Effects of fat deposition on the expression of insulin-signaling pathway and mTORC1 genes in Finnhorse mares

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Tiivistelmä — Referat — Abstract
Obesity and insulin resistance (IR) are key factors lead to equine metabolic syndrome and laminitis. Diet may play an important role in eliciting obesity by affecting insulin dynamics. Insulin-pathway signaling and mTORC1 genes may contribute to incred IR. The first objective of this study was to find and validate internal control genes for quantitative PCR method for adipose tissues in Finnhorse mares. The second aim was to quantitate the expression of mTORC1 and insulin-pathway associated genes after pasture season in two different treatment groups of Finnhorse mares and compare gene expression differences between treatment groups. In addition, gene expression differences were compared between two different adipose tissues.

Twenty-two mares were equally divided into eleven equal pairs, the two mares of each group were randomly grazed either on cultivated high-yielding pasture (CG) or on semi-natural grassland (NG) from the end of May to the beginning of September. Eight pairs of Finnhorse mares were selected for gene expression profiling. Subcutaneous adipose tissue (SAT) samples were collected from two groups of Finnhorse mares after pasture season. Gene expression of neck and tailhead SAT were determined with quantitative Real-Time PCR method (qPCR). The selected internal control genes were actin beta (*ACTB*), glucuronidase beta (*GUSB*) and mitochondrial ribosomal protein L39 (*MRPL39*). Candidate genes were mechanistic target of rapamycin (*MTOR*), sterol regulatory element binding transcription factor 1 (*SREBF1*), sterol regulatory element binding transcription factor 2 (*SREBF2*), *TBC1* domain family member 7 (*TBC1D7*), leptin (*LEP*), glucose transporter type 4 (*GLUT4*), monocyte chemoattractant protein-1 (*MCP-1*), retinol binding protein 4 (*RBP4*), tuberous sclerosis 1 (*TSC1*), tuberous sclerosis 2 (*TSC2*).

There were no distinct gene expression differences between NG and CG groups in both neck and tailhead SAT. However, *RBP4* had significantly (P=0.035) higher and *GLUT4* had a trend (P=0.064) to higher mRNA expression in CG group in neck SAT. *TSC1* had a trend (P=0.071) of higher expression in CG group in tailhead SAT. Gene expression differences were observed between tailhead and neck SAT. *SREBF1* and *GLUT4* had significantly (P=0.007 and P=0.026, respectively) higher expression levels in tailhead SAT compared to neck SAT. *RBP4* had a trend (P=0.066) to higher expression in neck SAT compared to tailhead SAT.

Minor differences in gene expression between NG and CG groups indicate that pasture-associated fat deposition may not considerably affect expression of insulin-pathway and mTORC1 genes associated to obesity and IR in studied subcutaneous adipose tissues. These results also provide additional evidence to our hypothesis that fattening resulting on unrestricted grazing on cultivated high-yielding pasture does not increase the risk of metabolic diseases in Finnhorse mares when they have normal body condition at the beginning of the grazing season.

Avainsanat — Nyckelord — Keywords
gene expression, molecular pathways, insulin, mTORC1, pastures, fattening, horse

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Content

ABBREVIATIONS AND CONCEPTS

ACTB	Actin beta
AMPK	Adenosine monophosphate-activated protein kinase
AT	Adipose tissue
CG	Cultivated high-yielding pasture group
Eff	PCR efficiency
EMS	Equine metabolic syndrome
GAP	GTPase-activating protein
GLUT4	Glucose transporter type 4
GTP	Guanosine triphosphate
GUSB	Glucuronidase beta
ICG	Internal control gene
IR	Insulin resistance
LEP	Leptin
MCP-1	Monocyte chemoattractant protein-1
MRPL39	Mitochondrial ribosomal protein L39
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
NEFA	Non-esterified fatty acids
NG	Semi-natural grassland group
PRAS40	Proline-rich Akt substrate 40 kDa
qPCR	Quantitative polymerase chain reaction
RBP4	Retinol binding plasma protein 4
Rheb	Ras homolog enriched in brain
SAT	Subcutaneous adipose tissue
SREBF1	Sterol regulatory element binding transcription factor 1
SREBF2	Sterol regulatory element binding transcription factor 2
TBC	Tre-2/Bub2/Cdc16
TBC1D7	TBC domain family member 7
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2

1 INTRODUCTION

Nowadays obesity has reached epidemic proportions worldwide due to changes in human lifestyle (Catenacci et al. 2009). Diet may play an important role in eliciting obesity by affecting insulin dynamics (Hoffman et al. 2003, Isganaitis & Lustig 2005). Increased adiposity is a major cause of insulin resistance (IR), dyslipidemia, hyperglycemia, and hypertension (Kahn & Flier 2000, Spiegelman & Flier 2001). The prevalence of obesity also has been risen dramatically in companion animals as well as in domestic horses and ponies (Giles et al. 2014). It has been reported that obesity is an increasing common disease and welfare issue in equines in developed countries (Thatcher et al. 2008, Wyse et al. 2008).

Equine metabolic syndrome (EMS) is an endocrinopathic disease of horses and ponies characterized by abnormal regional obesity, hyperinsulinemia and insulin resistance, altered reproductive cycling, laminitis, dyslipidemia and hyperleptinemia (Vick et al. 2008, Frank et al. 2010b). In horses, obesity is known as a key factor leading to an increased risk of insulin resistance, laminitis and metabolic syndrome (Hoffman et al. 2003, Treiber et al. 2006, Geor 2008). Obesity has been reported to associate with insulin resistance through inflammatory response and increased plasma lipid concentrations in equine and companion animals (Frank et al. 2006, Vick et al. 2007, Radin et al. 2009). However, the molecular mechanisms from obesity leading up to insulin resistance, laminitis or EMS have to yet been explained in detail.

mTORC1 genes and insulin-pathway genes may contribute to increased IR (Lamming et al. 2012). It is suggested that mTORC1 activation induces lipogenesis in adipose tissues (Ricoult & Manning 2013). In human and laboratory animal studies, it has been shown that certain pathways related to cytokines and adipokines are associated with obesity, insulin resistance and inflammation (Guiherme et al. 2008). In equidae, several studies underlined the role of adipose tissue in regulation of metabolism and homeostasis by various cytokines (Carter 2008, Burns et al. 2010, Frank et al. 2010a, Ungru et al. 2012). However, the

molecular mechanisms that link those cytokines, obesity and insulin resistance remain incompletely understood, which is of importance for further investigation.

2 LITERATURE REVIEW

2.1 Background of mTORC1

Rapamycin is a macrolide antibiotic produced by Streptomyces hygroscopicus. It was first extracted for its antifungal activity against Candida albicans (Vézina et al. 1975). The mechanistic target of rapamycin (mTOR, formerly known as mammalian TOR) is an atypical serine/threonine protein kinase which belongs to the phosphoinositide 3-kinase-related kinase family. TOR1 and TOR2 were identified in a screen of budding yeast Saccharomyces cerevisiae, and their role as transmitters of the toxic effect of rapamycin was discovered in early 1990s (Kunz et al. 1993, Cafferkey et al. 1993, Helliwell et al. 1994). Shortly after, mTOR was cloned and studied as target of rapamycin (Brown et al. 1994, Sabatini et al. 1994, Chiu et al. 1994, Sabers et al. 1995).

The mechanistic target of rapamycin complex 1 (mTORC1) is one of the distinct complexes that formed with mTOR and several protein components. It has been recently discovered that mTORC1 includes the catalytic mTOR subunit and five other known protein components (Dibble et al. 2013).

