle enzyme activities (Study IV)

Haematological values were measured from plasma within 3 hours of collection. CK and AST were measured from serum with standardised methods (Konelab, Vantaa, Finland).

4.6 Racing performance (Study I)

Racing information on the Thoroughbred horses was obtained from the archived data of the Racing Post (1 Canada Square, London E14 5AP, UK), which contains the race and performance history for racing Thoroughbreds involved in flat racing, National hunt and point-to-point racing in the United Kingdom. For a more detailed description of the parameters used, see Study I.

4.7 Statistical analysis

Normally distributed data are presented as means ± SD and non-normally distributed data as medians (with interquartile ranges). The statistical tests were performed using the original measurement data. Differences between groups were analysed using one way ANOVA with repeated measures (Studies II, III) or a Mann-Whitney U-test (Studies I, IV). Correlations were calculated with Spearman's rank correlation analysis. In study I, the frequencies were compared with the chi-squared test and the bimodality of distributions was tested with an F-test following curve fitting (Origin 7.5, OriginLab Corporation, Northampton, MA, USA). Differences were regarded significant at $p < 0.05$.

5 Results

The main results of Studies I-IV are presented below. For more detailed results, please see the original publications.

5.1 Expression of CD147 and lactate transporters MCT1 and MCT2 in three horse breeds (Study I)

The distribution of the amount of CD147 in Western blotting was bimodal ($p < 0.001$) in all three study breeds: Finnorse (FH), Standardbred (SB) and Thoroughbred (TB), and the horses could be divided into two groups. The high lactate transport activity group (HT) horses expressed CD147, while very little or no expression was detected among horses in the low lactate transport activity group (LT; Figure 3). The intensity of the band was higher ($p < 0.001$) in the HT horses than in the LT horses of all three breeds (Figure 3). Altogether, 85% of Finnhorses and 82% of Standardbreds expressed a high amount of CD147. In TB, 88% had a high level of CD147 expression and 11% low expression. More horses belonged to the HT group in the TB compared to SB ($p < 0.05$). There was no difference in the percentage of horses in the HT group in FH compared to SB or TB. One TB horse (1%) had intermediate expression of CD147 and could not be included in either group. Such an intermediate expression was not apparent in FH and SB.

Like CD147, the MCT1 bands were faint or absent in horses in the LT group and the intensity of the MCT1 bands was greater ($p < 0.001$) in the HT horses than in the LT horses in all three breeds (Figure 3). The amount of MCT1 followed the bimodal distribution of CD147, but was only statistically significant in the TB ($p < 0.05$). The amount of MCT1 correlated with the amount of CD147 in all breeds ($r = 0.569$; $p < 0.001$). Both HT and LT horses expressed MCT2 in equal amounts (Figure 3). There was no correlation between MCT2 and CD147 or MCT1. There was also no correlation between age and the amount of CD147, MCT1 or MCT2.

FH females had more MCT2 ($p < 0.05$) than males, while TB females had more CD147 ($p < 0.05$) than males. When all breeds were combined, no differences were detected between the sexes.
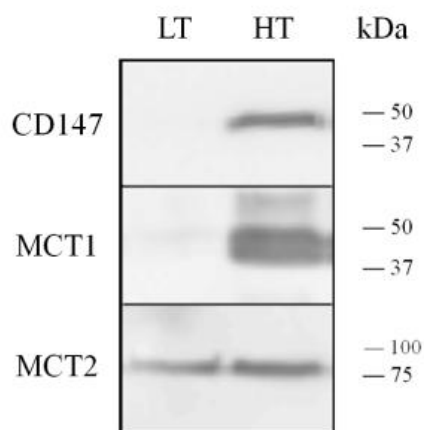
Figure 3. CD147, MCT1 and MCT2 Western blots of a high lactate transport activity (HT) and a low lactate transport activity (LT) Finnhorse.

5.2 Racing performance (Study I)

Racing performance data were available for 77 of the Thoroughbred racehorses. The best Racing Post ratings varied between 47-149 (median 89; IQR 110-70), the best official ratings varied between 40-149 (median 87; IQR 108-70), the best top speed varied between 16-137 (median 76; IQR 100-53) and career prize money varied between £0-414 872 (median £4 637; IQR £19 300-287). Colts and geldings had a higher best RPR, best TS and best OR compared to mares. The performance markers did not correlate with the amount of MCT1, MCT2 or CD147 in TB RBC membranes.