It was claimed that the molecular function of regulatory-associated protein of mammalian target of rapamycin (raptor) is to function as a binding partner of TOR (Hara et al., 2002). It was also argued that mTOR signals to the cell cycle progression and proliferation by interacting with raptor (Kim et al., 2002). Target of rapamycin complex subunit LST8, as known as mammalian lethal with SEC 13 protein 8 (mLST8), was reported as a positive regulator of the rapamycin sensitive pathway (Kim et al. 2003). Proline-rich Akt substrate 40 kDa (PRAS40) was found an insulin-regulated inhibitor of the mTORC1 kinase activity (Sancak et al. 2007, Wang et al. 2007). PRAS40 was proposed to regulate apoptosis by

interacting with mTOR (Thedieck et al. 2007). DEP domain containing mTOR-interacting protein (DEPTOR) was reported as a mTOR inhibitor (Peterson et al. 2009). Kaizuka et al. (2010) discussed the critical importance of Tti1/Tel2 complex as its role in regulating the stability of mTORC1.

2.2 Upstream and downstream of mTORC1 signaling pathway

From upstream, mTORC1 is activated by insulin and most other growth factors through either receptor tyrosine kinases or G-protein-coupled receptors at the cell surface (Loewith & Hall 2011). mTORC is regulated directly through the GTP-binding status of the Ras homolog enriched in brain (Rheb) (Huang & Manning 2008). The tuberous sclerosis complex 1/2 (TSC1/2, Tuberin and Hamartin) negatively regulate mTORC1 by acting as GTPase-activating protein (GAP) of Rheb (Tee et al. 2003). Nutritional signals such as glucose, amino acids and oxygen, or insulin, cytokines, growth factors and energy metabolism affect the GAP ability of the TSC-TBC complex through adenosine monophosphate-activated protein kinase (AMPK) and v-Akt murine thymoma viral oncogene (Akt) (Dibble et al. 2012). The phosphorylation of TSC2 by Akt can inhibit the TSC-TBC complex, thereby activating Rheb and mTORC1 kinase activity (Inoki et al. 2002). The mTORC1 signaling can also be activated without insulin by expression of constitutively active Akt or lack of either TSC1 or TSC2, meanwhile, it stimulates expression of SREBP1 and SREBP2 targets (Porstmann et al. 2008, Düvel et al. 2010).
Generally, mTORC1 signaling plays a multifunctional role in controlling mammalian lipid metabolism including lipid synthesis, storage and lipolysis, and adipocyte differentiation (Figure 1).

Activation of mTORC1 promotes biomass increase for cell growth and proliferation (Howell & Manning 2011, Laplante & Sabatini 2012). Through various downstream mechanisms, mTORC1 regulates lipid metabolism by promoting lipid synthesis and inhibiting lipid release, promoting the production of energy and reducing equivalents (Yecies & Manning 2011, Inoki et al. 2012, Ricoult & Manning 2013).

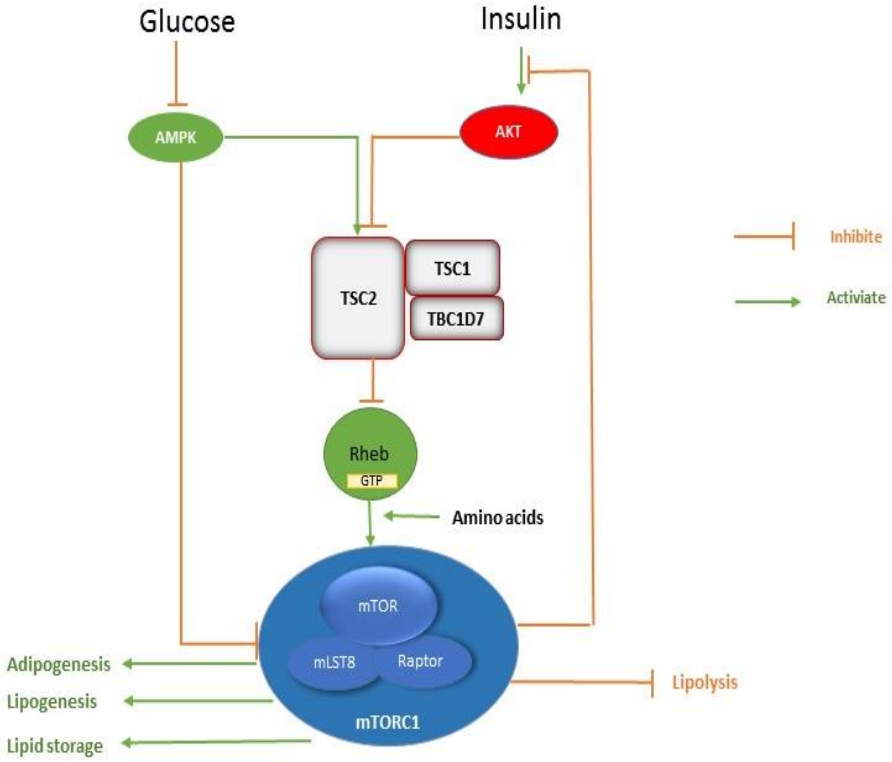

Figure 1. Upstream activation factors of mTORC1 and its downstream target regulation. Adapted from Ricoult & Manning (2013).

The activation of mTORC1 signaling stimulates lipogenesis through SREBP1 and SREBP2 which are major transcriptional factors (Porstmann et al. 2008, Düvel et al. 2010). Genetic models have suggested that mTORC1 plays a vital role in terminal adipocyte differentiation and adipogenesis (Zhang et al. 2009). Meanwhile, several studies suggested that mTORC1 signaling promotes fatty acids storage by inhibiting lipolysis (Morrisett et al. 2002, Zhang et al. 2009, Chakrabarti et al. 2010, Soliman et al. 2010). However, the molecular mechanisms by which mTORC1 stimulates adipocytes differentiation and regulates lipolysis have not yet been clarified.

It is claimed that mTORC1 may contribute to various metabolic diseases such as obesity, insulin resistance, diabetes and cancer via its important role in integrating cellular signals and regulating lipid synthesis, storage and transportation (Ricoult & Manning 2013). In human and laboratory animals studies, the role and molecular mechanisms of mTORC1 have been well established, while in horses the knowledge remains obscure.

2.3 The expression of adipokine genes in metabolic diseases

Retinol binding protein 4 (RBP4) is an adipokine secreted by adipocytes (Yang et al. 2005). In mice and human study, RBP4 contributes to insulin resistance in obesity and type-2 diabetes (Yang et al. 2005, Graham et al. 2006). Increased serum RBP4 levels were observed in humans with insulin-resistant states such as obesity, type-2 diabetes (Graham et al. 2006). Higher blood levels of RBP4 were detected in adiposity of type-2 diabetes (Klöting et al. 2007, Lee et al. 2007). It was also reported that down-regulation of GLUT4 can cause insulin resistance and increase the risk of developing diabetes (Abel, et al. 2001). Increased levels of serum RBP4 were detected in adipose-specific GLUT4 knockout (adipose-GLUT4 -/-) mice (Yang et al. 2005). However, in an equine study, RBP4 expression was claimed closely linked with adiposity, independent of other obesity factors such as insulin sensitivity (Ungru et al. 2012).

Leptin (LEP) regulates appetite, adipogenesis and energy homeostasis (Ingvartsen & Boisclair 2001). Previous equine study has reported that LEP is as an important endocrine signal of nutritional status and adipose tissue mass in the horse (Houseknecht et al. 1998). The plasma LEP concentration was positively correlated with adipose tissue mass, and strongly correlated with degree of insulin resistance (Buff et al. 2002, Kearns et al. 2006, Van Weyanberg et al. 2007).

MCP1 was reported to contribute to macrophage infiltration into adipose tissue and insulin resistance in obesity (Kanda et al. 2006). In human studies, the mRNA expression of MCP1 was up-regulated in adipose tissue in obese subjects (Christiansen et al. 2005). Increased circulating level of MCP1 was also observed in obese humans (Kim et al. 2006). MCP1

levels were higher in diabetic than in non-diabetic Afro-Caribbean subjects (Ezenwaka et al. 2009). Similarly, in mice studies, it was reported that elevated circulating concentration of MCP1 can lead to systemic insulin resistance (Tateya et al. 2010). However, Burns et al. (2010) did not find statistically significant differences have in mRNA expression of anti-inflammatory adipokine such as MCP1 between insulin resistant and insulin sensitive groups in obese horses, while in another recent study, a higher mRNA expression of MCP1 was detected in overweight group compared to normal weight group in Finnhorses (Selim et al. 2013).

3 RESEARCH OBJECTIVES

The objectives of this MSc thesis study were as follows: The first objective was to find and validate internal control genes for quantitative PCR method for adipose tissues in Finnhorse mares. DNA sequences for both control and candidate genes were analysed, primers were designed and tested for the utilization in this experiment. The second aim was to quantitate the expression of mTORC1 and insulin-pathway associated genes after pasture season in two different treatment groups of Finnhorse mares and compare gene expression differences between treatment groups. Treatments aimed to analyse if pasture-associated energy feeding difference have effect on adiposity and thus, on the gene expression of adipose tissue genes in Finnhorse mares. In addition, gene expression differences were compared between two different adipose tissues (neck and tailhead adipose tissues).

The hypotheses were that pasture-associated high energy feeding would result in accumulation of adiposity, which will lead to altered expression of mTORC1 and insulin-pathway genes in subcutaneous adipose tissue.

4 MATERIALS AND METHODS

4.1 Animals and experimental design

The experimental procedures followed the protocols approved by the National Animal Ethics Committee in Finland. Twenty-two mares were selected in to the study. Mares were equally divided into eleven equal pairs according to their pedigree, medication and reproductive history, age, diet, weight and body condition. The two mares of each group were randomly selected to graze either on cultivated high-yielding pasture (CG), or semi-natural grassland (NG) from the end of May to the beginning of September at MTT Agrifood Research Finland (currently Natural Resources Institute Finland (LUKE)) in Ypäjä. Eight pairs of mares were studied for gene expression profiling (Figure 2).

Figure 2. Finnhorse mares at the semi-nature grassland (NG) in Ypäjä (Photo: Kari Elo).

4.2 Adipose tissue collection

Neck and tailhead subcutaneous adipose tissue (SAT) samples were collected from two groups of Finnhorse mares before and after pasture season for gene expression profiling. A detailed description of collection of tissue samples is given in Selim et al (2015). Tissues were stored at -80 °C.

4.3 RNA extraction and cDNA synthesis

Total RNA of neck and tailhead SAT samples were extracted and purified using RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany). Approximately 50 mm^3 (about 55 - 65 mg) of SAT was cut from stored sample. Then the tissue was placed into a 5 ml tube on ice for disruption and homogenization with 1 ml QIAzol Lysis Reagent using the TissueRuptor for 30 seconds. After 5 min incubation at room temperature, 200 µl chloroform was added in to the tube for subsequent phase separation.

The tube was shaken vigorously for 15 seconds and then incubated at room temperature for 2-3 min before centrifugation at 12000 g for 15 min at 4 °C. The upper, colorless, aqueous phase (approximately 600 µl) was transferred to a new tube with addition of 1 volume (500 µl- 600 µl) of 70% ethanol and vortexed. Then the sample was transferred to RNeasy column in 2 ml tube for centrifugation at 8000 g for 15 seconds, after the centrifugation the flow-through was discarded. Then, 700 µl of buffer BW1 was added to the RNeasy spin column to wash the column membrane with centrifugation at 8000 g for 15 seconds. Then the spin column was carefully removed from the collection tube to avoid contacting the flow-through. The membrane was washed twice (15 seconds and 2 min, respectively) with 500 µl of buffer RPE and centrifugation at 8000 g. The spin column was centrifuged at 8000 g for 1 min in a new 2 ml collection tube to eliminate any possible carryover of buffer and residual flow-through. The spin column was placed in a new 1.5 ml collection tube to elute the total RNA, the membrane was eluted by 30 µl of RNase-free water and centrifuged at 8000 g for 1 min. Then, 3 µl of each extracted total RNA sample was taken

to 0.2 ml collection tube for quality verification. All of the samples were then stored in -80 °C until further use. One set of SAT samples (horse no.9, Sailori) from CG group was missing for RNA extraction, thus 15 animal samples (8 from NG group and 7 from CG group) were studied in the following steps.

The total RNA quality was verified prior to qPCR experiments. RNA concentration and RNA integrity number (RIN) were measured using Agilent Bioanalyzer 2100 chip electrophoresis system and using Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).

The RNA 6000 dye concentrate was equilibrated at room temperature for 30 min while preparing the gel. The gel was prepared by centrifuging 550 µl of RNA 6000 Nano gel matrix in a spin filter at 1500 g for 10 min at room temperature. Then, 65 µl of filtered gel was pipetted into a 0.5 ml RNase-free microfuge tubes. After vortexing for 10 seconds, 1 µl of concentrate dye was added into 65 µl of filtered gel. The tube was vortexed and then centrifuged at 13000 g for 10 min at room temperature. The RNA 6000 Nano chip priming station was set up in position C before loading the gel-dye mix. A new chip was placed on the chip priming station, 9 µl of gel-dye mix was pipetted into the well with mark G. The plunger was positioned at 1 ml and then pressed until held by the clip, and the clip was released after exactly 30 seconds. The plunger was slowly pulled back to 1 ml position after 5 seconds of waiting. Then 9 µl of gel-dye mix was pipetted into the wells marked ***G*.** After this, 5 µl of RNA 6000 Nano marker was pipetted into all 12 sample wells and into the ladder well. All RNA samples and RNA ladder was denatured by heating for 2 min at 70 °C and then kept on ice until further use. Then, 1 µl of prepared ladder was pipetted into well with ladder marker and 1 µl of prepared RNA samples were pipetted separately into each of 12 sample wells. When applicable, 1 µl of marker was added into unused sample well(s). The chip was placed horizontally in the IKA vortexer adapter and vortexed at 2400 rpm for 1 min before running in the Agilent 2100 Bioanalyzer. The running was started within 5 minutes from the end of vortexing.

Synthesis of cDNA was completed with Anchored-Oligo (dT) 18 primer using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). All reagents were kept on ice while setting up the reactions. The template-primer mix was made by mixing 12 µl of each total RNA sample with 1 µl of Anchored-Oligo (dT)18 primer (50 pmol/ µl) in a sterile, nuclease-free, thin-walled PCR tube. All the template-primer mixtures were denatured by heating for 10 min at 65 °C and then kept on ice until further steps. The master mix was prepared for 35 reactions by pipetting 140 µl of Transcriptor Reverse Transcriptase Reaction Buffer, 17.5 µl of Protector RNase Inhibitor (40 U/ µl), 70 µl of Deoxynucleotide Mix, 17.5 µl of Transcriptor Reverse Transcriptase (20 U/ µl). Then, 7 µl of the master mix was added to each sample's template-primer tube to form a total of 20 µl mixture for reaction. After brief centrifugation, the tube was placed in a thermal block with a heated lid, then incubated for 30 min at 55 °C and 5 min at 85 °C for Transcriptor Reverse Transcriptase inactivation. The reaction was stopped at 4 °C. Synthesized cDNA samples were stored at -20 °C until further use for Real-Time PCR.

4.4 Primer design

Primer sequences and lengths for PCR products of selected genes were designed using the online Primer3 software program (Rozen & Skaletzky, 2000). Main criteria used in primer design were the length of PCR product (accepted range from 100 bp to 250 bp), the nearest neighbor temperature (same annealing temperature (60 °C) for all primer pairs) and absence of sequence-based hybridization problems. Exon-exon junctions were included in primer sequences of selected genes. The uniqueness of primer sequences was determined using NCBI database and BLASTN tool.

Primers were designed for six internal control genes (ICG)(Table 1). The selected internal control genes were actin beta (*ACTB*), glucuronidase beta (*GUSB*), mitochondrial ribosomal protein L39 (*MRPL39*). The mRNA abundances of *ACTB, GUSB and MRPL39* were evaluated together with candidate genes through different groups and adipose tissues. Then, the expression stabilities of these three genes were tested with NormFinder software (Andersen et al. 2004).