5.3 Immunohistochemical staining of the middle gluteal muscle fibres with MCT1 and CD147 antibodies (Studies II, III)

MCT1 antibody stained both membranes and cytoplasm, particularly in oxidative type *I* and type *IIA* fibres, and to a lesser degree in type *IIAB* fibres. Type *IIB* fibre cytoplasm and membranes stained faintly or not at all. The results were similar in both breeds examined, Standardbred and Norwegian-Swedish Coldblood trotters. In Study II, when all fibre types were combined, the staining intensity of MCT1 in both the cytoplasm and the membranes correlated with the staining intensity of NADH tetrazolium reductase ($r = 0.246$ for cytoplasm ($p < 0.05$) and $r = 0.376$ ($p < 0.01$) for membranes).

The amount of MCT1 in the membrane of type *I* fibres was 3.1 ± 1.2 times ($p < 0.001$), in type *IIA* fibres 3.1 ± 1.1 times ($p < 0.01$), and in type *IIAB* fibres 2.2 ± 0.9 times ($p < 0.05$) as high as that in the *IIB* fibre membrane (Study II). The differences between *I, IIA* and *IIAB* were not significant (Study II).

CD147 antibody stained the membranes and cytoplasm of all muscle cells. The amount of CD147 in the membrane of type *I* fibres was 1.1 ± 0.4 times, type *IIA* 1.4 ± 0.6 times

and type *IIAB* 1.2 ± 0.8 times as high as that in the *IIB* fibre membrane (Study II). In study II, no differences were seen between the fibre types, but in Study III, fibre types *IIA* and *IIAB* had more CD147 expression in their sarcolemma compared to type *IIB* fibres. A similar trend ($p = 0.06$) was seen in type *I* fibres.

Cytoplasmic expression of both CD147 and MCT1 was higher in fibre types *I, IIA* and *IIAB* compared to *IIB* fibres. The amount of MCT1 in the cytoplasm of type *I* fibres was 1.11 ± 0.05 times ($p < 0.001$), type *IIA* fibres 1.09 ± 0.05 times ($p < 0.001$) and type *IIAB* fibres 1.04 ± 0.29 times ($p < 0.01$) as high as that of *IIB* fibres (Study II). The differences between type *I* and *IIAB*, and *IIA* and type *IIAB* were also significant ($p < 0.001$ for both), but there was no difference between the staining of type *I* and type *IIA* fibres (Study II). The amount of CD147 in the cytoplasm of type *I* fibres was 1.03 ± 0.04 times ($p < 0.05$), type *IIA* 1.05 ± 0.04 times ($p < 0.001$) and type *IIAB* 1.04 ± 0.03 times ($p < 0.01$) as high as that of *IIB* fibres (Study I).With all fibre types combined, the amount of CD147 in the cytoplasm correlated with the respective amount of MCT1 ($r = 0.431$; $p < 0.001$; Study II).

In Study II, the capillaries showed pronounced MCT1 staining in immunohistochemistry. Electron microscopic images of gluteus muscle showed grouping of mitochondria around the capillaries.

The horse MCT4 antibody failed to stain fibres in immunohistochemistry, despite the fact, that it has previously worked in Western blotting of horse muscle (Koho et al. 2006).

5.4 The effect of training on MCT1 and CD147 expression in different fibre types of the horse gluteus muscle (Study III)

No significant changes were identified in paired observations in the relative distribution of MCT1 and CD147 in membranes of different fibre types. The relative cytoplasmic content of MCT1 and CD147 seemed to increase with training in fibre types *I, IIA* and *IIAB*, but the changes were only significant for MCT1 in *IIAB* fibres and for CD147 in *IIA* fibres ($p < 0.05$ for both).

5.5 Histochemical staining of gluteal muscle fibres and the effect of training (Studies II, III)

In study II, horses had $15 \pm 14\%$ of type *I* fibres, $45 \pm 10\%$ type *IIA* fibres, $8 \pm 5\%$ type *IIAB* fibres and $32 \pm 12\%$ type *IIB* fibres. The intensity of NADH tetrazolium reductase staining in type *I* fibres was 1.7 ± 0.2 times ($p < 0.001$), in type *IIA* 1.6 ± 0.2 times ($p < 0.001$) and in type *IIAB* 1.5 ± 0.2 times ($p < 0.001$) as high as that of *IIB* fibres. The differences between type *I* and *IIA* ($p < 0.01$), type *I* and type *IIAB* ($p < 0.001$) and type *IIA* and type *IIAB* ($p < 0.05$) were also statistically significant.

In study II, the percentage of type *IIB* fibres decreased and that of type *IIAB* increased during the training period ($p < 0.05$ for both). The relative distribution of NADH tetrazolium reductase staining did not change with training.