Table 1. Primer sequences (5'-3') of internal control genes, GenBank accession numbers and lengths of PCR products.

Gene	Sequence (5'-3')	GenBank	Length (bp)
ACTB	For.TGACCCAGATCATGTTTGAGACC	NM_001081838	139
	Rev.ATGGGCACAGTGTGGGTGA		
GUSB	For.GCTGACATCCGAGGGAAGG	XM_001493514	148
	Rev.CCACAATCCCATAGCGGTCA		
MRPL39	For.CCGGCTGGAGATTTATAGCA	XM_001496687	225
	Rev.CACTCAAATGCATGGCACA		
GAPDH	For. ACCATCTTCCAGGAGCGAGATC	NM_001163586	104
	Rev.CCTTCTCCAAGGTAGTGAAGACACC		
HSP90AB1	For. AGCATTTATGGAGGCTCTTCAGGC	NM_001081938	107
	Rev.TGATCACAACCACCTTCTCTGCC		
RPS2	For. CCGAGAAAACACCAAATGGCGG	XM_001497974	110
	Rev.CCACTGCCGAAGCCTCCG		

Candidate genes were mechanistic target of rapamycin (*MTOR*), sterol regulatory element binding transcription factor 1 (*SREBF1*), sterol regulatory element binding transcription factor 2 (*SREBF2*), *TBC1* domain family member 7 (*TBC1D7*), leptin (*LEP*), Glucose transporter type 4 (*GLUT4*), monocyte chemoattractant protein-1 (*MCP-1*), retinol binding plasma protein 4 (*RBP4*), tuberous sclerosis 1 (*TSC1*), tuberous sclerosis 2 (*TSC2*) (Table 2).

Table 2. Primer sequences (5'-3') of candidate genes, GenBank accession numbers, lengths of PCR products and mean amplification efficiencies (Eff).

Gene	Sequence (5'-3')	GenBank	Length (bp)	Eff
MTOR	For.GGAGAGAGGCTATCCGTGTGTT	XM_001492351	111	1,94
	Rev.ACAGGCTGACAGCAGAAGCA			
SREBF1	For.GGCCTTTACAGACCCTGGTG	XM_001918214	149	1,93
	Rev.GTGGGCTGTGCGCTTCTC			
SREBF2	For.CAGGTTCTGGGGGCTGGT	XM_005606691	146	1,94
	Rev.GAAGGTGACTGAGGAGCGTGA			
TBC1D7	For.CCAGTTTACAGAGGGTTTGGGATA	XM_001492275	128	1,91
	Rev.TGTTATCTTCTCGGCACTGTTCA			
LEP	For. CACACGCAGTCAGTCTCCTC	NM_001163980	176	1,94
	Rev.CGGAGGTTCTCCAGGTCAT			
GLUT4	For.GCCCCACAGAAGGTGATTGAA	NM_001081866	200	1,91
	Rev.CAGCATTGCCCTTTTCCTTCC			
MCP-1	For.GGCTCAGCCAGATGCAATTA	NM_001081931	141	1,92
	Rev.ATGGTCTTGAAGTTGGGACACT			
RBP4	For.TGATCTCTCACAACGGTTATTG	NM_001081951	152	1,92
	Rev.GGAGAAGAGAGGGCCAAACT			
TSC1	For.CGCAGAATAGCTATGGGAGTGC	XM_001498348	108	1,95
	Rev.GTGTCGGTGGGGAACTCAGA			
TSC2	For.GGAAGAAGAACTGGCTGAGTTTG	XM_005599067	131	1,94
	Rev.GGACCATCGATGCGATGTATT			

4.5 Quantitative Real-Time RT-PCR

Quantitative PCR (qPCR) reactions were prepared using LightCycler 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany). The volume in qPCR reaction was 10 µl (2.5 µl of cDNA and 7.5 µl of master-mix), and each sample was run in triplicate. All the samples and reagents were kept on ice while setting up the reaction. Each cDNA sample was diluted 1:4 to 80 µl (20 µl cDNA diluted into 60 µl of Dnase/Rnase free water) for 24 reactions on 96-well plate.

The final concentration of primers in qPCR was 5 pmol/ µl/each; therefore, 65 µl of forward primer (100 pmol/ µl) and reverse primer (100 pmol/ µl) were mixed to get 50 pmol/ µl primer-mix dilution. The master-mix was prepared by mixing 64 µl of primer-mix, 96 µl of Dnase/Rnase free water and 320 µl of Roche's 2x master-mix, and then the solution was divided into 8 wells on 96-well plate. The plates of master-mix and diluted cDNA were centrifuged at 1500 g for 2 min. PCRs were pipetted into optical 384-well plates using epMotion 5075 pipetting robot (Eppendorf AG, Hamburg, Germany), and then centrifuged at 1500 g for 2 min before incubation for 1 hour at 4 °C. In this study, five sets of 384-well plates were prepared for 3 internal control genes and 10 candidate genes

PCR reactions were performed in the LightCycler 480 Real Time PCR instrument (Roche Diagnostics GmbH, Mannheim, Germany). The temperature profile of qPCR in this study was as follows: initial denaturation step for 5 min at 95°C, followed by 45 amplification cycles for 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C.

Output results were first preprocessed with the LightCycler® 480 Software (Figure 3). All samples with crossing point (Cp) cycle values higher than 35 were excluded from data collection. After preprocessing, all valid data was saved as text files for further statistical analyses. Saved data included fluorescence measurements at each cycle and Cp values. Data were imported into Excel software.

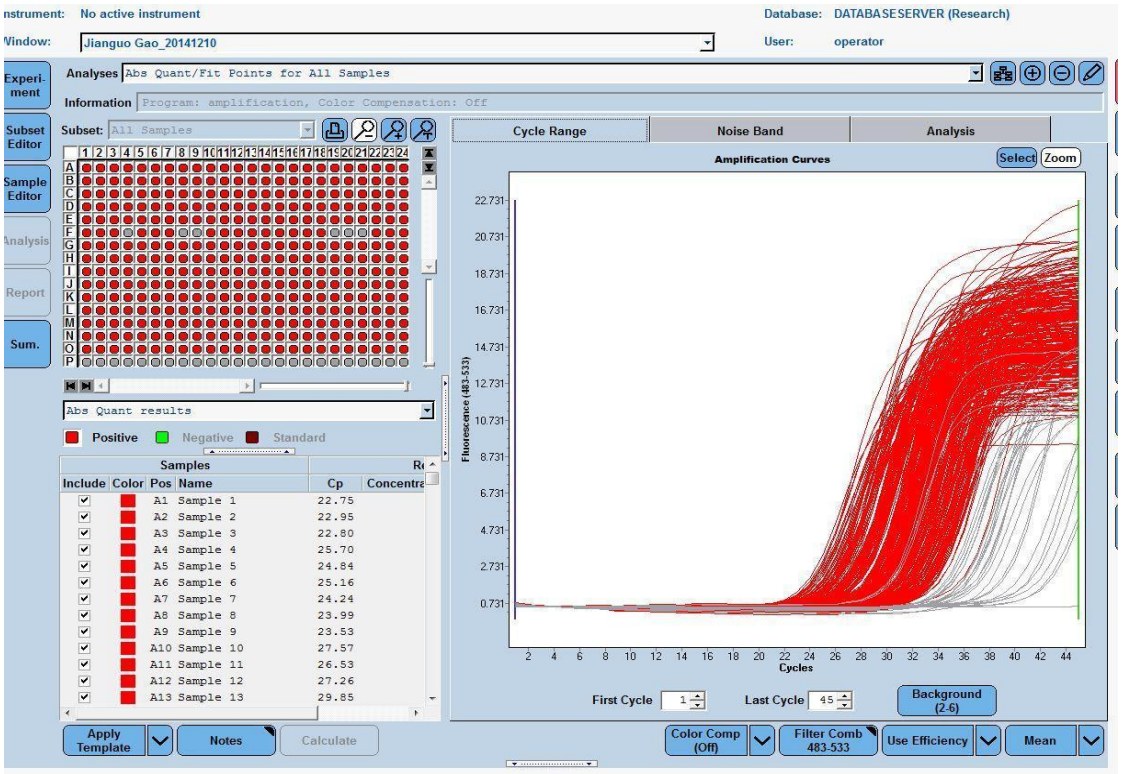

Figure 3. Snapshot of results output sheet from the LightCycler® 480 Software.

4.6 Statistical analysis

The calculation of mRNA abundance was based on delta cycle threshold (ΔCt) values. The method of Livak and Schmittgen (2001), so called $2^{-\Delta\Delta Ct}$ method, was used in analyses of relative gene expression data. LinRegPCR (2004) software was used to calculate PCR efficiency corrected Ct values (Ramakers et al. 2002, Ruijter et al. 2009). Then, the Cq values were the PCR efficiency corrected Ct values. In addition, PCR efficiencies (Eff) were calculated with LinRegPCR software for all primer pairs.

Relative mRNA abundance of genes between NG and CG groups in different SAT groups and between SAT groups were presented as 14-ΔCq values. Cq values were also used in gene expression fold change (2-ΔΔCt) analyses when target group were compared to control group.

The mean Cq values of both internal control genes and candidate genes were calculated as averages of replicates. The geometric mean of the three internal control genes ($Cq._{ICG.}$) was used to calculate mRNA abundance of candidate genes by accessing the relatively difference between their Ct values (ΔCq). ΔCq value was determined by equation:

$$\Delta Cq = Cq._{candidate} - Cq._{ICG}$$

The fold change in expression of the candidate gene relative to the internal control gene between groups was studied using $2^{-\Delta\Delta Ct}$ method. For the samples in control group, ΔΔCq equals zero and $2^{-\Delta\Delta Ct}$ was one. The fold change was calculated by equation:

$$\Delta\Delta Cq = (Cq._{candidate} - Cq._{ICG})_{Target} - (Cq._{candidate} - Cq._{ICG})_{control.}$$

Final statistical analyses were conducted using R statistical software package (http://www.r-project.org/). Distribution of variables was analyzed using the Shapiro Wilk normality test with histogram and Q-Q plot. The Wilcoxon rank sum test was used to analyse gene expression differences between different pasture groups and tissue groups.

5 RESULTS

5.1 Stability of internal control genes

ACTB, GUSE and *MRPL39* were selected as internal control genes ((ICG) in this study according to the stability validation conducted using NormFinder software (Table 3). The mRNA abundances of these three genes were stable between groups and different adipose tissues compared to candidate genes and thus, neither energy feeding or SAT tissue type does not affect their expression.

Table 3. Output results of gene stability from NormFinder. The lower the stability value, the more stable is the expression of the studied gene.

Gene name	Stability value
ACTB	0.015
GUSB	0.019
MRPL39	0.011
MTOR	0.033
SREBF1	0.030
SREBF2	0.025
TBC	0.009
INSR	0.028

5.2 Total RNA quality

The average RIN value of prepared total RNA samples was 7.7, average RNA concentration was 99.1 ng/ µl. Electropherograms for all samples of neck SAT (Figure 4) and tailhead SAT (Figure 5) reveal acceptable and rather uniform quality among all total RNA samples.

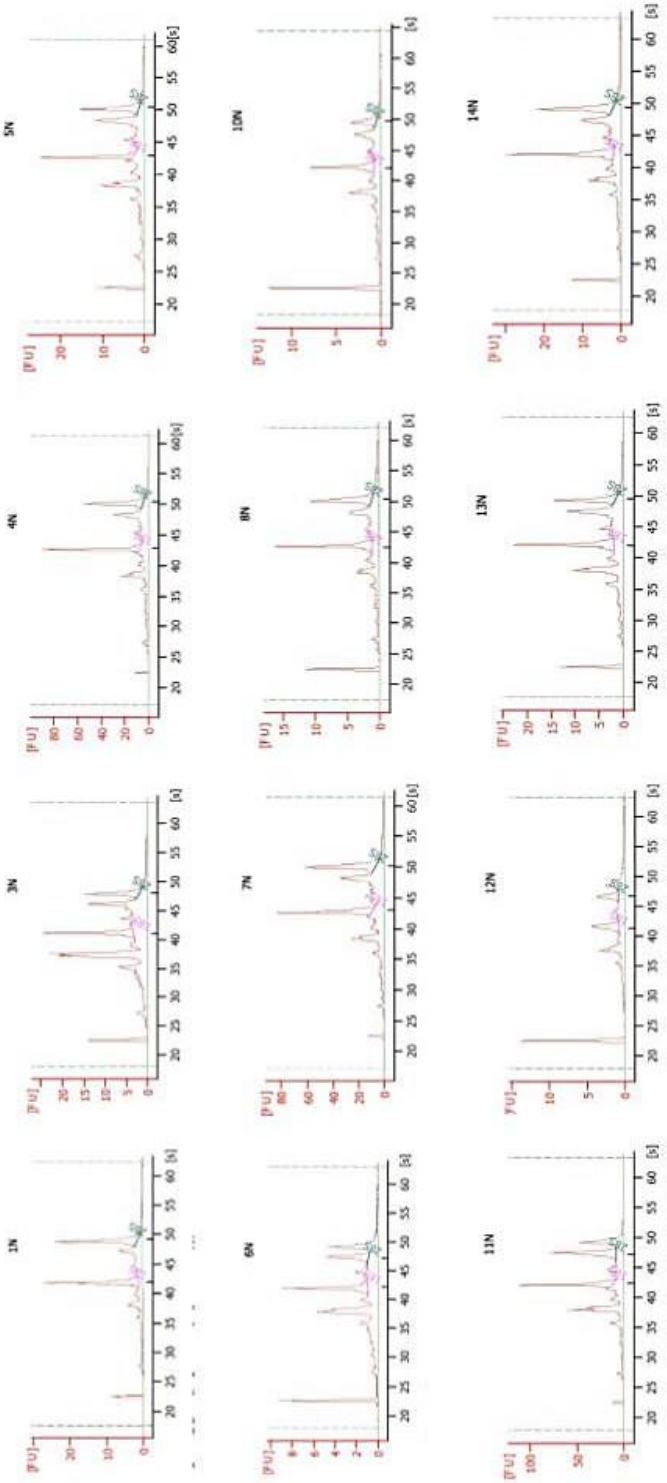

Figure 4. Electropherogram results of neck adipose tissue samples from Agilent 2100 Bioanalyzer. The Y-axis presents the fluorescence intensity (in arbitrary fluorescence units) and the X-axis presents run time in electrophoresis (in seconds).