5.6 Sequence variations in MCT1, MCT4 and CD147 (Study IV)

The PCR fragments studied covered 99% of MCT1 cDNA and amino acids 5-500 from the N-terminus, including the whole C-terminus of MCT1. In 31 of the 42 horses, there was 100% homology to the database entry of horse MCT1 full length cDNA AY457175.1. In MCT1, two single nucleotide sequence variations caused an amino acid change. A 1498G>A nucleotide sequence variation was found in 10 horses, causing a heterozygous V432I mutation (accession no. AAR21622) in a trans-membrane region closest to the C-terminus of the protein. Five of these horses were healthy and 5 suffered from myopathy. In one myopathy horse, a heterozygous 1573A>C nucleotide sequence variation was found, causing a K457Q mutation in the C-terminal cytoplasmic domain of MCT1.

The whole MCT4 cDNA was sequenced and in 23 of the 42 horses there was 100% homology to the database entry of horse MCT4 full length cDNA EF564279.2. Several sequence variations were found in both healthy horses and horses with myopathy, but none of them caused a change in the amino acid sequence.

The PCR fragments studied covered 97% of CD147 cDNA and amino acids 9-272, which includes most of the protein except for part of the Ig-like domain distal to the membrane in the extracellular N-terminus. In 19 of the 42 horses there was 100% homology to the database entry of horse full length cDNA EF564280.1. In 10 horses, an 389A>G nucleotide sequence variation was found, causing a M125V mutation in the extracellular Ig-like domain proximal to the membrane. Two of these horses were healthy and 8 were horses with signs of myopathy.

5.7 Blood chemistry and muscle PAS-amylase staining (Study IV)

Haematocrit (HCT) and haemoglobin (Hb) values were higher in the myopathy group compared to the control group. Standardbreds were over-represented in the myopathy group compared to Finnhorses. When control and myopathy horses were examined according to breed, Finnhorses (n = 12) had lower HCT and Hb values (38 ± 4% and 133 ± 13 g/L) compared to Standardbreds (n = 30) (42 ± 4% and 150 ± 15 g/L; $p < 0.01$ and $p < 0.01$). The CK activity was higher ($p < 0.01$) in the myopathy group (median 272; IQR 859-373) compared to the control group (median 194; IQR 417-310). The horses in the myopathy group were younger compared to control horses ($p < 0.01$). All the muscle sections were negative for PSSM in PAS-amylase staining.

5.8 RBC MCT1 and CD147 Western blotting in control and myopathy horses (Study IV, unpublished data)

The amount of MCT1 and CD147 in the RBC membrane was used to estimate the lactate transport activity in muscle (Koho et al. 2006). Seven of the 42 horses showed very little or no expression of both MCT1 (Figure 4) and CD147 (see Study IV) in Western blots.

There was no difference in the expression level of these proteins between the myopathy and control groups (Figure 4).
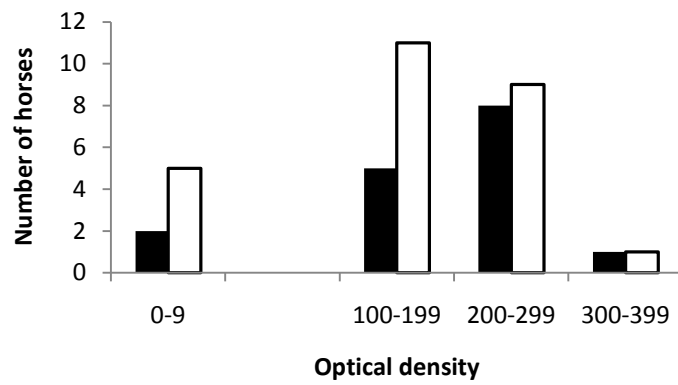
Figure 4. Distribution of the intensity of staining in MCT1 Western blots between myopathy (white bars) and control groups (black bars).