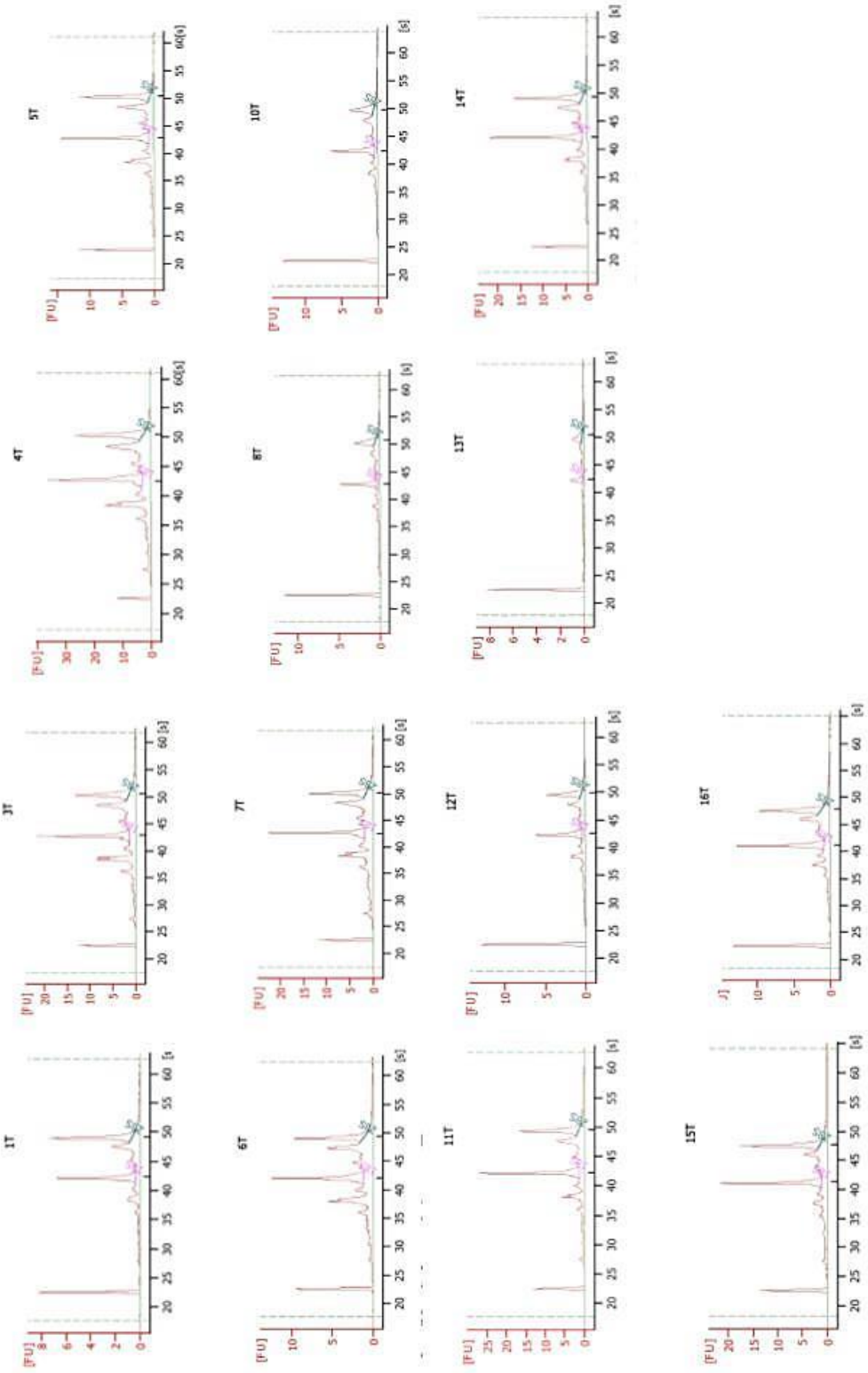

Figure 5. Electropherogram results of tailhead adipose tissue samples from Agilent 2100 Bioanalyzer. The Y-axis presents the fluorescence intensity (in arbitrary fluorescence units) and the X-axis presents run time in electrophoresis (in seconds).

5.3 Gene expression between treatment groups

There were no obvious gene expression differences between NG and CG groups in both neck and tailhead SAT based on fold changes (Figure 6). All candidate genes except *TSC2* had slightly higher expression in CG group than in NG group in neck SAT when using Wilcoxon rank sum test (Table 4). However, the differences were not obvious ($p>0.05$), only *RBP4* had significantly higher gene expression ($p<0.05$) (Table 4).

CG group showed lower mRNA abundance in tailhead SAT except *TSC1* and *TSC2* (Table 5). The difference of *TSC1* between NG and CG group in tailhead SAT was obvious ($p<0.05$).

Table 4. Relative mRNA abundances (14-ΔCq value) of genes between NG and CG groups in neck SAT and statistical significances of differences between groups based on Wilcoxon rank sum test.

	NG	SEM	CG	SEM	P-value
MTOR	11.49	0.81	11.85	0.53	0.418
SREBF1	9.89	0.42	10.78	0.44	0.203
SREBF2	12.47	0.55	12.51	0.56	0.817
TBC1D7	10.61	0.13	11.04	0.34	0.475
LEP	11.75	0.64	12.96	0.66	0.482
GLUT4	9.05	0.41	10.33	0.44	0.064
MCP1	7.86	0.40	8.93	0.60	0.225
RBP4	9.09	0.44	11.57	0.89	0.035
TSC1	11.60	0.12	11.65	0.23	0.488
TSC2	7.34	0.49	7.07	0.50	0.643

Table 5. Relative mRNA abundances (14-ΔCq value) of genes between NG and CG groups in tailhead SAT and statistical significances of differences between groups based on Wilcoxon rank sum test.

	NG	SEM	CG	SEM	P-value
MTOR	10.64	0.42	10.51	0.74	0.910
SREBF1	11.62	0.48	11.61	0.39	1.000
SREBF2	11.80	0.37	11.50	0.67	0.817
TBC1D7	11.15	0.27	10.23	0.47	0.133
LEP	13.51	0.58	13.28	0.36	0.848
GLUT4	10.42	0.31	11.24	0.63	0.302
MCP1	8.31	0.49	8.05	0.59	0.796
RBP4	9.30	0.78	8.23	0.29	0.475
TSC1	11.41	0.10	11.74	0.16	0.071
TSC2	7.28	0.55	7.52	0.69	0.699

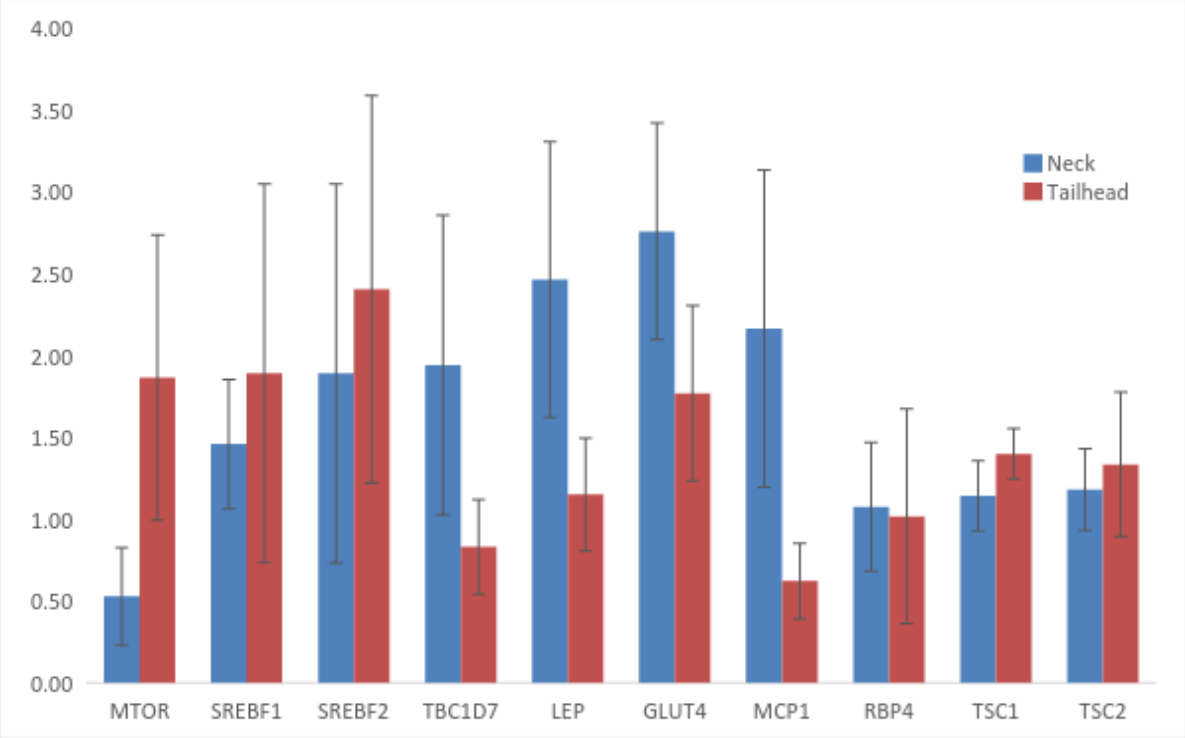

Figure 6. Fold change in gene expression ($2^{-\Delta\Delta C}$) of CG group using NG group as covariates in both Neck and Tailhead SAT (gene expression in NG group = 1).