6 Discussion

6.1 Methodological considerations

Information on horse transcriptome sequences has made it possible to design and raise horse-specific antibodies. MCT1, MCT2, MCT4 and CD147 antibodies used in Studies I-IV were horse-specific and designed against the C-termini of the equine proteins. In MCT1, MCT2 and CD147, this sequence consists of 13-17 amino acids and is not identical to the respective human or rat sequence. In this C-terminal area, the horse MCT1 sequence differs by 5 and MCT2 by 9 amino acids compared to the human sequences. The specificity of the antibody in the species studied is crucial to the reliability of the results. In earlier studies, our laboratory group found that the MCT1 antibody designed against human protein was not specific to horse MCT1. The human-designed MCT1 antibody gave different results in Western blotting compared to the horse-specific antibody that was later introduced to our laboratory protocol (Koho et al. 2002, 2006). However, the specificity of an antibody cannot always be determined by comparing the protein sequence homology with other species. For instance, the CD147 protein sequence is known to vary considerably between species (Reeben et al. 2006). The C-terminus of horse CD147 differs by 4 amino acids compared to the respective human sequence. Nevertheless, the human CD147 antibody has shown horse specificity in the Western blots of previous studies (Koho et al. 2002, 2006). Despite species specificity, antibodies can still behave unpredictably. The human and rat MCT4 C-terminal sequence is identical to horse MCT4. Previously, antibodies designed against this homologous sequence have been successfully used to stain rat and human muscle (Wilson et al. 1998; Pilegaard et al. 1999b). However, in Studies II and III, the equine MCT4 antibody failed to work in immunohistochemistry, although it gives a single band in Western blots of horse muscle (N. Koho, personal communication).

In Study I, the molecular weight of both MCT1 and CD147 bands was approximately 50 kDa, which is in accordance with earlier reports from other species (Kasinrerk et al. 1992; Poole and Halestrap 1992; Garcia et al. 1994a). MCT2 is reported to be of a similar size to MCT1 (Garcia et al. 1995). However, in Study I, the molecular weight of the MCT2 band was significantly greater, almost 90 kDa. This indicates that the protein was either in a dimeric form or attached to its ancillary protein in the Western blots of Study I. Previously, it has been suggested that such a dimer of membrane proteins might be stable enough to withstand the denaturing conditions of SDS-PAGE (Wilson et al. 2005).

The method used to measure the intensity of the immunohistochemical staining in Studies II and III was to set the least oxidative fibre type *IIB* cytoplasm as a baseline. This technique was chosen to minimize the variation due to variable amount of antibody per section area and the photographic technique. The slides were photomicrographed with automatic light exposure, which caused marked differences in the intensity of the background. While this method made it possible to reliably compare different fibre types within a sample, it did not allow us to compare the expression of these proteins between different horses or repeated samples from the same horse. This was an unfortunate

shortcoming, especially in Study III, which failed to show increases in MCT1 membrane expression as the training progressed. The problem could have been overcome by placing sections from different samples of the same individual on the same slide and by staining them together.

6.2 Bimodal MCT1 and CD147 expression in different horse breeds

Standardbreds can be divided into two groups based on lactate transport into red blood cells (RBCs; Figure 2; Väihkönen and Pösö 1998). Previously, this has been shown to be due to two expression levels of CD147 (Koho et al. 2002). An abundance of CD147 is expressed in the RBC membrane in horses with a high lactate transport activity (HT), while only little or no CD147 is expressed in horses with a low lactate transport activity (LT; Koho et al. 2002). In previous studies, the expression level of MCT1 has not varied between the two groups (Koho et al. 2002, 2006). However, in Study I, the amount of CD147 correlated with the amount of MCT1, and it is therefore likely that the expression of both MCT1 and CD147 is needed for RBC lactate transport activity. The discrepancy between the Western blot results in Study I and the earlier work of our laboratory team is probably due to the previous use of human MCT1 antibody. If CD147 is not expressed, MCT1 is not transported to the cell membrane and accumulates in the endoplasmic reticulum (Kirk et al. 2000). There is also evidence that the same happens *vice versa*. If MCT1 expression is inhibited by siRNA, CD147 is not expressed on the cell membrane (Deora et al. 2005). The fact that CD147 expression is dependent on MCTs has also been shown in a cancer cell line, in which the silencing of MCT4 expression resulted in the accumulation of CD147 in the endoplasmic reticulum (Gallagher et al. 2007). Based on the Western blot results in Study I, we cannot conclude whether the transcription or translation of one or both of these proteins is low in LT horses, since the expression levels of both of the proteins are mutually dependent. Interestingly, recent studies have shown that several external stimuli can simultaneously upregulate the expression of both MCT1 and CD147 (Fanelli et al. 2003; Benton et al. 2008; Kirat et al. 2009). In these studies, the effect was not verified at the mRNA level, but there is evidence to suggest that the actual transcription of the two proteins can also be simultaneously upregulated. König et al. (2010) demonstrated that the two proteins share at least one common regulatory element: a nuclear receptor, PPAR-α, can upregulate the expression of both MCT1 and CD147 mRNA.

In the horse, the vast majority of total lactate transport into RBCs is due to MCTs (Skelton et al. 1995; Väihkönen and Pösö 1998). Up to 50% of blood lactate can be found in horse RBCs after intense exercise (Pösö et al. 1995; Väihkönen et al. 1999), whereas in human athletes the respective percentage is around 20% (Juel et al. 1990; Smith et al. 1997). It has been speculated that the influx of lactate from the plasma into RBCs sustains the gradient between muscle cells and the plasma, enabling more lactate to be produced in the muscle cells (Pösö et al. 1995; Juel et al. 2003). The horse has a large splenic reserve of up to 50% of the red cell volume (Persson and Lydin 1973). When the reserve pool of

erythrocytes is released from the spleen during exercise, this increased red cell volume, as well as the ability to use these RBCs as a lactate sink, might be beneficial in high intensity exercise.

In our study, we found that 82% of the Standardbreds studied belonged to the HT group, which is in accordance with earlier results (Väihkönen and Pösö 1998). The percentage of Thoroughbred racehorses in the HT group (88%) was greater when compared to Standardbreds. However, almost as many Finnhorses (85%) belonged to the HT group as Thoroughbreds. This was an unexpected finding, since Finnhorses race at lower speeds and the blood lactate concentration after a race is lower compared to the lighter racing breeds, Standardbred and Thoroughbred (Pösö et al. 1983; Harris and Snow 1988). One possible explanation is that when the number of Finnhorses declined from 400 000 to only 14 000 horses within the thirty years after the Second World War, the horses with a high lactate transport activity might have been favoured in breeding by coincidence. If a high lactate transport activity is favourable for exercise, as previously suggested (Pösö et al. 1995; Juel et al. 2003), it is also possible that these horses were favoured in breeding due to better performance as sport horses.

The bimodal distribution of lactate transport activity is a unique feature of the horse, since it has not been reported in other species studied, namely humans, dogs and reindeer (Skelton et al. 1995; Väihkönen et al. 2001). In previous studies, the two groups could already be distinguished in foals and horses were found to remain in their group in adulthood (Väihkönen and Pösö 1998; Väihkönen et al. 2002). It has been suggested that in the Standardbred, a low lactate transport activity is inherited as an autosomal recessive trait in a single locus (Väihkönen et al. 2002). Previously, training has been shown to increase RBC lactate transport activity in reindeer and sled dogs, but not in horses (Väihkönen et al. 2001). In humans, the effect of training is controversial, as both an increase in RBC lactate transport activity and no effect has been reported (Skelton et al. 1995; Väihkönen et al. 2001). In Study I, age did not affect the levels of lactate transport proteins in any breed. Since older horses can be assumed to have undergone more training, this finding supports the argument that there is no effect of training on RBC lactate transport protein expression in the horse.

6.3 Expression of MCT1 and CD147 in different fibre types

The names given to different fibre types vary between species. When antibody stains are used to identify myosin heavy chains in small mammals, three different fast fibre types exist, type *IIA*, *IIX* and *IIB* (Schiaffino et al. 1989). The type *IIB* fibres identified in the ATPase staining of equine muscle correspond to *IIX* fibres in small animals (Karlström and Essén-Gustavsson 2002). In Study II, the fast fibre type was named type *IIB* based on ATPase staining. In Study III, another version of the naming system was chosen and the same fibres were named type *IIX*, based on the fact that the *IIX* myosin antibody stains these fibres. The hybrid fibres, *IIAB* in Study II, corresponded to *IIAX* fibres in Study III. These hybrid fibres, which are in the process of transforming from *IIB* (or *IIX*) to *IIA*, show the expression of both myosin types (Rivero and Piercy 2008). Many of these hybrid

fibres were found in the young horses in our studies. The intermediate oxidative capacity as well as the expression of MCT1 in these fibres shows that the expression of MCT1 is gradually upregulated as the fibre transition progresses.

As far as I am aware, Study II was the first to examine the muscle expression of MCT1 and its ancillary protein CD147 in the horse using immunohistochemistry. Both MCT1 and CD147 were detected in the membranes and cytoplasm of horse muscle fibres. Staining of MCT1 in the cytoplasm and sarcolemma correlated with the oxidative capacity of the fibre type and was higher in the oxidative type *I* and *IIA* fibres than in the less oxidative *IIAB* hybrid and *IIB* fibres. This finding indicates that the distribution of MCT1 among fibre types in the horse is similar to that in humans and rats (Fishbein et al. 2002; Hashimoto et al. 2005). Therefore, conclusions about the function of MCTs drawn from the results in other species can be applied to horses. MCTs can transport lactate both into and out of cells, and the direction of transport is determined by the proton gradient (Deuticke 1982). MCT1 is probably responsible for lactate influx to oxidative fibres for oxidation during rest and aerobic muscle work, when the muscle lactate concentration remains low (Wilson et al. 1998). During intense exercise, oxidative fibres also produce lactate, and MCT1 is then likely to change the direction of transport and remove lactate from the cell. During intense work, an abundance of lactate is formed in type *IIB* cells, which are glycolytic muscle fibres. Type *IIB* muscle fibres in man only express the MCT4 isoform and therefore this isoform is likely to be responsible for lactate efflux during heavy exercise (Wilson et al. 1998). In the horse, MCT1 is not present in *IIB* cells, so MCT4 is also likely to extrude lactate from these cells in this species.