5.4 Gene expression between neck and tailhead subcutaneous adipose tissue

Two candidate genes had statistically significant differences in the gene expression between neck and tailhead SAT groups (Table 6). *SREBF1* and *GLUT4* had significantly higher expression ($p<0.05$), and *RBP4* had a trend of higher expression in tailhead adipose tissue ($p<0.10$). *MTOR, SREBF2, TBC1D7, LEP, TSC1, TSC2* and *MCP1* had approximately same expression levels between different SAT groups.

Table 6. Relative mRNA abundances (14-ΔCq value) of genes between neck and tailhead SAT group and statistical significances of differences between groups based on Wilcoxon rank sum test.

Genes	14-ΔCq (Neck)	SEM	14-ΔCq (Tailhead)	SEM	P-value
MTOR	11.66	0.48	10.58	0.39	0.191
SREBF1	10.31	0.32	11.61	0.30	0.007
SREBF2	12.49	0.38	11.66	0.36	0.206
TBC1D7	10.81	0.17	10.72	0.28	0.765
LEP	12.35	0.47	13.39	0.33	0.183
GLUT4	9.65	0.34	10.77	0.33	0.026
MCP1	8.39	0.38	8.20	0.36	0.927
RBP4	10.33	0.59	8.81	0.45	0.066
TSC1	11.62	0.12	11.55	0.09	0.359
TSC2	7.22	0.34	7.38	0.42	0.760

Relative mRNA fold change (determined by $2^{-\Delta\Delta C}$) values between neck and tailhead group are presented in Figure 7. The neck group was referred as control group (gene expression =1) and then expression levels in tailhead group were compared to expression levels in control group (Figure 7). The fold change values of *MTOR, SREBF2, TBC1D7, RBP4, TSC1* and *TSC2* were around 1.00, which demonstrates that gene expression levels were about the same between different SAT groups. Although *SREBF1, LEP, GLUT4* and *MCP1* had relatively high expression with fold change values 2.63, 2.10, 2.33, and 2.18 respectively, the differences between SAT groups were not statistically significant.

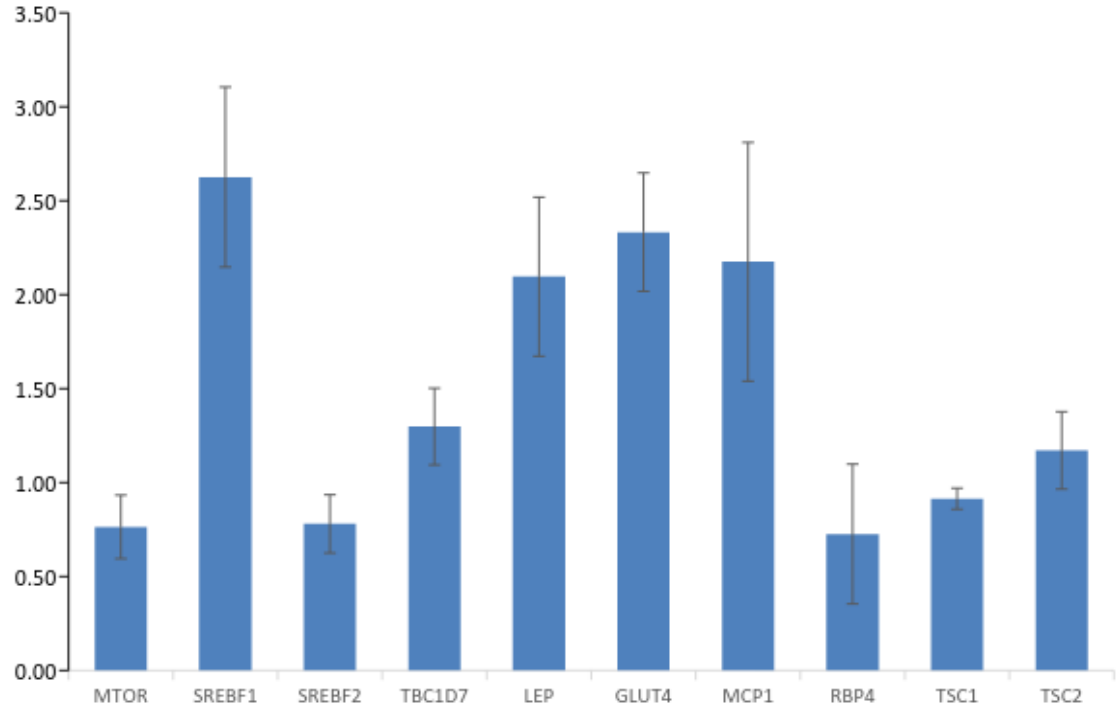

Figure 7. Fold change in gene expression ($2^{-\Delta\Delta C}$) in tailhead SAT compared to neck SAT (neck gene expression = 1).

6 Discussions

6.1 Testing and validation of internal control genes

For gene expression profiling of small number of genes the qPCR is an accurate and sensitive method. The method requires data normalization to account for analytical errors that introduce variation to measurements (Dheda et al. 2005, Valasek & Repa 2005). Data normalization can be done by using reference genes or ICGs (Dheda et al. 2005). Therefore, introducing ICG is the most reliable approach in qPCR analysis (Bionaz & Loor 2007). The expression of ICG should not associate with experimental treatment, or vary between investigated tissues (Vandesompele et al. 2002). It has been claimed that careful evaluation and validation of is standard requirement when working with qPCR technology (Bustin et al. 2009).

In this study, primers for six ICG genes were designed in the first place. However, according to the desired length of product and the nearest neighbor temperatures, only expression of *ACTB, GUSB and MRPL39* were tested together with candidate genes. The expression stabilities of the selected ICG were evaluated through treatment groups and adipose tissues with NormFinder software (Andersen et al. 2004). Finally, the geometric mean of the three internal control genes was used in the statistical analyses in this study.

6.2 mRNA expressions between CG and NG group

In this study, RBP4 had significantly higher and GLUT4 had a trend to higher mRNA expression levels in CG group than at NG group in neck SAT. While, only TSC1 had trend to higher expression in CG group at tailhead SAT. Gene expression of SREBF1, SREBF2, TBE1D7, TSC2 at both neck and tailhead SAT were not different between treatment groups. RBP4 is an adipokine that contributes to insulin resistance in obesity and type-2 diabetes (Yang et al. 2005, Graham et al. 2006). In the mouse model, serum RBP4 levels in obesity and insulin resistance groups were significantly higher than in the control group, and the GLUT4 gene knockout mice had obviously higher insulin sensitivity than the wild-type control group mice (Yang et al. 2005). Expression of GLUT4 was reported down-regulated selectively in adipocyte and not in skeletal muscle in insulin-resistant states (Shepherd & Kahn 1999). Down-regulation of GLUT4 can cause insulin resistance and increase the risk of developing diabetes (Abel, et al. 2001). Increased serum levels of RBP4 have been detected in adipose-specific GLUT4 knockout (adipose-GLUT4 -/-) mice (Yang et al. 2005).

Thus, in mice adipose tissue, RBP4 expression was inversely correlated with GLUT4 expression because adipocytes sense glucose uptake and regulate systemic glucose metabolism through RBP4. However, in equine adipocyte studies, no statistical differences were detected in mRNA expression of RBP4 between insulin resistant and insulin sensitive groups of ponies in tailhead SAT (Ungru et al. 2012). RBP4 expression levels closely linked with adiposity, but they were independent of other obesity factors such as insulin sensitivity (Ungru et al. 2012).

It was claimed that RBP4 might be regulated differently in mice and humans (Janke et al. 2006). In human subjects, a positive correlation was found between RBP4 and GLUT4 expression levels (Janke et al. 2006). Increased serum RBP4 levels were observed in humans with in insulin-resistant states such as obesity, type-2 diabetes (Graham et al. 2006). In adipose tissue, GLUT4 expression was the only determinate factor of RBP4 expression.