In addition to the cell membrane, MCT1 was abundant in the cytoplasm of oxidative fibres. While MCT1 is probably expressed in various parts of the muscle cell, such as the sarcoplasmic reticulum, other intracellular membranes and the T-tubules, the most likely explanation is that the staining is due to mitochondrial MCT1 (Brooks et al. 1999; Bonen et al. 2000; Benton et al. 2004; Butz et al. 2004). The intensity of NADH dehydrogenase staining, which indicates the number of mitochondria in the cell, correlated with the amount of MCT1. The functional role of MCT1 in the mitochondria is probably to transport pyruvate into the mitochondria, where it is decarboxylated to acetyl-CoA, which can then enter the tricarboxylic acid cycle. Capillaries stained intensely with MCT1 antibody, which can be explained by the accumulation of sub-sarcolemmal mitochondria near the capillaries in equine muscle (Study II; Hoppeler et al. 1987; Kayar et al. 1988).

6.4 Effects of training on MCT1 and CD147 expression

Study III focused on following the expression of these two proteins during the first two years of training in Coldblood trotters. The first samples were taken when the horses were approximately 2 years old and training had not yet begun. The training protocol included 45- to 60-minute training sessions of a gradually increasing intensity 4-5 times a week for the subsequent two years. The last samples were taken when the horses were 3.5 years old and race fit. The expression of MCT1 and CD147 was similar in this heavier racing breed to the Standardbred. There was a tendency for the cytoplasmic expression of MCT1 and

CD147 to increase more in fibre types *I, IIA* and *IIAB* compared to the reference fibre type *IIB*. This was possibly due to the fact that training increases the number of mitochondria within the cell (Tyler et al. 1998). While type *IIB* fibres can also increase their oxidative capacity with training, based on our results it seems that the effect on the expression of MCT1 and CD147 is stronger in the more oxidative fibre types (Snow and Valberg 1994; Karlström et al. 2009).

In humans and rats, training induces MCT1 expression in the muscle cell membrane (Juel 2008). The same effect has not been previously reported in the horse (Kitaoka et al. 2010). Here, the distribution of MCT1 and CD147 in the sarcolemma in Study III did not change with training. This could be due to the fact that the expression of these proteins increased in all fibre types evenly. The method used only allows fibre types be compared within a muscle section, and it was not therefore possible to measure the absolute change in the expression of these proteins. Furthermore, the time of sampling might have influenced the results. An acute reduction is seen in sarcolemmal MCT1 expression in humans, but not in rats immediately post-exercise (Coles et al. 2004; Bishop et al. 2007). The samples in Study III were taken shortly after the horses had exercised on the treadmill, and it is therefore possible that less MCT expression was present compared to muscles at rest after a longer period of recovery. Training induces changes towards more oxidative fibre types (Ronéus et al. 1992; Pette and Staron 1997; Tyler et al. 1998). In Study III, type *IIB* fibres, which have the lowest MCT1 expression, decreased with training, indicating in an indirect way that the overall MCT1 expression in the muscle probably increased with training, as the other fibre types with greater MCT1 expression became more common. Increasing the proportion of fibre types expressing a great deal of MCT1 would mean more lactate could be transported to the muscles for oxidation during submaximal work. This finding is in accordance with the higher lactate threshold observed after training in Study III. However, using Western blotting, Koho et al. (2006) failed to show that membrane expression of MCT1 and CD147 increased with age or training. Unfortunately, the number of horses used in that study was small and the antibodies were not horse specific.

Studies II and III extended earlier findings by determining the fibre type distribution of CD147, which is the ancillary protein for MCT1 and indispensable for its activity in muscle as well as in red blood cells (Kirk et al. 2000). The equal expression of CD147 in membranes of all fibre types is not surprising. CD147 is a chaperone that forms a complex with MCT1, but it is also a chaperone for MCT4 (Gallagher et al. 2007). In other species, MCT4 is predominantly expressed in the sarcolemma of type *IIB* and most *IIA* fibres (Pilegaard et al. 1999a; Fishbein et al. 2002; Hashimoto et al. 2005). MCT4 is also expressed in horse muscle membranes, but the fibre type distribution of the expression is not known (Koho et al. 2006). The co-expression of CD147 with both MCT1 and MCT4 would explain the equal staining of the sarcolemma in all fibre types. Furthermore, CD147 is expressed on the cell membrane together with several other proteins, such as integrins and caveolin-1, which provides a possible further explanation for the equal staining of CD147 among different fibre types (Huet et al. 2008).

6.5 Sequence variations in MCT1, MCT4 and CD147

In humans, mutations in MCT1 have been shown to influence the lactate transport capacity and cause variable signs of myopathy (Merezhisnkaya et al. 2000; Cupeiro et al. 2010). Therefore, we took samples from horses that had repeatedly shown signs of exercise-induced myopathy. We ruled out a well-known inherited muscle disease, polysaccharide storage myopathy (PSSM), with the PAS-amylase stain (Valberg et al. 1992). In very young horses, this method of detection of PSSM may reveal false negative results (Firshman et al. 2006). However, it was unlikely in this study, since the youngest horse in the myopathy group was three years old.

Two sequence variations that cause a change in the amino acid sequence were found in the coding sequence (cDNA) of horse MCT1 in Study IV. The K457Q mutation in MCT1 was only found in a horse that showed signs of myopathy. This mutation replaces a positively charged lysine with a neutral glutamine residue. A change in charge might affect protein structure and function. However, Reeben et al. (2006) previously found the same mutation in a healthy horse. Therefore, it is unlikely that this mutation would affect protein function and cause signs of exercise-induced myopathy. The novel V432I mutation in MCT1 was found in the 12th transmembrane domain of MCT1 in 10 horses, 5 of which suffered from myopathy. The hydrophobic interactions of the transmembrane domain of CD147 have been reported to stabilize the MCT1-CD147 complex (Finch et al. 2009). The mutation in horses changes a hydrophobic valine to hydrophobic isoleucine, and it thus remains to be shown whether this mutation is physiologically significant. Furthermore, the mutation was found in both healthy horses and horses with myopathy, indicating that it is not linked to myopathy. Neither of the mutations found in MCT1 were the same as those that have been reported to occur in human subjects (Merezhinskaya et al. 2000; Lean and Lee 2009).

In Study IV, a DNA sequence variation was found in 10 horses in the CD147 Ig-like domain proximal to the membrane. This sequence variation causes a M125V amino acid change and it has been previously reported by Reeben et al. (2006). This sequence variation was also found in both breeds studied. As far as I am aware, mutations in CD147 have not been examined in other species. In CD147, the M125V mutation was found in 10 horses, 8 of which showed signs of myopathy. Interestingly, the remaining 2 horses showed a very low level of CD147 expression. However, we could not conclude that the M125V mutation has physiological significance, because in the earlier work of Reeben et al. (2006), this mutation was found equally in both healthy horses and horses with signs of myopathy, and was not associated with a decreased lactate transport activity.

Study IV was the first to sequence horse MCT4. No nucleotide sequence variations were found in the horse MCT4 cDNA that would cause an amino acid change in the protein. As far as I am aware, sequence variations in MCT4 have not previously been studied in any other species. If MCT4 is responsible for the removal of lactate from the muscle fibre during intense exercise, it is reasonable to assume that impaired function of this protein might lead to an abnormally rapid accumulation of lactate in muscle and cause signs of myopathy.

In the present study, horses with a high and low expression level of MCT1 and CD147 were distributed evenly between the myopathy group and the control group. Therefore, no association between recurrent exercise-induced myopathy and the membrane expression of these proteins could be found. However, the number of horses studied was small and lactate transporters may still have a role in myopathy, as in humans (Merezhinskaya et al. 2000). This argument is supported by a case of postanaesthetic myopathy, a well known complication in horses (Klein 1990). The affected horse had a novel V51I mutation in MCT1. After the induction of anaesthesia, the horse, unlike the other 23 horses that underwent the same operation, had a significantly higher plasma lactate concentration (2.6 mmol/L) at 60 minutes into anaesthesia and CK (832 IU/L) at four hours after a the 2-hour operation (N. Koho, personal communication). This might indicate that mutations in MCT1 can also impair muscle function in the horse and in this case make the horse susceptible to an anaesthetic complication.