These results may indicate that regulation and expression of RBP4 in horse adipose tissue may be more similar with expression in adipose in human than in mice. It should be noted that subjects of this study were mature Finnhorses while Ungru et al (2012) studied ponies. Comparing results of this study and Ungru et al. 2012, it is assumed that the function of adipokines in metabolism may be different in ponies and horses, which is of interests for further study.

LEP serves as an important endocrine signal of nutritional status and adipose tissue mass in the horse (Houseknecht et al. 1998). It regulates appetite, homeostasis and adipogenesis (Ingvartsen & Boisclair 2001). In horses, studies have reported that the plasma LEP concentration was positively correlated with adipose tissue mass (Buff et al. 2002, Kearns et al. 2006). Other study has indicated a strong correlation between LEP concentration and degree of insulin resistance (Van Weyanberg et al. 2007).

In my study, the relatively higher expression of LEP in CG group may indicate greater fat mass due to overfeeding, which associates with risk of obesity or insulin resistance. This result agreed with the recent study that reported up-regulation of LEP expression in overfed dairy cow (Ji et al. 2014). However, the mRNA expression of LEP had no statistically significant difference between CG and NG mares in either neck or tailhead SAT.

MCP1 is a type of inflammatory cytokine with chemotactic function which might be related to the monocytes associated inflammatory process (Kanda et al. 2006). Obesity is associated with macrophage accumulation in adipose tissue (Weisberg et al. 2003). MCP1 contributes to macrophage infiltration into adipose tissue and insulin resistance in obesity

(Kanda et al. 2006). In obese human subjects, the mRNA expression of MCP1 was up-regulated in adipose tissue, which was correlated directly with adiposity (Christiansen et al. 2005). Increased circulating levels of MCP1 were observed in obese humans and they associated with body mass index (Kim et al. 2006). MCP1 levels were higher in diabetic than in non-diabetic Afro-Caribbean subjects (Ezenwaka et al. 2009). Tateya et al (2010) also reported that elevated circulating concentration of MCP1 caused systemic insulin resistance in mice.

However, only a trend of higher mRNA expression of MCP1 in CG group than in NG group was observed in this study, yet expression difference between treatment groups was not significant. A higher mRNA expression of MCP1 was detected in CG group compared to NG group in tailhead SAT (Selim et al. 2013). It has been reported that there were no statistically significant differences in mRNA expression of anti-inflammatory adipocytokine such as MCP1 between insulin resistant and insulin sensitive groups from visceral and various SAT in obese horses (Burns et al. 2010).

These findings may suggest that the mares might have to undergo a prolonged period of energy feeding to get greater fat reserves and obesity levels, before the up-regulation of MCP-1 or LEP in SAT will be detectable. Alternatively, grazing on high yielding pasture may not cause risk of metabolic diseases that associated with mTOR1 pathway regulation when the horses were with healthy body condition score before the grazing season.

Besides, the Finnhorses in this study were grazed in quite high latitude (60.795ºN and 23.315 ºE). Finnhorse mares' hormone secretion, e.g. alpha-melanocyte stimulating hormone (alpha-MSH) and metabolism activity may have been adapted to accumulation of adipose tissue during the grazing season (III), in order to adapt to the winter season. Hoggard et al. (2004) claimed that increased alpha-MSH inhibited LEP gene expression. It is also suggested that even higher or prolonged energy feeding period would be needed for the onset insulin-resistant states and this kind of experiment could be interesting for further study.

6.3 mRNA expressions between neck and tailhead SAT

In equidae, adipose tissue distributed especially on the crest of the neck might contribute to hyperinsulinemia, insulin resistance and/or an increased risk of laminitis (Carter et al. 2009, Frank et al. 2010b). Interestingly, selected mRNA expression profile in nuchal ligament SAT was found more active than other fat depots including tailhead SAT (Burns et al. 2010). Neck SAT was claimed to have outstanding and unique role in horse adipobiology processes and in metabolism related diseases such as equine metabolic syndrome (Burns et al. 2010).

It has been shown in horses that expression differences of glucose transporters were associated to different adipose tissues (Waller et al. 2011). Total GLUT4 expression was significantly greater in visceral adipose tissue compared to SAT in both insulin sensitive and resistant groups (Waller et al. 2011). In this study, significantly higher expression of GLUT4 was found in tailhead SAT compared to neck SAT ($P<0.05$).

LEP mRNA expression was found to be significantly higher in neck than in mesenteric SAT samples that were collected from different breeds, ages and varying body condition (Lien et al. 2013). Since previous study has indicated a strong correlation between LEP concentration and degree of insulin resistance (Van Weyanberg et al. 2007), it was assumed that enlarged nuchal adipose tissue could be a key risk factor for metabolic diseases (Carter et al. 2009, Frank et al. 2010b). However, higher but not significant expression of LEP was observed in tailhead SAT (0.61) compared to neck SAT (1.65) in this study.

The unexpectedly higher expression of SREBF1, GLUT4 and LEP in tailhead may indicate that tailhead SAT accumulated more fatness than neck SAT after the grazing season. A previous study by Särkijärvi et al (2012) reported that the differences of fat deposition in neck area were small, despite that CG group had more fatness during grazing season. However, in tailhead adipose, the fat thickness was significantly different between groups in the end of grazing season (Särkijärvi et al. 2012). The mRNA expression of SREBF1, GLUT4 and LEP may be upregulated in response to the significantly greater fat

accumulation in tailhead SAT. In contrast, the fatness difference in neck SAT may not be great enough to affect the mRNA expression of studied genes.

A trend of higher mRNA expression of RBP4 was shown in neck SAT compared to tailhead SAT ($P<0.10$). It may indicate different roles of neck and tailhead SAT in regulation of metabolism, which is of interests to compare expression from different adipose tissue locations in further studies. No significant mRNA expression differences were observed in MTOR, SREBF2, TBC1D7, TSC1 and TSC2 genes between neck and tailhead SAT. The result is in line with a previous study that no differences were detected in pro-inflammatory cytokines and adipokines in mRNA expression between insulin resistant and insulin sensitive horses (Burns et al. 2010).

6.4 Hypotheses about mTORC1 pathway genes

Our hypotheses were that mares in CG would have increased fat deposition in SAT including neck and tailhead area, which might lead to higher mRNA expression in insulin and mTORC1 pathway genes. However, Särkijärvi et al. (2012) claimed no statistical differences between NG and CG in neck SAT before and after grazing. In May, there were no significant body weight differences between groups, while CG group had significantly higher body weight after grazing in September (Särkijärvi et al. 2012). It may indicate that although the body weight significantly increased due to high energy feeding in CG during the grazing season, the fat deposition in neck SAT was not affected enough to upregulate expression of mTORC1 pathway genes. In tailhead SAT, CG mares had smaller fat thickness in May and this difference disappeared in September (Särkijärvi et al. 2012). It may explain that no mRNA expression differences in tailhead SAT were detected between groups.

In addition, Selim et al. (2015) presented that no differences were detected in plasma glucose and basal NEFA levels between CG and NG groups. Similarly, it has been reported that high energy feeding over 16 weeks had no effect on plasma NEFA concentrations despite it induced obesity in horses (Carter et al. 2009). However, increased plasma NEFA

concentration was reported in obese horses due to insulin resistance (Frank et al. 2006). It is suggested that mares in CG group may need prolonged feeding period or higher energy feeding difference between groups before distinct differences can be detected in expression levels of studied mTORC1 genes in adipose tissues.

7 CONCLUSIONS

The lack of distinct expression differences between groups may indicate that pasture associated fat deposition may not considerably affect expression of insulin pathway and mTORC1 genes in neck and tailhead adipose tissue in Finnhorse mares. These results also provide additional evidence to our hypothesis that fattening resulting on unrestricted grazing on cultivated high-yielding pasture does not increase the risk of metabolic diseases in Finnhorse mares when they have normal body condition at the beginning of the grazing season.

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