6.6 Future perspectives

To my knowledge, the horse is the only species in which MCT2 has been found in RBC membranes (Koho et al. 2002, 2006). In Study I, the expression of MCT2 varied between individuals, but there was no difference between HT and LT groups. This finding is in accordance with earlier results (Koho et al. 2002, 2006). In other species, MCT2 has been shown to have gp70 as an ancillary protein instead of CD147 (Wilson et al. 2005). This is consistent with our finding of no correlation between the expression level of CD147 and MCT2. MCT2 has lower K_m values for lactate, whereas the V_{max} of MCT1 is several fold higher compared to MCT2 (Bröer et al. 1999). Therefore, it is likely that MCT2 transports lactate at low concentrations in horses, while MCT1 is more important during exercise (Koho et al. 2002). Nevertheless, the functional role of this protein in the horse erythrocyte membrane remains unclear and further studies are warranted to determine its physiological significance. Future work could compare non-athletic horse breeds and donkeys to examine whether the variations are still present throughout the equidae, or if this feature is something that has been selected for along with racing potential.

MCT4 is expressed in horse muscle, but the distribution between fibre types remains unknown (Koho et al. 2006). Other antibodies or different immunohistochemical staining techniques should be tested in order to visualize the distribution of this protein in horse muscle. An increase in MCT1 and MCT4 expression during training is well documented in humans (Juel 2008). In order to investigate whether the same happens in the horse, a more quantitative method, possibly Western blotting or ELISA, should be used. A single fibre Western blot would reveal how the level of MCT expression changes with training in each fibre type separately.

A substantial number of horses, at least in the three studied breeds, showed differences in the expression MCT1 and CD147 in their RBC membranes. One of them, the Finnhorse, represents a heavier breed originally bred for work in the fields and forest, but which nowadays competes in trotting races under similar conditions to Standardbreds. The Finnhorse bloodline has not been mixed with the Standardbred or any other breed since

the early 1900s. This indicates that the mutation underlying the trait dates back more than a hundred years. Additional breeds and possibly other equids should be studied to map when the mutation first occurred in evolution. If high lactate transport activity is beneficial to exercise, it would be interesting to understand why horses with a low lactate transport activity have survived in evolution and breeding. The expression of CD147 is upregulated in various inflammatory conditions and, for instance, smokers have been reported to have an increased amount of CD147 in bronchoalveolar lavage. Horses suffer from a chronic inflammatory disease called recurrent airway obstruction (RAO), which affects more than 50% of ageing horses. The disease shares some pathology with smoking-induced chronic obstructive pulmonary disease (COPD) in humans (Robinson 2001). Treatment with anti-CD147 antibody can reduce inflammation, which is at least in part due to decreased leukocyte activation (Deeg et al. 2001). The very low level of CD147 expression in the LT horses might therefore be beneficial in hindering the onset of respiratory inflammation.

Räsänen et al. (1995) found that horses with high amounts of lactate in their RBCs after a trotting race had better performance indices than horses with low amounts of lactate in RBCs. However, in Study I, we could not show any correlation between racing success and the level of lactate transporting proteins in Thoroughbreds. This finding is similar to that in the study of Väihkönen et al. (1999). The variation between results highlights the challenge in developing appropriate performance markers in horses. The current performance markers all depend on age, and the development of racing indices that take age into account would allow better comparison of individuals. The number of factors influencing racing performance, such as psychological factors, training and racing conditions, is not small. Furthermore, the differences among individuals in a highly selected breed are relatively small and the effect on performance would perhaps be better demonstrated when comparing a non-athletic breed with an athletic one. One possibility to overcome some of these problems is to compare individuals with a standardized maximal exercise test on a treadmill. However, the treadmill environment never corresponds to the actual race track, where the horses need to perform.

7 Summary of findings

Two groups of horses differing in lactate transporter (MCT1 and CD147) expression in red blood cell membranes were present in all three horse breeds studied: Finnhorse, Standardbred and Thoroughbred. The greatest proportion of horses with a high expression of MCT1 and CD147 was recorded in the TB. Unexpectedly, a large number of Finnhorses were also found with a high expression of MCT1 and CD147.

No correlation was observed between the amount of lactate transporters in the RBC membrane and markers of racing performance in the Thoroughbred.

The expression of MCT1 in the cytoplasm and membranes of different fibre types in the horse muscle resembles that of humans and rats, and is highest in oxidative fibres. Capillaries were pronounced in MCT1 staining.

The amount of CD147 in the horse muscle cytoplasm correlates with the amount of MCT1, but CD147 is evenly expressed in the sarcolemma of all muscle fibre types.

The expression pattern of MCT1 and CD147 in muscle is similar in Coldblood trotters to that in the Standardbred.

Mutations in the coding sequences of MCT1 and CD147 were found in both Standardbreds and Finnhorses, while no mutations were detected in MCT4.

Mutations were detected in both healthy individuals and horses with myopathy, and thus the association of these mutations with clinical signs remains unclear. Furthermore, the mutations could not be linked to the level of MCT1 or CD147 expression.

8 Acknowledgements

9 References

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